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PATHOBIOLOGY IN FOCUS

Melanoma stem cells: not rare, but well done

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Since the identification of self-renewing cells in the hematopoietic system, stem cells have transformed the study of medicine. Cancer biologists have identified stem-like cells in multiple malignancies, including those of solid organs. This has led to the development of a stem cell theory of cancer, which purports that a subpopulation of self-renewing tumor cells is responsible for tumorigenesis. This contrasts with the stochastic model of tumor development, which advances that all tumor cells are capable of tumor formation. Within the field of melanoma, the identity and existence of cancer stem cells has been the subject of recent debate. Much of the controversy may be traced to differences in interpretations and definitions related to the cancer stem cell theory, and the use of dissimilar methodologies to study melanoma cells. Accumulating evidence suggests that cancer stem cells may exist in melanoma, although their frequency may vary and they may be capable of phenotypic plasticity. Importantly, these primitive melanoma cells are not only capable of self-renewal and differentiation plasticity, but also may confer virulence via immune evasion and multidrug resistance, and potentially via vasculogenic mimicry and transition to migratory and metastasizing derivatives. Therapeutic targeting of melanoma stem cells and the pathways that endow them with virulence hold promise for the design of more effective strategies for amelioration and eradication of this most lethal form of skin cancer.
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"*The keys to nature's puzzles are merely hidden, not scarce*"
Anon

THE CONTROVERSY

'Melanoma, the deadliest form of skin cancer, does not follow the conventional cancer stem cell model'.[1] For those even peripherally interested in the pathogenesis of the more virulent forms of human cancer, this statement is potentially of critical scientific and therapeutic importance. If true, it suggests that melanoma may represent a key exception to the growing number of malignant neoplasms that conform to the cancer stem cell concept. In this focus on pathobiology, we will critically examine and dissect many of the data and related issues that concern the possible existence of melanoma stem cells with the intent of determining whether melanoma is, in keeping with many other cancers, likely to be driven by a subpopulation of self-renewing, aggressive cells. This inquiry is crucial in determining whether melanoma may indeed be ultimately subject to therapeutic intervention by way of uncovering and targeting a protean stem-cell 'Achilles' heel' that confers clinical virulence to this potentially most deadly form of human cancer.

WHAT IS A 'STEM CELL'?

In 1868, the term 'stammzelle' or 'stem cell' first appeared when Ernst Haeckel, a German biologist, used it to refer to both a 'unicellular ancestral organism from which he presumed all multicellular organisms evolved' and 'the fertilized ovum that gives rise to all of the cells of an organism'.[2] Three decades later, American cell biologist Edmund B Wilson used the term to describe 'the unspecialized mother cell of the germline'.[2,3] Since its early usage, the term 'stem cell' has been invoked to describe a variety of behaviors, concepts, and applications. Today, we recognize at least four types of stem cells: embryonic stem cells, adult or somatic stem cells, engineered stem cells, and cancer stem cells. Although all these share certain fundamental characteristics, they differ in several important respects.

Of the four stem cell types, adult or somatic stem cells historically were the first to be identified in the 1960s in the hematopoietic system.[3,4] Regarded as the prototypical somatic stem cell, they are characterized by their ability to self-renew and differentiate into heterogeneous, functionally mature progeny. Thus, these cells may proliferate nearly indefinitely as well as give rise to a diverse array of specialized

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cells that comprised the hematopoietic system. Since the first identification of hematopoietic stem cells, similar cells of mesodermal, ectodermal, and endodermal lineages have been recognized in adult tissues. In skin, for instance, epithelial stem cells have been found in the basal layer of the epidermis and in the bulge or 'wulst' region of hair follicles,[5] mesenchymal stem cells are scattered within the dermis,[6] and melanocytic progenitors are present in dermis[7] and hair follicles.[8] These adult stem cells are relatively rare, give rise to cells of the tissue type in which they are found, and function in tissue repair and maintenance as well as potentially in regenerative responses. Their eventual depletion may also relate to the poorly understood yet ubiquitous and ultimately lethal phenomenon of chronological aging.

Approximately three decades after the discovery of adult/somatic stem cells, embryonic stem cells were isolated, first from mouse embryos[9,10] and subsequently from human embryos.[11] These are pluripotent cells found in the inner cell mass of the blastocyst stage, capable of giving rise to an entire multicellular organism. Like somatic stem cells, embryonic stem cells self-renew and proliferate extensively. However, the differentiation potential of embryonic stem cells by far exceeds that of somatic stem cells. Because the isolation of embryonic stem cells requires destruction of human embryos, political, religious, and ethical controversies have limited their use in biomedical research. Efforts to continue the advancement of stem cell research while abiding by federal regulations for use of human embryos have fueled the development of genetically engineered stem cells, also known as induced pluripotent stem (iPS) cells,[12] as a potential alternative stem cell source. These iPS cells are embryonic stem-like cells created from somatic cells via insertion and expression of genes important in maintaining embryonic stem cell properties. Such iPS cells resemble embryonic stem cells in that they have extensive differentiation capacity and give rise to all three germ layers.

CANCER STEM CELLS

Whereas adult and embryonic stem cells are integral to a functioning healthy organism, another type of stem cell, the cancer stem cell, promotes a disease state. Simply stated, cancer stem cells are the subset of cells found in a malignancy that is responsible not only for the formation of tumors, but also for their inexorable progression. These tumorigenic cells are like adult or embryonic stem cells in that they have the ability to self-renew (produce new stem cells) and give rise to a diversity of cells that differentiate and after a finite number of divisions, eventually succumb to programmed cell death. However, cancer stem cells differ from adult stem cells in that their division results in tumor initiation and growth, whereas adult stem cells divide and differentiate in response to physiologic signals to replenish cells in the organs in which they reside. Thus, although these two types of stem cells have functional and phenotypic overlap, cancer and adult stem cells are not coincident.

The origin of cancer stem cells and their relationship to adult stem cells remain elusive. Some have posited that cancer stem cells arise from mutations of physiologic adult stem cells,[13] arguing that transformed or mutated adult stem cells may lead to tumorigenesis via unregulated self-renewal. Although this is likely the case in certain malignancies, not all tumor cells that arise from mutations in long-lived physiologic adult stem cells are inevitably cancer stem cells. Conversely, cancer stem cells need not necessarily derive from adult stem cells.[14] For instance, it has been speculated that cancer stem cells arise from oncogenic transformation of transit amplifying cells,[15] committed progenitor cells,[16,17] or even terminally differentiated cell types.[18] It has also been suggested that differentiated somatic cells can reacquire stem cell-like characteristics by reactivation of signaling pathways that facilitate self-renewal and are associated with malignant transformation.[19,20] Finally, some have averred that fusion of mutated somatic cells with normal stem cells may give rise to cancer stem cells.[21]

Like adult/somatic stem cells, cancer stem cells were first identified in the hematopoietic system. Studies in leukemia and multiple myeloma in the 1960s and 1970s showed that only a small subset of tumor cells were capable of extensive proliferation.[22,23] These cells were referred to as leukemic stem cells, as their clonogenicity resembled that of adult hematopoietic stem cells. In the 1990s, leukemic stem cells were isolated in studies of acute myeloid leukemia (AML) and found to have a unique cell surface phenotype ($CD34+CD38-$).[24,25] These relatively rare tumor-forming cells were exclusively capable of both initiating human AML and reconstituting the heterogeneous phenotype of the original tumor when transplanted into irradiated non-obese diabetic (NOD)/severe combined immunodeficient (SCID) mice. Furthermore, the $CD34+CD38-$ phenotype of these cells was similar to that of normal hematopoietic stem cells, suggesting that leukemic stem cells may have arisen from mutated physiologic adult stem cells.[24] Since their recognition in hematopoietic malignancies, cancer stem cells have been identified in a variety of solid tumors, including cancers of the breast,[26] brain,[27] colon,[28,29] and skin.[30]

As a result of the identification of stem cells in numerous cancers, a potential paradigm for tumor development that varies from the more traditional stochastic model has gained considerable attention. According to the stem cell theory of human cancer, a subpopulation of malignant cells capable of self-renewal is responsible for tumor initiation, progression, and generation of phenotypic heterogeneity. Thus, although the stochastic model of tumor development postulates that all tumor cells are theoretically capable of tumor formation and progressive growth via the process of random mutation and clonal selection,[31] the stem cell model proposes that only a discrete subpopulation of cells are responsible[32] (Figure 1). However, these two models are not necessarily mutually exclusive, and elements of the stochastic model may be at play within the cancer stem cell subpopulation.[33,34]

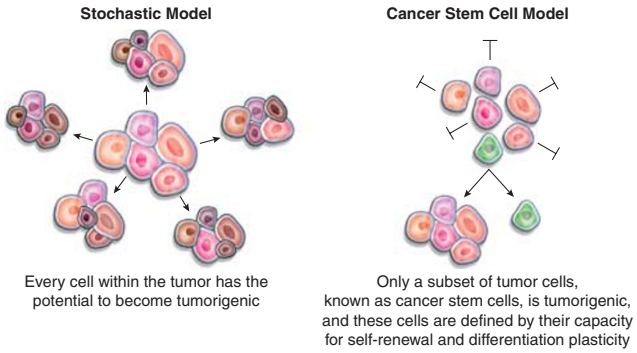

Figure 1 Stochastic *versus* cancer stem cell model of tumorigenesis. According to the stochastic model, every cell within the tumor has the potential to become tumorigenic. In contrast, the cancer stem cell model purports that only a subset of tumor cells, known as cancer stem cells, is tumorigenic, and these cells are defined by their capacity for self-renewal and differentiation plasticity.

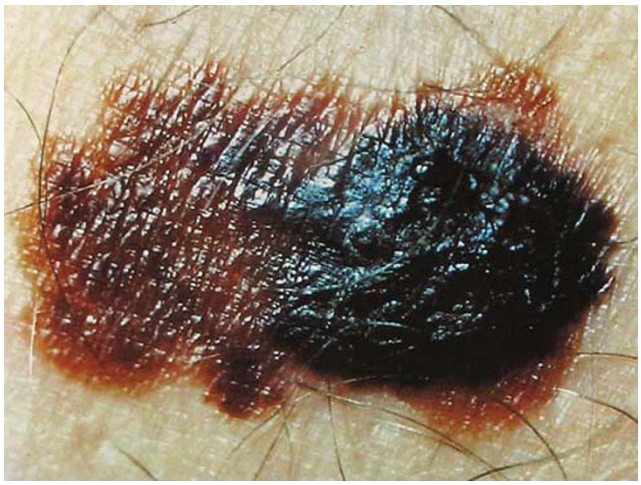

Figure 2 Clinical presentation of superficial spreading melanoma. Most lesions grow initially as relatively flat, variably pigmented, and irregular patches and plaques that after many months or even years ultimately develop raised papules or nodules signifying 'tumorigenic' growth and heralding acquisition of potential for metastasis. (Figure 8–5C, from Elder and Murphy.[165] Used with permission).

There is considerable disparity, though, regarding the specifics of the cancer stem cell model and the very definition of a cancer stem cell.[19] A major source of confusion potentially comes from the multiplicity of features that have been ascribed to the identity of the cancer stem cell. Substantial variation exists among studies as to which sets and combinations of criteria are used to define the cancer stem cell, and researchers may emphasize certain characteristics to describe cancer stem cells in the context of their hypotheses and related findings in the laboratory. Given the wide diversity of characteristics attributed to the cancer stem cell, the American Association for Cancer Research (AACR) in 2006 sought to unify conceptions of this cell type. In their working definition, the AACR determined that a cancer stem cell is 'a cell within a tumor that possesses the capacity to *self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor*'.[14] Thus, the two hallmark features of a cancer stem cell are self-renewal and differentiation.

MELANOMA STEM CELLS

The study of cancer stem cells in one solid tumor, human malignant melanoma, has recently elicited great interest as well as considerable debate. As the most lethal form of skin cancer, melanoma is a highly aggressive tumor whose incidence is currently on the rise.[35] Melanoma is unique in that it is generally readily visible on the surface of the body (Figure 2). However, although its superficial location and small volume suggest benevolence, its appearance may be deceiving, particularly when it evolves from a relatively flat lesion to one with expansile growth and invasion. Indeed, some primary melanomas may metastasize and prove fatal when they have grown to a volume no larger than that of a grain of rice!

The virulence of melanoma relates to the phases of tumor growth. In the radial growth phase, the malignant cells are confined to the epidermis and superficial dermis, and the tumor has little potential to metastasize. At this stage, the melanoma often appears as an irregular, pigmented, relatively flat lesion, and complete surgical excision generally results in cure. In the more advanced, vertical growth phase, melanoma cells extend to the dermis and proliferate to form a tumor mass. The vertical growth phase component of the lesion appears as a papule or nodule and the melanoma cells now have the capacity to metastasize. Importantly, prognosis correlates with tumor thickness that increases over time. Although melanoma is highly curable if identified during the radial growth phase, it is markedly resistant to therapy at more advanced stages; for example, in one study, whereas the 5-year survival rate for tumors <0.76 mm in thickness was 97.9%, it fell to 57.5% for those >3.6 mm.[36]

Because primary tumors in the vertical phase of growth are potentially extremely virulent, melanoma serves as an instructive model for metastatic behavior. In addition, it is an informative tumor because of its genetic, histological, and phenotypic heterogeneity[37,38] and its resistance to conventional anticancer therapy. Accordingly, melanoma is a potentially useful model system for the study of stem cell biology and carcinogenesis. Moreover, its cutaneous location makes it accessible and easily observable, and the developmental biology of melanocytes is relatively well understood. However, although the observed correlation between melanoma tumor thickness and prognosis has been well characterized, the cells responsible for tumor initiation, invasion, and metastases are only now beginning to be recognized.

Early evidence of a stem cell-like population in human melanoma came in 2005 from experiments with melanoma cells isolated from patient metastases as well as from estab-

lished cell lines.[39] A subpopulation of melanoma cells was defined experimentally by their ability to proliferate as nonadherent spheres when cultured in medium suitable for human embryonic stem cells (hESCs). These 'spheroid-forming' melanoma cells were enriched in the CD20+ surface marker, a hematopoietic phenotype normally associated with mature B lymphocytes. The cells were determined to be capable of self-renewal and differentiation into melanocytic, adipocytic, osteocytic, and chondrocytic lineages. These spheroid-forming melanoma cells also displayed increased tumorigenicity compared with adherent cells when xenografted into SCID mice, although the difference in tumor-forming capacity between nonadherent, spheroid-forming cells and non-spheroid-forming adherent cells was only modest. Nevertheless, the properties of self-renewal, differentiation plasticity, and tumorigenesis suggested that a subset of stem cells may exist in human melanoma.

Additional support for melanoma stem cells evolved 2 years later when the surface marker, CD133, a cancer stem cell marker previously applied to tumors of the brain[27] and colon,[28,29] was employed to isolate a subset of stem-like melanoma cells from patient biopsies.[40] Cells that expressed CD133 had enhanced tumorigenic potential upon transplantation to NOD/SCID mice, and thus were considered to potentially represent 'cancer stem/initiating cells'. In a long-term melanoma cell line, CD133 was highly expressed and CD133+ cells displayed increased tumorigenicity upon xenotransplantation. Moreover, CD133+ cells were able to differentiate into mesenchymal as well as neurogenic lineages, grow as spheres under serum-free culture conditions, and express angiogenic and lymphoangiogenic markers thought to contribute to virulence. The authors of the study did not examine whether CD133+ cells were capable of self-renewal, a defining quality of cancer stem cells, and differentiation plasticity was only shown for CD133+ cells from cell lines and not studied in patient biopsies. Of interest, the marker CD133 was highly coexpressed in the melanoma cell line studied with ABCG2, a member of the ATP-binding cassette (ABC) transporter family associated with dye efflux capabilities. A positive correlation between CD133 expression and melanoma progression was subsequently found in immunohistochemical staining of tissue microarrays.[41] In a supporting study, downregulation of CD133 *in vitro* and *in vivo* resulted in slower melanoma cell growth and decreased ability to metastasize,[42] further indicating a potential role for this biomarker in melanoma progression.

However, it is now clear that a seminal development in understanding the pathobiology of melanoma came earlier, in 2003, interestingly in a study concentrating on cell fusion.[43] At the time it was known that co-culture of pluripotent embryonic and mesenchymal stem cells with lineage-committed cell types produced hybrids as a result of cell fusion, and that these hybrids could generate differentiated progeny *in vitro* and *in vivo*.[44–48] Although such fusion events were thus considered to represent a model whereby progenitor cells express plasticity and renewal, the mechanism for this process was unknown. One candidate, however, was the ABC superfamily of active membrane transporters known to be expressed on stem/progenitor cells[49,50] and to mediate dye efflux capacity and multidrug resistance,[51–65] as well as influence membrane fluidity and potential.[66] Reasoning an ABC transporter that had been newly cloned, ABCB5 (ATP-binding cassette, subfamily B, member 5), might hold the key to understanding progenitor cell fusion; it was found that ABCB5 marks CD133+ progenitor skin melanocytes and also determines the propensity for fusion.[43] Furthermore, ABCB5 expression was detected in cells from an established human melanoma cell line. Thus, by defining a molecular mechanism for stem cell fusion that resulted in cell growth and differentiation, the stage was set for determining the potential relevance of this marker to human melanoma.

The investigators next considered the role of related members of the ABC transporter family in the phenomenon of melanoma multidrug resistance (MDR). Taking into account the drug resistance conferred by the ABC transporter proteins, along with the newly identified regulatory function of ABCB5 in progenitor melanocyte cell fusion and its expression in a melanoma cell line, it was speculated that ABCB5 may confer chemoresistance to primitive melanoma cells. Indeed, ABCB5 was identified as a drug transporter and chemoresistance mediator in human melanoma, as well as a molecular marker for a subpopulation of chemoresistant tumor cells with stem cell phenotypic markers among melanoma bulk populations.[67] Specifically, blockade of ABCB5 reversed resistance of melanoma cells to doxorubicin, an agent that is ineffective in clinical melanoma therapy, to enhance cytotoxic efficacy. Given the newly found expression of ABCB5 in melanoma cells with a progenitor, fusion-oriented phenotype, the task ahead was to further characterize ABCB5+ melanoma cells in functional assays.

A pivotal publication appeared in 2008 when ABCB5 was shown to be a functional biomarker of melanoma stem cells.[30] In contrast to previous[39,40] and some subsequent studies,[68,69] this report examined serial xenotransplantation of prospectively isolated subpopulations of melanoma cells, *in vivo* genetic lineage tracking, and proof-of-principle targeting of melanoma cells using a monoclonal antibody to ABCB5 to demonstrate the existence of ABCB5-expressing melanoma stem cells.[30] ABCB5+ melanoma cells, but not their ABCB5– counterparts, not only proved capable of tumorigenesis, but also of self-renewal and differentiation into a heterogeneous population when primary patient-derived tumor cells were serially transplanted into NOD/SCID mice. *In vivo* genetic lineage tracking was performed by stable transfection of the human melanoma cell line, G3361, with either red fluorescent protein (DsRed) or yellow-green fluorescent protein (EYFP) to generate two novel melanoma cell lines: G3361/DsRed and G3361/EYFP. Magnetic bead cell

sorting then isolated ABCB5+ cells from the G3361/DsRed cell line and ABCB5− cells from the G3361/EYFP cell line. Xenotransplantation of ABCB5$^+$/DsRed and ABCB5$^-$/EYFP cells to NOD/SCID mice revealed a tumor hierarchy in which ABCB5− cells displayed no differentiation capacity and gave rise only to ABCB5− cells, whereas ABCB5+ cells restored both subpopulations. This work suggested that two fundamentally different phenotypes of cells existed within the melanoma tumors. Although the relative proportions of these cells could potentially vary and the phenotypes might not be rigidly fixed *in vivo*, only one cell type was capable of self-renewal and differentiation. The aggregate findings were thus strongly supportive of the predictions of the cancer stem cell model.

One additional step was to define a potential clinical correlation between ABCB5 expression and melanoma progression. A positive correlation between ABCB5 immunoreactivity and melanoma clinical evolution was identified using tissue microarrays.[30] Specifically, there was higher expression of ABCB5 in primary melanoma than nevus, thick primary melanoma compared with thin primary melanoma, and lymph node metastases than primary melanoma, suggesting that ABCB5 may be a biomarker of melanoma progression. Subsequent studies have provided confirmatory evidence of a clinical correlation between ABCB5 expression and melanocytic tumor progression.[70,71] Whereas one study of patient melanomas described increasing expression of the ABCB5 protein with progression from nevi to primary melanoma to metastases,[70] another reported escalating expression of ABCB5 mRNA with more pathogenic melanomas.[67] Moreover, induction of terminal differentiation of melanoma cells has been shown to decrease expression of the protein ABCB5.[72]

If the melanoma stem cell model and related findings involving ABCB5 were valid, then ablation of this subpopulation would be anticipated to inhibit tumor development. Administration of anti-ABCB5 monoclonal antibodies into nude mouse melanoma xenografts impaired tumor initiation and growth and slowed progression of established tumors via antibody-dependent cell-mediated cytotoxicity directed against the ABCB5+ melanoma population.[30] Thus, specific targeting of this subpopulation inhibited tumor growth, providing important 'proof-of-principle' with translational therapeutic implications.

Corroborative support for the stem cell properties of ABCB5+ melanoma cells has also come from *in vitro* study of patient melanoma samples.[73] Melanoma cells were sorted by flow cytometry according to expression of multidrug-resistance gene product 1 (MDR1), which is coexpressed with ABCB5 and ABCC2. Limiting dilution assays of isolated MDR1+ and MDR− cells and serial replating revealed that the MDR1+ fraction contained more clonogenic cells than MDR− cells, were preferentially enriched with self-renewing cells, and demonstrated higher anchorage independence than MDR1− cells when grown on ultralow attachment plates. Thus, *in vitro* substantiation of the stem cell properties of melanoma cells expressing ABCB5 was established in a setting that was independent of the mouse microenvironment.[73]

FURTHER EVIDENCE OF MELANOMA STEM CELLS

Additional evidence that melanoma follows a cancer stem cell model has evolved since the identification of the melanoma stem cell biomarker, ABCB5.[74] Serial xenotransplantation of sorted melanoma cells into T-, B-, and natural killer (NK)-deficient Rag2−/−γc−/− (RG) mice further confirmed that not all melanoma cells are equally adept at tumor initiation. Melanoma cells from primary and metastatic tumors that expressed CD271 (nerve growth factor receptor), a surface marker of neural crest stem cells, displayed a markedly enhanced capacity for self-renewal and differentiation plasticity when compared with CD271− melanoma cells. When tumorigenicity was further examined using humanized mice with engrafted human skin or bone, CD271+ melanoma cells, but not CD271− cells, formed tumors. Finally, tumors formed by CD271+ cells had the ability to produce metastases, whereas no tumors or metastases were observed in matching mice injected with CD271− cells isolated from the same patient. This study suggests using highly relevant humanized local growth microenvironments that not all melanoma cells are equally tumorigenic; rather, as previous studies had suggested,[39,30] a distinct subpopulation of melanoma stem cells exists that are capable of self-renewal and differentiation plasticity. Interestingly, CD271 and ABCB5 were recently found to be preferentially coexpressed on the same tumor subpopulation in clinical human melanoma specimens.[75]

In another report, a rare subpopulation of melanoma cells identified by high aldehyde dehydrogenase (ALDH) activity displayed enhanced tumorigenicity and capacity for self-renewal over ALDH-negative cells when serially transplanted into NOD/SCID and IL-2R$\gamma^{-/-}$ NOD/SCID mice.[76] The existence of a stem cell-like population in melanoma was additionally fortified by a recent study of the effect of the ECM glycoprotein tenascin-C on melanoma progression; these data demonstrated that tenascin-C in the microenvironment encouraged manifestation of a stem cell-like phenotype in melanoma cells that included the expression of ABCB5.[77] Most recently, melanoma cells that expressed the receptor activator of NF-κB (RANK) demonstrated enhanced tumorigenicity compared with RANK-negative melanoma cells in IL-2R$\gamma^{-/-}$ NOD/SCID mice.[78] Moreover, RANK was coexpressed with ABCB5 and CD133 on melanoma cells, and preferentially expressed by peripheral circulating melanoma cells, primary melanomas, and metastases from stage IV melanomas compared with stage I melanoma patients.[78]

SKEPTICISM REGARDING MELANOMA STEM CELLS

Soon after the identification of ABCB5 as a melanoma stem cell marker, the existence of cancer stem cells in human

melanoma was questioned. Data were presented indicating that tumor-initiating cells in melanoma might not be as rare as had been previously believed and that non-stem cells may also be tumorigenic.[69] Whereas the original report observed only 1 in 10^6 human melanoma cells to be tumorigenic in NOD/SCID mice at 8 weeks post inoculation,[30] the subsequent study found that when unsegregated melanoma cells exposed to Matrigel were co-injected into interleukin-2 receptor γ chain-null ($Il2rg^{-/-}$) NOD/SCID (NSG) mice, an average of 1 in 9 human melanoma cells formed tumors at 8 weeks.[69] Moreover, in single-cell transplants, an average of 27% of unsorted human melanoma cells initiated tumors under these specific conditions.

Most recently, using similar experimental approaches, it has been reported that among multiple surface markers studied, including ABCB5 and CD271, no marker distinguished tumorigenic from nontumorigenic melanoma cells, and surface markers appeared to be reversibly expressed by tumorigenic melanoma cells.[68] Furthermore, a relatively high proportion of melanoma cells were tumorigenic (28%) and all tumorigenic melanoma cells appeared to be capable of 'unlimited proliferation'.[68] If cancer stem cells are defined to be a distinct, fixed, hierarchical subpopulation of rare cells, then such data were interpreted as shedding doubt on the assertion that melanoma conforms to the classical cancer stem cell model.[79–81]

WHY DO MELANOMA STEM CELL STUDIES SEEM TO DISAGREE?

The dissonance regarding melanoma adherence to the cancer stem cell model may be in large part attributable to disparate interpretations of the model itself, as well as variation in the methodology used to study melanoma stem cells. Although a consensus definition of cancer stem cells has been promulgated,[14] multiple additional definitional interpretations persist regarding this important cell type. In the field of melanoma, there are at least four fundamental points of disagreement among researchers that may account for much of the controversy regarding the existence of melanoma stem cells. These relate to perceptions that stem cells are rare, reflective of growth rate, defined solely by experimental tumorigenicity, and phenotypically immutable, and are embedded in the following queries:

Must All Cancer Stem Cells Be Rare?

The notion that rarity is a required feature of melanoma stem cells may be traced to descriptions of rarity in other types of stem cells, such as the prototypical hematopoietic stem cell.[13] It follows logically that if one type of stem cell is rare, then another type may be rare as well. However, rarity is not included in the AACR consensus definition of cancer stem cell.

Although it is true that in certain malignancies, such as particular forms of leukemias,[24] stem cells are indeed extraordinarily rare, the identification of higher numbers of stem cells in certain other neoplasms *ex vivo* does not negate the existence or the application of the stem cell model.[19,82] This is because the proportion of stem cells to non-stem cells is a complex function of cell kinetics, self-renewal, differentiation, and cell death within a given tumor; it is not defined solely by the presence or absence of a hierarchical population of stem-like cancer cells.[68,69] Moreover, the tumor microenvironment and host immune system in which the cells are studied may affect the frequency of cancer stem cells.[19,82] As discussed by Dr Robert A Weinberg, a leading scholar in cancer biology, several aspects of host biology, including vascularization at sites of implantation, extracellular matrix (ECM) constitution, growth factor availability, and host immunocompetence, influence cancer cell engraftment rate.[19] For instance, xenotransplantation into mice may underestimate the frequency of tumorigenic cells because the foreign extracellular microenvironment differs significantly from that of humans and may interfere with essential interactions of tumor cells with support cells.[83] Conversely, an increased frequency of tumorigenic cells was observed when melanoma cells were co-injected with a solution containing growth factors (Matrigel) compared with co-injection with vehicle in identical serial xenotransplant models.[69] Similarly, when tumorigenicity of melanoma cells was compared in more *versus* less immunocompromised mice under otherwise identical experimental conditions, an increased frequency of melanoma-initiating cells was observed in the most profoundly immunocompromised mice,[69] suggesting that the number of cells in a tumor that become tumorigenic is dependent, in part, upon the immune status of the host.[83,84] Thus, the frequency of cancer stem cells in a tumor is not necessarily an inherent characteristic of cancer stem cells. Rather, it is dependent upon the kinetics of the non-cancer stem cell component of the tumor as well as the tumor microenvironment and host immune system. In the case of melanoma, it must always be remembered that the only authentically relevant microenvironment for melanoma stem cells is that of the living patient.

Must Rate of Tumor Growth Correlate with Cancer Stem Cell Number?

It has been suggested that the cancer stem cell model implies that aggressively growing tumors contain higher frequency of cancer stem cells.[68] Although this statement may at first seem logical, it does not withstand critical scrutiny because tumor growth is only partially dependent on the frequency of cancer stem cells. Rather, cancer growth rate involves complex interplay between stem cells, host/microenvironmental factors, and non-stem cell kinetics, the latter involving factors such as the rates of differentiation and apoptosis. The survival characteristics of the non-stem cell component of a cancer may thus profoundly affect tumor growth rates without any specific correlation with stem cell frequency.

Although it is true that certain markers for cancer stem cells are more robustly expressed with clinical tumor progression,[30,70,71] this is not tantamount to equating stem cell number and tumor growth rate.

Is Tumorigenicity Tantamount to Self-Renewal?

The capacity for self-renewal is the fundamental property of a cancer stem cell.[14] It is generally accepted that the 'gold standard' assay for the identification of cancer stem cells is serial transplantation into immunodeficient murine models that are analyzed at various time points for tumor formation.[14] The exclusive use of tumorigenicity assays to identify cells capable of self-renewal has led to some confusion in semantics, as terms such as 'tumorigenic' and 'tumor-initiating cell' have been used to describe putative cancer stem cells.[14] Although it is true that self-renewing cancer cells inexorably replicate in a manner ultimately to produce tumors in the form of clinically observed nodules, not all clinical tumors develop from stem cells with the capacity for self-renewal. In benign moles (melanocytic nevi), for example, tumors form and then stabilize in a manner that implies limited renewal potential. Thus, although tumors should be expected to form as a result of a subpopulation of cells with self-renewal capabilities (hence the use of tumorigenicity as one measure of 'stemness'), not all tumors should be expected to result from such cells.

In naturally occurring human melanoma, mutated melanocytes form tumors in potentially hostile epidermal and dermal environments that often involve robust host immune responses. These lesions often evolve to clinically detectable nodules only after many months to several years.[85] In the subcutaneous layers of highly immunocompromised mouse models, such as the NSG mouse, in contrast, substantial tumors may evolve from patient-derived malignant cells within less than a month. Does production in such an experimental animal of a tumor derived from a single melanoma cell devoid of stem cell biomarkers refute the cancer stem cell model?[68] One needs to only consider the hypothetical fate of a single non-stem cancer cell (one not capable of self-renewal and thus with limited lifespan) that divides but once daily in the non-physiologically permissive subcutis of the NSG animal. Such a single cell with a theoretical lifespan limited to 1 month needs to divide only once daily in order to exceed one billion (10^9) cells, the number commonly equated with the formation of a 1 cm tumor! It becomes clear that a tumorigenicity assay over a limited period of time and in the context of potentially permissive and stimulatory experimental conditions cannot stand alone as a barometer for whether or not melanoma adheres to the cancer stem cell model. Thus, experimental tumorigenicity is best interpreted in combination with additional assays such as *in vivo* lineage tracking in order to validate its utility in defining self-renewing melanoma stem cells.

Is Cancer Stem Cell Plasticity Incompatible with a Cancer Stem Cell Hierarchy?

There exists at least two forms of cancer stem cell plasticity. The first is *differentiation* plasticity (discussed later), whereby primitive stem cells may express genes that induce differentiation characteristics that theoretically may confer a selective growth or invasion advantage (eg, plasticity along endothelial[37,86] or mesenchymal lines[87–89]). The second involves plasticity with regard to expression of stem cell biomarkers themselves. Does the possibility of a non-stem cell, perhaps as a function of microenvironment or epigenetic factors, acquiring the phenotypic and functional characteristics of a stem cell (or vice versa) invalidate the cancer stem cell model? Certainly, if the majority of cells expressing stem and non-stem cell biomarkers are in a constant state of flux, it is difficult to envision a hierarchy of stemness, and at least one recent study advances this opinion.[68] Cancer stem cell phenotype and function, however, are not likely to be independent of discrete, regulatory stem cell niches.[90] A tumor in which fixed microenvironmental niches dynamically regulate phenotype and behavior of a minority component of stem cells does not preclude that at any given point in time, the composite tumor subpopulations do not adhere to metrics that define a reproducible hierarchy. Based on recent studies in breast cancer,[91–93] the cancer biologist, Dr Robert A Weinberg has acknowledged that non-cancer stem cells may be able to differentiate into cancer stem cells in response to certain contextual signals from the microenvironment.[19] Admittedly, such 'bidirectional interconvertibility' between cancer stem cells and non-stem cells is at variance with a portrayal of stem cells, in which they may give rise to non-stem cells, but not the reverse.[19] Yet, Weinberg avers that plasticity within cancer populations does not undermine the cancer stem cell model, as 'cancer stem cells and non-cancer stem cells retain their distinct identities in the sense that they can be distinguished phenotypically and functionally at any moment in a cancer cell population'.[19] Indeed, a recent report demonstrates that stem-like melanoma cells can switch phenotypes through epigenetic changes,[94] suggesting 'stem-like cells may be more plastic than previously thought'.[90]

Although this is not an exhaustive list of the disparate interpretations of the cancer stem cell model, it highlights some of the potential misconceptions that could easily influence conclusions. As alluded to above, variations in methodologies may influence actual experimental results. These variations with reference to melanoma stem cell biology may be clustered into at least four main categories: (1) immune status of animal models, (2) co-injection of growth factors and stimulants, (3) length of time to follow-up measurement of tumor formation, and (4) stage of patient melanoma from which cells were obtained for study. As already indicated, although the traditional assay used for the identification of cancer stem cells is serial xenotransplantation into NOD/SCID mice,[14] several groups have begun using

more highly immunocompromised mice, such as NSG[68,69] and RG[74] mice that lack NK cell activity. Some studies have also co-injected tumor cells with Matrigel,[68,69] a solution containing melanoma growth factors and stimulants,[95] whereas earlier reports did not.[30] A careful comparison of methodologies used in different studies could be the basis for apparently disparate results.[19] Clearly, if melanoma stem cells are derived from more advanced tumors in which their proliferation is more robust, if they are exposed to potential mitogenic stimulants such as laminin[96] contained within Matrigel, and if they are grown as xenografts in animals permissive to stem cell dominance, the stage has been set for cancer stem cells to appear to be more abundant. Perhaps most significant, however, is the use of profoundly immuncompromised animal bearers of xenografted tumors, an experimental condition seemingly disconnected from the physiological setting where melanoma, arguably one of the most immune-provoking neoplasms, develops in the setting of an immunologically normal host. Importantly, even in studies where use of more profoundly immunocompromised animals resulted in data interpreted to invalidate the cancer stem cell model for melanoma due to lack of stem cell scarcity, when conditions identical to previous studies were used, the same low frequency of tumorigenic melanoma cells were detected (1 in 10^6 (Schatton *et al*[30]) *vs* 1 in 837 000 (Quintana *et al*[69])).

Of course, the limit to all experimental models is that they do not allow for precise replication of the human stromal and immune microenvironment, and immunosuppressed animals are critical for *in vivo* modeling approaches. However, because no single assay in itself provides conclusive evidence of stemness, studies that utilize multiple corroborative experimental approaches to the melanoma stem cell issue may be most informative. One cancer stem cell pioneer, Peter Dirks, is responsible for identifying rare cancer stem cells in brain tumors[27] that, like melanoma, share a common neural crest lineage. In a recent commentary,[34] Dirks offers two fundamentally important insights regarding the current controversies that surround melanoma stem cells. He begins by stating: 'The question is whether a distinct subpopulation of the cells drives tumour growth and generates cellular variation. To answer this, the data must be interpreted carefully'. And he concludes with: 'Each study must be carefully assessed, particularly when considering the experimental methodology and models used. One point that should always be borne in mind is the relevance of a study to tumour growth in patients'. Mindful of these key tenets, we are better equipped to decipher the rapidly proliferating database regarding melanoma stem cells in the interest of determining how this information may be put to translational utility.

Ultimately, disagreements among melanoma researchers are positive for the field in that they stimulate debate and scrutiny of results. More work is needed toward unifying conceptions of the cancer stem cell model, particularly as it pertains to melanoma, and standardizing the methodology used by different groups to study melanoma cells. It is important to recognize that it is not the raw data presented in conflicting studies that are problematic, but the incongruities in the experimental designs used to produce such data, and the divergent interpretations of such data based on pre-existing conceptions of the cancer stem cell model.

The current controversy over the application of the cancer stem cell model to melanoma is more than just an academic debate. If melanoma follows a cancer stem cell model, the translational implication is that therapy must be designed to include this subpopulation, as well as the majority of non-stem cells, within the tumor.[19] Even though melanoma stem cells may ultimately prove in humans to be more common than in other cancers (eg, as abundant as 1 in 4 tumor cells[69]) or capable of occasional morphing between stem cell and non-stem cell phenotypes (eg, as a function of microenvironment), the ability to therapeutically and lethally target the only self-renewing tumor component could be of key translational importance. This is because removal of the most aggressive melanoma cells, regardless of their numbers or ability to change phenotype, would be of considerably more benefit than targeting solely the majority population of non-stem cell tumor cells predestined to undergo eventual differentiation and programmed cell death (as will be discussed below, melanoma stem cells may be impervious to certain therapies, and thus the therapeutic benefit of eliminating non-stem cells tends to be short lived). Therapeutic elimination of these cells at the hierarchical nadir of clinical virulence, regardless of some degree of ongoing physiologic plasticity in the human host, may well be a highly effective manner to ablate cancer stem cells in afflicted patients.

BEYOND SELF-RENEWAL: FURTHER ATTRIBUTES OF MELANOMA STEM CELLS

To this point we have discussed the different types of stem cells, highlighted the fundamental properties of cancer stem cells, and compared the cancer stem cell model with the stochastic model of cancer development. We have also discussed markers of melanoma stem cells, and reviewed recent controversy regarding the existence of melanoma stem cells. What remains to be explored are the heterogeneous, adaptive properties that have been attributed to melanoma stem cells and may contribute to their survival in hosts. Based on the AACR consensus definition of cancer stem cells, we have defined melanoma stem cells on two key tenets—self-renewal and differentiation plasticity. However, melanoma stem cells may also possess several additional virulence factors. These are not primary, defining properties of cancer stem cells, but secondary features of melanoma stem cells that may help them survive in the host environment. These additional properties, which are likely to be expressed by certain subsets of melanoma stem cells, reflect the dynamic relationship that exists between melanoma stem cells and their microenvironment. Accumulating evidence suggests that primitive

melanoma cells may confer virulence via several mechanisms, including immune evasion, MDR, so-called 'vasculogenic mimicry', and metastasis.

How Melanoma Stem Cells May Evade and Modulate Host Immunity

For years, clinicians have recognized that immunocompromised patients have a heightened risk of developing cancer.[97] More recently, a negative correlation has been observed between host immunocompetence and tumor initiation in models of human malignant melanoma, as fewer melanoma cells were required to initiate tumor growth in more severely immunocompromised recipients.[69] When immunogenic cancers like melanoma are studied in immunocompromised hosts, such as IL-2R$\gamma^{-/-}$ NOD/SCID mice, the frequency of tumor-initiating cells may be overestimated because aspects of antitumor immunity have been eliminated in the host.[98] Although a relatively large number of melanoma cells are capable of initiating tumors in severely immunodeficient mice, a limited number of melanoma cells are able to evade host antitumor immunity and initiate melanoma in IL-2Rγ^{WT} NOD/SCID mice with relatively intact immunity. This observation suggests that a subpopulation of melanoma stem cells may exist that is capable of immune evasion and modulation.[99]

Several of the mechanisms by which melanoma stem cells may evade antitumor immunity have recently been identified. One method of immune evasion may be decreased expression of melanoma-associated antigens. MART-1 (melanoma antigen recognized by T cells-1) is one such antigen that is associated with melanocyte differentiation and recognized by T cells. In fact, T cells specific for and reactive against tumor antigens, such as MART-1, have been identified in melanoma patients.[100,101] By downregulating tumor-associated antigens such as MART-1, tumors may escape anticancer immune responses.[102] Melanoma cells that express ABCB5+, a marker of melanoma stem cells,[30] have been found to have decreased expression of MART-1 in cell lines[98] and patient samples,[103] as well decreased expression of additional tumor-associated antigens, including ML-IAP, NY-ESO-1, and MAGE-A.[103] Similarly, CD271+ melanoma cells either lacked or had deceased expression of TYR, MART, and MAGE (C1-C2) in patient samples.[74] Through downregulation of these antigens, melanoma stem cells may evade antitumor immune response directed at tumor antigens associated with a more differentiated malignant phenotype, thus achieving a state of immune privilege via masking their antigenic identity.[98] This could explain the relative ineffectiveness of CD8 tumor-reactive T cells in achieving antitumor response.[100]

Another mechanism by which melanoma stem cells may evade the immune system is via altered expression of major histocompatibility complex (MHC) class I molecules. Normally found on all nucleated cells, MHC class I molecules present antigenic peptides to CD8+ T lymphocytes to activate the adaptive immune response. ABCB5+ melanoma cells have significantly reduced expression of MHC class I molecules, and some ABCB5+ melanoma cells completely lack these molecules.[103] Without MHC class I expression, the adaptive immune system cannot recognize melanoma cells and antitumor immunity is thwarted. Indeed, reduced MHC class I expression has been described as one of the principal mechanisms used by tumor cells to evade host antitumor immunity.[102,104] Clinically, decreased MHC class I expression is associated with melanoma progression, therapeutic failure, and poor clinical outcome.[105–107]

Interestingly, although suppression of MHC class I expression by melanoma stem cells may enable evasion from CD8+ T lymphocytes of the adaptive immune response, low MHC class I expression would be predicted to increase their susceptibility to NK cells of the innate immune system, which are normally inhibited by MHC class I molecules. However, it is conceivable that melanoma stem cells may escape NK cells by altering their expression of additional surface molecules, such as NK cell ligands, which have activating or inhibitory effects on NK cells. Indeed, studies in colon carcinoma and glioblastoma have shown that cancer stem cells display decreased expression of the activating NKG2D (NK group 2, member D) ligands, MICA and MICB (MHC class I-related proteins A and B), and ULBP 1–4 (UL 16-binding proteins 1–4).[108,109] Additionally, melanoma stem cells may potentially evade NK cells by affecting the expression of activating or inhibitory receptors on the surface of NK cells themselves. For instance, mesenchymal stem cells are capable of downregulating the expression of activating surface receptors on NK cells.[110] To our knowledge, interactions between melanoma stem cells and NK cells have not been studied, but investigation into this important facet of antitumor immunity is warranted and will be necessary to fully appreciate how melanoma cells survive in the face of both innate and adaptive immune responses.

In addition to reduced expression of melanoma-associated antigens and MHC class I molecules, ABCB5+ melanoma stem cells may resist immune-mediated rejection by inducing tolerance of melanoma-specific T cells. The ligand, B7.2, and the ligand receptor, PD-1, can both serve as negative costimulatory molecules of the CD28/B7 superfamily that downregulate immune responses by inducing T-cell anergy and activating regulatory T cells (Treg cells).[111,112] Both B7.2 and PD-1 are overexpressed on ABCB5+ melanoma cells in established xenografts and clinical tumor specimens,[103] suggesting that melanoma stem cells may evade antitumor immunity via downregulation of tumor-specific T cells by negative costimulatory molecules. Indeed, B7.2 expressed by ABCB5+ tumor subsets induces CD4+CD25+FoxP3+ Treg cells and regulates their secretion of the immunosuppressive cytokine, IL-10.[103,113]

Finally, melanoma stem cells may evade antitumor immunity by inhibition of IL-2 production, a critical cytokine for early T-cell activation and proliferation. In immune

activation assays, melanoma stem cells inhibited human peripheral blood mononuclear cell proliferation and IL-2 production more efficiently than ABCB5– melanoma cell populations.[103] The preferential inhibition of IL-2 production by ABCB5+ melanoma cells may explain observed differences in frequency of tumorigenic melanoma cells in IL-2R$\gamma^{-/-}$ compared with IL-2Rγ^{WT} NOD/SCID murine hosts.[30,69,103] More specifically, tumorigenicity assays conducted in the absence of IL-2 signaling may overestimate the frequency of tumor-initiating cells because the host environment has impaired antitumor immunity and may therefore permit tumor bulk populations, which do not normally initiate tumors in immunocompetent hosts, to generate tumors.[98] Indeed, if one of the methods by which melanoma stem cells distinguish themselves to evade antitumor immunity is inhibition of IL-2 production, xenotransplantation into a murine model that is IL-2 receptor null is not an appropriate environment in which to determine frequency of tumor-initiating cells in melanoma because host immunity is abnormally impaired.[98] In sum, there are a variety of mechanisms by which melanoma stem cells may evade the host immune system to promote tumor growth, including, but not limited to, downregulation of melanoma-associated antigens, decreased expression of MCH I molecules, induction of tolerance in T cells, and inhibition of IL-2 production. Thus, the immunoevasive and immunomodulatory functions of melanoma cells should be a consideration in the design of tumorigenicity experiments so as to improve the quality and applicability of results.[98]

How Melanoma Stem Cells May Thwart Anti-Cancer Drug Therapy

Another virulence-conferring feature that has been attributed to melanoma stem cells is their MDR. The term MDR describes the impairment of cancer chemotherapeutic efficacy by either intrinsic or acquired tumor resistance to multiple, structurally unrelated therapeutic drugs with different mechanisms of action.[114] MDR can result from several distinct mechanisms, including alterations of tumor cell cycle checkpoints, impairment of tumor apoptotic pathways, repair of damaged cellular targets, and reduced drug accumulation in tumor cells.[114] One mechanism of particular interest in melanoma is decreased intracellular drug accumulation, which is accomplished by energy-dependent efflux pumps, known as ABC transporters that translocate solutes across the cellular membranes.[115] The ABCB5 transporter and melanoma stem cell marker mediates melanoma resistance to the chemotherapeutic agent doxorubicin,[67,116,117] and is also a molecular marker of melanoma stem cells.[30] Blockade of ABCB5 significantly enhances intracellular drug accumulation and reverses resistance of melanoma cells to doxorubicin.[67] Another ABC transporter, MDR1, is also preferentially expressed in human melanoma cells with stem cell properties.[73] The expression of ABC transporter proteins in melanoma cells capable of self-renewal and differentiation identifies MDR as another potential mechanism by which melanoma stem cells may promote tumor growth.

How Primitive Melanoma Cells May Encourage 'Vasculogenic Mimicry'

In addition to immune evasion and modulation and MDR, primitive melanoma cells may promote tumor growth via vasculogenic mimicry (VM), a phenomenon first described by Hendrix in 1999 as the ability of aggressive melanoma cells to form ECM-rich, extravascular, patterned networks in three-dimensional cultures.[118] Histologically, VM appears as multiple, laminin-rich networks that are periodic acid-Schiff (PAS) positive and surround clusters of tumor cells.[119] The patterned channels formed in VM are not lined by true, CD31-positive endothelial cells, as in the case of conventional tumor angiogenesis. Rather, they are associated with tumor cells expressing CD144, also known as vascular-endothelial (VE) cadherin. The tubular structures formed in VM resemble primary sinusoidal networks formed during embryonic development. These endothelial-independent networks in tumors are hypothesized to serve as sites of nutritional exchange and dissemination routes for metastasis, which may be independent of or function in concert with angiogenesis.[37] Although the specific functions that underlie VM remain unclear and at times controversial, VM is clearly associated with tumor aggressiveness, poor clinical outcome, and high risk of recurrence.[118,120,121] Furthermore, conventional anti-angiogenic therapies, such as endostatin, have been ineffective at inhibiting VM.[122]

The melanoma cells that form the patterned vascular channels characteristic of VM are highly invasive, multipotent cells that express primitive genes.[118] These cells express genes associated with multiple cell lineages, including those of endothelial, epithelial, pericyte, fibroblastic, hematopoietic, kidney, neuronal, muscle, and several other cell types,[123,124] suggesting that they are capable of differentiating into a variety of cellular phenotypes. Furthermore, molecular analyses have revealed that these aggressive tumor cells express several genes associated with embryonic stem cells. One such gene is *Nodal*, a potent embryonic morphogen from the transforming growth factor (TGF)-β family. Nodal has been shown to maintain a stem cell-like phenotype in melanoma cells,[38] and may prove to be an important marker of melanoma stem cells. Interestingly, Nodal is required for VM and tumor growth,[38] and Nodal-expressing melanoma cells are spatially associated with formation of the channel-like networks that characterize VM.[125] These findings suggest a key role for primitive, stem cell-like melanoma cells in the genesis of VM. Inhibition of Nodal signaling impairs the ability of aggressive melanoma cells to form vasculogenic networks on a three-dimensional collagen network, reduces melanoma cell invasiveness, and promotes transition of melanoma cells toward a more differentiated, melanocytic phenotype.[38] Clinically, Nodal is positively correlated with melanoma tumor progression.[38]

Whereas stem cell-like genes, such as *Nodal*, are increasingly expressed in aggressive melanoma cells that encourage VM, melanocytic-specific markers are downregulated. Genes specific to their parental melanocytic lineage, such as the melanin biosynthesis-pathway related genes *melan-A*, *microphthalmia-associated transcription factor* (*MITF*), and *tyrosinase*, *tyrosinase-related protein 1* (*TYRP1*), are reduced by 22-fold, 34-fold, and 37-fold, and over 100-fold, respectively, in aggressive melanomas compared with their poorly aggressive counterparts.[86] Furthermore, reduced tyrosinase levels have been correlated with poor clinical outcome.[126] The reduction in melanocyte-specific genes reflects the de-differentiation of more aggressive melanoma cells.

Based on their gene and protein expression profile, aggressive melanoma cells involved in VM resemble undifferentiated, primitive, embryonic-like stem cells,[37] suggesting that melanoma stem cells may give rise to the patterned networks that typify VM. Indeed, ABCB5+ melanoma cells have demonstrated preferential expression of the vasculogenic differentiation markers tyrosine kinase with Ig-like and EGF-like domains 1 (TIE-1), CD144, and bone morphogenetic protein receptor type 1A (BMPR1A),[30] but do not express CD31, identifying them as distinct from mature tumor vessels. Furthermore, ABCB5+ melanoma cells were recently found to be spatially associated with laminin-positive, patterned channel-like networks that are characteristic of VM in clinical and experimental human melanomas. Moreover, such cells preferentially express vascular endothelial growth factor receptor-1 (VEGFR-1), which was shown via knockdown experimentation to be required for laminin production, VM, and efficient tumor growth *in vivo*.[75]

The current model of signaling pathways involved in VM highlights the dynamic interaction between melanoma cells and their microenvironment. Primitive, aggressive melanoma cells that encourage VM upregulate several endothelial-associated genes, including VE-cadherin (CD144), the receptor protein tyrosine kinase erythropoietin-producing hepatocellular carcinoma-A2 (EphA2), and laminin 5 γ2-chain, a major component of the basement membranes.[119,127,128] These proteins are expressed only by aggressive melanoma cells, not poorly aggressive or nonaggressive melanoma cells, and downregulation of VE-cadherin, EphA2, or laminin 5γ2 results in inability of melanoma cells to form vasculogenic-like networks in three-dimensional cultures.[119,127,128] One model of the signaling pathway in VM suggests that VE-cadherin and EPHA2, which are colocalized at the cell membrane, activate signal transduction pathways whose downstream effect is the cleavage of the laminin 5γ2 chain by matrix metalloproteinases (MMPs).[86] The resulting cleavage fragments then induce poorly aggressive melanoma cells to express vascular-associated genes, assume a more migratory phenotype, and form vasculogenic-like networks.[86,119] Thus, a cycle may exist by which aggressive melanoma cells expressing endothelial-type genes promote molecular changes in the nearby matrix that, in turn, stimulates additional melanoma cells to express endothelial-type genes and participate in VM.

Epithelial-Mesenchymal-Transition (EMT) and Metastasis: Is There a Role for Primitive Melanoma Cells?

The phenomenon of VM highlights the plasticity of certain primitive subpopulations of melanoma cells. In VM, aggressive melanoma cells transdifferentiate from a multipotent, stem-like phenotype into a more endothelial phenotype that forms vasculogenic-like networks.[86] Another example of the plasticity of melanoma cells may be seen during metastasis of melanoma, which involves dissemination of cells to distant sites via lymph or blood vessels, followed by extravasation, and survival in a new microenvironment. According to the cancer stem cell model, it is the melanoma stem cells that travel to new sites during metastasis that will have clinical significance, as only these cells are capable of self-renewal and differentiation into the heterogeneous phenotypes that comprise the tumor. Indeed, melanoma circulating tumor cells have recently been shown to contain an ABCB5+ subpopulation, and the presence of circulating ABCB5+ tumor cells was predictive of metastatic progression in tumor xenotransplantation models.[129]

Migration and metastasis of melanoma cells may mimic a physiologic process, known as EMT, in which polarized, immotile epithelial cells lose cell–cell and cell–matrix connections and acquire the motile, migratory properties of mesenchymal cells. In states of malignancy, EMT confers metastatic and invasive properties to carcinomas,[130] and some studies have suggested that it is a major determinant of disease progression and metastasis in melanoma.[87–89] At a molecular level, EMT involves changes in cell adhesion and cytoskeletal proteins. Environmental influences, such as the fibroblast-secreted growth factor TGF-β,[131,132] encourage downregulation of the cell adhesion protein, E-cadherin, and upregulation of N-cadherin.[133] Cytoskeletal proteins are also rearranged during EMT;[134] for example, in certain epithelial cancers a characteristic increase in vimentin expression and loss of cytokeratins is observed.[135] An intermediate filament in melanoma that may be of significance in EMT is nestin, a biomarker of multilineage progenitor cells that is expressed by migrating and proliferating neural crest stem cells during embryogenesis.[136] Nestin expression is associated with cell migration and metastasis in prostate cancer,[137] and tumor progression and deceased survival in melanoma.[41,138–142] Nestin and SOX2,[143] an embryologic stem cell transcription factor that binds an enhancer region on the nestin gene,[144] are preferentially coexpressed in metastatic melanomas when compared with nevi or primary melanomas.[145] Moreover, SOX2-positive melanoma cells tend to be more spindle-shaped cells and to have a more peripheral nestin pattern, which may represent a motile, more mesenchymal phenotype.[145] Such preliminary observations suggest a

possible role for nestin and SOX2 in melanoma metastasis and EMT.

Thus, although VM involves transdifferentiation of primitive melanoma stem cells to endothelial-like cells, epithelial-like cells may transform into a spindle-like, mesenchymal phenotype in EMT. Interestingly, like VM, EMT occurs during embryogenesis, involves cell–matrix interactions, and is associated with disease progression. Given their plasticity, there is likely a link between primitive melanoma cells and EMT, although the precise relationship is still under investigation. Breast cancer trials recently demonstrated that EMT enriches the cancer stem cell population.[91,92] However, whether this is the result of de-differentiation of mature cells, or accelerated division and increased proportion of self-renewing division in pre-existing cancer stem cells, remains to be elucidated.[146]

To summarize, primitive melanoma stem cells may confer virulence via a variety of mechanisms, including immune evasion and modulation, MDR, and VM (Figure 3). They also may play a role in EMT and melanoma metastasis. Taken together, these observations suggest that although all melanoma stem cells share key features such as self-renewal and differentiation capacity, this group of cells may be a heterogeneous subpopulation with a variety of survival-promoting mechanisms. The microenvironment in which melanoma stem cells reside may influence their additional, survival-promoting properties. For example, signals from the ECM may induce melanoma cells to express endothelial-type or mesenchymal genes, depending on whether the molecular signal is laminin fragments or TGF-β, respectively. We speculate that there is plasticity among the different phenotypes of melanoma stem cells, and identifying factors that confer switches in phenotype will be an important area of future research.

TRANSLATIONAL RELEVANCE OF MELANOMA STEM CELLS

Melanoma is an exceedingly difficult cancer to treat once it has entered advanced stages of growth because of its resistance to conventional therapies. The cancer stem cell model may help explain the failure of conventional anticancer therapeutics, as these agents are primarily aimed at bulk tumor population, which have different properties than the cancer stem cell subpopulation. It has been hypothesized that cancer stem cells represent a pool of resistant cells in cancer patients.[99] In melanoma, cancer stem cells have been described as chemoresistant[30,43] and immunoevasive,[103] and have been associated with metastases[147] and the

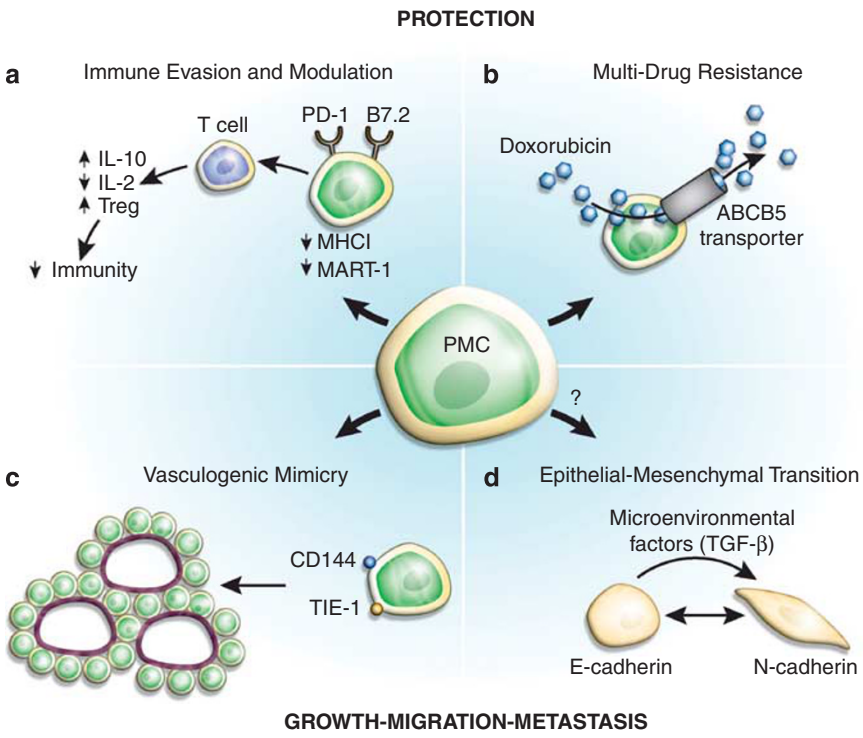

Figure 3 Virulence-conferring mechanisms of primitive melanoma cells (PMCs). (**a**) Melanoma stem cells may evade and modulate host immunity by stealth (downregulation of MHCI and MART-1), thus masking their antigenicity, and immune modulation (expression of negative costimulatory molecules B7.2 and PD-1), thus encouraging regulatory T (Treg) cells and IL-10 secretion, and inhibiting T-cell activation and IL-2 secretion. (**b**) Melanoma stem cells are chemoresistant, and use the multidrug resistance efflux pump, ABCB5, to transport agents (eg, doxorubicin) out of cells. (**c**) PMCs expressing endothelial-type genes *CD144* and *TIE-1* contribute to the phenomenon 'vasculogenic mimicry' by inducing the formation of laminin-associated networks. (**d**) Speculative role of primitive melanoma cells in EMT; stromal microenvironmental mediators such as TGF-β may preferentially regulate stem cell plasticity that governs a more migratory mesenchymal phenotype conducive to invasion and metastasis.

virulence-associated phenomenon of VM.[37,38,118,123] These protective properties for the melanoma stem cell translate into tumor aggressiveness directed against the patient. It follows that therapeutic targeting of the survival mechanisms employed by primitive melanoma cells may increase the efficacy of anticancer therapies and decrease the risk of relapse and progression. In a twist of irony, targeting the very protective mechanisms that have permitted melanoma cells to evade detection and destruction may prove to be the most efficacious way to ultimately thwart this deadly disease.

There are several possible approaches to targeting melanoma stem cells. These include tagging anti-stem cell bioprobes with cell toxins, negating stem cell-specific mediators of chemoresistance, inhibiting self-renewal and stimulating differentiation, ameliorating stem cell strategies for immune evasion, and abrogating plasticity pathways that may promote tumor growth (eg, VM).[99] One key molecular marker to target is ABCB5. Importantly, ABCB5 is not only a biomarker of melanoma stem cells, but also provides a mechanism for chemoresistance. Several potential therapies against ABCB5 have been explored, including monoclonal antibodies[30] and short hairpin (sh)RNA-mediated knockdown.[116] As previously discussed, selective killing of ABCB5-expressing melanoma stem cells via antibody-dependent cell-mediated cytotoxicity inhibited experimental tumor growth.[30] Moreover, both anti-ABCB5 antibody[67] and siRNA gene silencing[116] reversed melanoma resistance to the chemotherapeutic agent doxorubicin, and gene silencing increased the sensitivity of melanoma cells to the chemotherapeutics 5-fluorouracil and camptothecin.[117] Finally, shRNA-mediated knockdown of CD133, which is expressed on a subset of ABCB5+ melanoma stem cells,[67] decreased growth rate and impaired metastatic capacity of melanoma cells.[42]

In addition to targeting molecular markers and reversal of resistance mechanisms, another potential strategy to eradicate melanoma stem cells is the induction of a more differentiated phenotype. It has been recognized for several decades that differentiation of primitive cells within a malignancy may result in tumor deterioration.[148] Differentiation therapy has proved efficacious in human glioblastoma where targeting of morphogen-driven signaling pathways by bone morphogenetic protein BMP4 inhibited tumor growth.[149] BMP4-dependent differentiation strategies may also be an effective method of targeting melanoma, as BMPR1A is preferentially expressed on ABCB5+ melanoma stem cells.[30] Another potential target in melanoma is the embryonic morphogen Nodal given its role in maintenance of a stem cell-like phenotype in melanoma cells.[38] Interestingly, both BMP4 and Nodal are also potential targets in anti-VM therapy (see below), as downregulation of both proteins impaired VM and reduced expression of genes involved in VM such as VE-cadherin.[38,150] In addition to morphogen-driven signaling pathways, melanoma stem cells may be induced to differentiate by altering gene expression profiles using small non-coding microRNA[151] or epigenetic differentiation therapy via inhibition of DNA methyltransferases and/or histone deacetylases,[152] both of which have been shown to induce differentiation of cancer stem cells in breast cancer.

More indirect therapies aimed at VM and immune evasion and modulation are also promising strategies against melanoma, as both have been described as survival-promoting mechanisms employed by primitive melanoma cells. As melanoma immunotherapy is a robust area of current research, we will narrow our discussion to immunotherapeutic strategies as they relate to immune evasion and modulation by primitive melanoma cells. Immunotherapeutic strategies of relevance to this discussion include the Food and Drug Administration-approved use of the immunomodulatory cytokines IL-2 and IFNα-2b, as well as newer therapeutic developments, such as tumor vaccinations and specific targeting of the molecules that induce immune tolerance to melanoma. The efficacy of IL-2 therapy is of interest given that melanoma stem cells suppress IL-2 production by T cells as a mechanism of immune evasion.[103] By overcoming melanoma suppression of this cytokine, the anticancer immune response is invigorated. Another immunotherapy, tumor vaccination, has been less efficacious.[153] Vaccines composed of known markers of melanocyte differentiation may have limited clinical effectiveness because of the downregulation of melanocyte-associated antigens by melanoma stem cells to impair T-cell recognition.[103] Nonetheless, the possibility that future vaccines could sensitize host immunity against biomarkers selectively expressed by melanoma stem cells merits future investigation.

Another immunotherapeutic strategy currently under investigation is specific molecular targeting of regulatory elements involved in immune tolerance. Negative regulators of the adaptive immune response, such as B7-2 and PD-1, and inhibitory cytokines, such as IL-10, are employed by melanoma stem cells to downregulate T cells,[103] and are potential molecular targets for immunotherapy. CLTA4 (cytotoxic T-lymphocyte antigen 4) is a ligand receptor associated with B7.2, and functions as a negative regulator of T-cell activation. Two anti-CTLA4 monoclonal antibodies, tremelimumab and ipilimumab, have shown promise in initial clinical trials,[154–157] and a randomized phase III trial recently demonstrated a 3.7-month survival benefit in patients with metastatic melanoma who received ipilimumab *versus* glycoprotein 100 peptide vaccine.[158] As more is learned about the anticancer immune response and the mechanisms of immune evasion and modulation utilized by primitive melanoma cells, further immunotherapeutic targets will be identified.

Anti-VM therapy is another indirect strategy to target primitive melanoma cells. Therapeutic targeting of the molecular pathways responsible for the generation and maintenance of VM may be a particularly promising therapy

against melanoma because there is no known physiologic analog of VM in children or adults, and therefore anti-VM therapies should have minimal effects on normal physiological processes.[159] It is noteworthy that inhibitors of angiogenesis are ineffective against the tubular networks seen in VM[122] as it is an angiogenesis-independent phenomenon. It has been suggested that an efficient anti-VM therapy focuses on three aspects: remodeling of the ECM and tumor microenvironment, blocking biochemical and molecular signaling pathways of VM, and inhibiting plasticity of tumor cells.[160] Genes involved in the molecular signaling pathways of VM, such as *MMP-2*, *MMP-9*, *VE-cadherin*, *EphA2*, and *laminin 5γ2*, can be downregulated with therapeutic agents that have been approved for other medical indications. In *in vivo* models of murine melanoma, thalidomide inhibited MMP-2 and MMP-9 expression and VM channel formation, and promoted melanoma cell necrosis.[161] Doxycycline also reduced MMP-2 and MMP-9 expression, impaired VM formation, and inhibited tumor growth in murine melanoma models.[162] In three-dimensional culture, a chemically modified tetracycline, COL-3, inhibited the expression of VM-associated genes in aggressive melanoma cells, including *MMP-2*, *MMP-9*, *MT1-MMP*, *TIE-1*, and *VE-cadherin*, and impaired resultant VM.[163] Furthermore, COL-3 prevented the induction of VM in poorly aggressive cells seeded onto an aggressive cell-preconditioned matrix by inhibiting MMP expression and preventing production of laminin 5γ2 chain promigratory fragments in the ECM. Additional methods of impeding VM may employ monoclonal antibodies, antisense oligonucleotides, or gene knockout of key proteins in signaling pathways. In addition, VEGF, a major mediator of tumor angiogenesis, and its receptors may prove to be future targets against VM, as VEGFR-1 has recently been shown to be expressed by ABCB5+ melanoma cells and required for VM and tumor growth *in vivo*.[75] Studies in osteosarcoma have demonstrated that VEGF-siRNA silencing suppresses vasculogenic mimicry, inhibits proliferation, and induces apoptosis *in vitro*.[164]

LOOKING FORWARD

The stem cell concept has far-reaching implications in the fields of embryology, hematology, regenerative medicine, and cancer biology. Since their identification in hematopoietic malignancies, cancer stem cells have increasingly been isolated from solid tumors. The early twenty-first century has been an exhilarating period in melanoma research, as we have begun to identify and characterize a subpopulation of melanoma cells capable of self-renewal and differentiation plasticity, the two defining features of cancer stem cells. We have learned that melanoma stem cells may be present within tumors in varying frequencies, depending upon their microenvironment, the host immune system, and the kinetics of the non-stem cell tumor population, and may even be capable of some degree of phenotypic and functional plasticity in response to epigenetic or microenvironmental factors. This subpopulation of melanoma cells can be isolated via identifying the drug transporter ABCB5 or the neural crest stem cell marker CD271. Melanoma stem cells may engage in several survival-promoting and virulence-conferring mechanisms, including chemoresistance and immune evasion and modulation. Moreover, melanoma stem cells may participate in VM, an incompletely understood phenomenon associated with melanoma progression, and may play a role in tumor metastasis. Although melanoma stem cells have not been definitively linked to EMT, the plasticity of the cells that participate in EMT, the occurrence of EMT in embryology, and the stem cell model of cancer growth all suggest that primitive melanoma cells may play a role in these important processes.

The survival mechanisms employed by melanoma stem cells and primitive melanoma cells are targets for future anticancer therapies. In addition, therapies that directly target molecular markers on melanoma stem cells or induce differentiation of these immature cells may prove beneficial. Substantial progress in melanoma research has been made in the past decade. Now, as additional melanoma stem cell markers are identified and virulence-conferring mechanisms of melanoma stem cells are elucidated, novel antimelanoma therapies are likely to emerge. The cancer stem cell model has not only provided a new paradigm for understanding the pathobiology of melanoma, but also may well translate into strategies to ultimately conquer this arguably most virulent form of human cancer.

ADDENDUM

Since the submission of this "Pathobiology in Focus" article, an important study has been published by Civenni *et al*[166] that bears directly on the role of experimental methodology for accurate and translationally-relevant demonstration of melanoma stem cells.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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