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Melanocytes, melanocyte stem cells, and melanoma stem cells

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Abstract

Melanocyte stem cells differ greatly from melanoma stem cells; the former provide pigmented cells during normal tissue homeostasis and repair, while the latter play an active role in a lethal form of cancer. These two cell types share several features and can be studied by similar methods. Aspects held in common by both melanocyte stem cells and melanoma stem cells include their expression of shared biochemical markers, a system of similar molecular signals necessary for their maintenance, and a requirement for an ideal niche microenvironment for providing these factors. This review provides a perspective of both these cell types and discusses potential models of stem cell growth and propagation. Recent findings provide a strong foundation for the development of new therapeutics directed at isolating and manipulating melanocyte stem cells for tissue engineering or at targeting and eradicating melanoma specifically, while sparing non-tumor cells.

Keywords

melanocyte; stem cell; cancer stem cell; melanoma; cancer therapy

An introduction to melanocytes, melanocyte stem cells, and melanoma

Our bodies are constantly under threat from external hazards. Skin, the main protection against many such environmental insults, is composed of three layers: the epidermis, dermis, and subcutaneous tissue. The main specialized cell types of the outermost layer, the epidermis, are the keratinocyte, Langerhans cell, and melanocyte. To guard against hazardous ultraviolet radiation (UVR) from sunlight, the melanocyte contains a unique organelle, the melanosome, that produces the pigment, melanin, to provide photoprotection.

The melanocyte is an appealing model for studying cellular function and differentiation, because they work as a single-cell unit and have a distinctive differentiation product in the form of melanosomal organelles and melanin. Interest in melanocytes and skin pigmentation can be traced back many centuries to Asia, where fancy mice were bred for their different coat colors (1). For many of these mouse strains, it was eventually discovered that the variation in coat color was the result of differences in genes involved in the production of

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melanin and the function of the melanocytes. These studies provide a foundation for our yet incomplete understanding of melanocyte function.

Melanocyte stem cells have specific qualities that make them an excellent model for the study of stem cells generally. First, they can be relatively non-invasively harvested from the skin. Within the skin, melanocyte stem cells are most likely located within a specific anatomic niche, making them easy to find and isolate; specifically, in murine skin, melanocyte stem cells reside within the bulge region of the hair follicle (2). A second valuable trait of the melanocyte stem cells is that expansion and differentiation of daughter cells are closely coupled to the hair growth cycle (at least in murine systems). Quiescence or growth of melanocytes may be easily controlled through hair depilation. Lastly, dysfunction of this population is easily identified by the resulting defects in pigmentation. Improper maintenance of melanocyte stem cells, such as through increased apoptosis as a consequence of Bcl2 deficiency, causes stem cell loss and hair graying (3).

Several intrinsic qualities of melanocytes in general are useful for any studies or techniques that require manipulation of these cells. These traits include the relatively long life of melanocyte stem cells in comparison to other skin populations, such as keratinocytes. In addition, melanocytes work as a single-cell unit, and growth and differentiation are tightly controlled through signals from surrounding cells. In addition, melanocytes are derived from the neural crest, a very plastic embryonic tissue.

Due to their developmental origins, melanocyte stem cells' multipotency and ability to migrate readily to new locations may be innate properties. The multipotent and highly prolific melanocyte stem cells provide an important stem cell source for regenerative medicine applications. Such cells could be utilized for gene therapy through *ex vivo* gene delivery and re-transplantation. These excellent qualities of the melanocyte stem cell, including its long lifespan, ability to be manipulated *in vivo* and *ex vivo*, plasticity regarding its differentiation choices, and capability to migrate *in vivo*, open the doors to great opportunities for stem cell therapy.

The same traits that make the melanocyte stem cell so promising for stem cell therapeutics may also help explain why melanomas (tumors derived from melanocytes) are often so aggressive and deadly. Melanoma tends to metastasize early in the disease process and is often fatal. The incidence of melanoma, unlike that of many other cancer types, has been rising steadily for over half a century (4).

Therapeutic choices for melanoma are limited, and most treatments fail to improve quality of life or survival in a meaningful way (4, 5). In the past year, the development of a small compound (PLX4032/RG7204) targeting a specific V600E point mutation found in B-Raf kinase has produced exciting results, with an amazing 77–81% response rate for patients whose melanomas harbor this particular mutation (6, 7). These studies demonstrate that malignant melanoma can respond very well to targeted therapy. Unfortunately, PLX4032 is unhelpful or even harmful to the approximately 50% of melanoma patients whose tumors lack the V600E B-Raf mutations, and nearly all responding patients develop drug resistance followed by rapid disease progression (8–12). Additional molecular targets need to be identified and tested to develop new therapeutics to fight this highly lethal tumor.

Because some melanoma cells may be more able to promote and drive tumor formation than others, it would be more advantageous to target those tumor cells therapeutically to block cancer progression. Several years ago, it was discovered (or, more accurately, rediscovered (13)) that a small subset of tumors contains cancer cells that have qualities like their tissue-specific stem cells (14). These experiments determined that a small sub-population of acute myeloid leukemia cells could re-establish tumors in severe combined immunodeficiency

(SCID) mice, while the majority of the tumor cells could not. This study laid the foundation for the cancer stem cell hypothesis, which states that a sub-population of the tumor cells can self-renew, differentiate, and initiate tumor formation. While these putative cancer stem cells may have properties similar to the stem cell population of their tissue of origin, the tumor need not arise from the normal stem cell pool. If melanoma cancer stem cells indeed exist, new therapeutics may be designed to specifically target these cells.

Many current chemotherapeutics target rapidly dividing cells, but cancer stem cells may be quiescent most of the time, thereby evading these treatments. While there is evidence that melanoma follows a cancer stem cell model for tumor development and a hierarchical model for tumor growth and progression, there is also support for a model wherein melanoma cells progress in a stochastic manner (Figure 1). Although there is still a debate on which model (or combination of models) melanoma follows, this point is crucial for designing new drug targets and therapeutics.

This review provides an overview of the rapidly progressing fields of melanocyte stem cells and melanoma stem cells. While these two cell types are not interchangeable, some of the lessons learned for one may provide insights into the other.

Melanocyte stem cells

Adult stem cell definitions and criteria for determining stem cells

There is strong evidence to support that adult tissues harbor adult stem cells and that they are similar to their embryonic stem cell precursors. Broadly defined, stem cells have two essential characteristics (15, 16). First, stem cells self-renew; as they divide, they maintain the stem cell population. Second, stem cells provide the specialized and differentiated daughter cells of their specific tissue type. While the defining traits of self-renewal and multipotency are common to all stem cells, the degree of plasticity and the markers expressed differ between adult and embryonic stem cells, as well as between adult stem cells of different tissue types.

The mechanisms by which adult stem cells renew themselves and give rise to daughter cells are not well characterized and may be distinctive for each tissue type. In addition, circumstances in which the stem cells undergo division, for example during development, in response to environmental stress or injury, or in the course of normal tissue turn-over and remodeling, may also affect how the stem cell yields daughter cells. For example, the division of the stem cell may be asymmetrical or symmetrical (17) (Figure 1). In asymmetrical (or hierarchical) stem cell division, the stem cell yields two daughter cells, one of which maintains the stem cell qualities of the parent cell, while the other differentiates in order to perform the tasks of the tissue. The cells' fates may be influenced by internal cues, with or without signals from the environment. In symmetrical (or stochastic) stem cell division, both daughter cells have the chance to be either a stem cell or a differentiating cell; again, this process maybe regulated through the stem cell environment. It is believed that many stem cells reside within a specific microenvironment called a "stem cell niche." This specialized location may contain various cell types, including multiple kinds of stem cells as well as supportive cells that provide scaffolding or signaling to the stem cells (18). Most stem cells probably can undergo both asymmetric and symmetric division, and the method selected may be dependent on cell type, environmental signals, and circumstances (such as during development or injury) (17).

In order to realize the promise of stem cell therapeutics, it will be important to be able to identify and characterize tissue specific stem -cells. Multiple methods exist for these purposes, each with its own strengths and limitations; these include measurement of

quiescence, behavior of the cells in culture, the ability to transplant the cells and observe them behaving as stem cells, *in vivo* lineage tracing, and the use of specific markers and anatomical location of cells (19). Cellular quiescence is a trait often attributed to stem cells, although it is unrelated to the major defining stem cell characteristics of self-renewal and multipotency. In terms of quiescence, it is often linked with stem cells along with self-renewal and multipotency; indeed, many adult stem cells exist in an overall quiescent state (20–23). Methods for marking cells that are non-proliferative and long-lived include label uptake and retention, most often through the incorporation of DNA analogues such as bromo-deoxyuridine (BrdU) (21); however, some stem cell populations are reportedly long-lived yet highly proliferative (24, 25). Growth-arrested and terminally differentiated cells with relatively long life spans may also retain BrdU (26). For these reasons, although a relatively long lifespan and quiescence are often associated with stem cells, quiescence and label retention should not be used as sole criteria for identifying stem cells.

A second method for identifying and characterizing stem cells is the study of isolated stem cells *ex vivo*. The advantage of this method is that the cells may be observed and tested in a controlled environment. The multipotency of the cells, as well as their responses to external stimuli, may be directly determined in cell culture. Many stem cells depend on the microenvironment within their natural niche; loss of the niche microenvironment may alter their natural behavior. In addition, stem cells grown on a totally artificial matrix, such as plastic, may undergo permanent alterations in phenotype and behavior (27).

A third standard method is to transplant donor stem cells into recipient mice. The advantage of this method is that it assesses both self-renewal and multipotency; however, once again, it removes the stem cells from their natural environment and the cells may behave differently. For example, in the skin, epithelial stem cells that endogenously provide only one or two of the cell types of the main skin structures (epidermis, hair follicle, and sebaceous gland) can yield all three cell types after transplantation (28–31). Perhaps, stress or injury induced by the transplantation leads to a different response that is seen in normal homeostasis. Indeed, some findings from the transplant studies are similar to responses seen after full-thickness skin wounds (28–34).

A fourth method for identifying and characterizing stem cells is lineage tracing; this method often relies on transgenic mouse models to genetically label a cell population (35). An example of this is an inducible Cre recombinase enzyme expressed from a gene or a genetic promoter that is active in stem cells. The inducible Cre will be expressed in the stem cell, but will become active only after exposure to an inducing agent. An example of an inducible Cre system is the $CreER^{T2}$ expression gene, where the Cre enzyme is fused with a modified estrogen receptor moiety (36). Another example is CrePR1e, where Cre is fused to a modified progesterone receptor (37, 38). The resulting $CreER^{T2}$ or CrePR1e enzymes will be sequestered in the cytoplasm until exposed to exogenous tamoxifen or anti-progestin homologues, resulting in a change in conformation of the protein and permitting entry into the nucleus. In addition to the Cre allele, the mice also possess some type of marker gene that will become active and express reporter protein after modification with active Cre enzyme. This method will label cells when the Cre enzyme is active as well as their resulting progeny. A main advantage of this method is that it tests stem cells within their physiological niche. The putative stem cells may be labeled, persistence of marker may be recorded as a test of self-renewal or quiescence, and the daughter cells can also be traced to measure multipotency. Because the cells may be labeled with a marker, cells may be isolated for *ex vivo* cultures or for gene expression experiments. While this method is a powerful tool for stem cell biology, it is limited by the promoter or gene locus used for the transgenics. It is difficult to find genes expressed specifically in the stem cell: often these genes are also expressed in daughter cells or other cell types.

While the four assays mentioned above test the main criteria of self-renewal and/or multipotential, other methods utilize protein expression or microanatomical location; these include the expression of potential stem cell markers and the location of cells within a stem-cell niche. Cell expression markers are valuable tools for stem cell biology, although expressing a marker cannot be used as a sole criterion, because it does not demonstrate either self-renewal or multipotency. Identification of markers from cells shown to have stem cell characteristics provides a short cut for the identification, isolation, and labeling of stem cells; however, markers may be expressed transiently in the stem cell population; only during certain conditions such as tissue injury or remodeling,; or in daughter cells and differentiated cells as well.

Unique stem cell markers are rare and are often not linked to stem cell function. For this reason, markers should be used stringently and only after being thoroughly tested biologically for being co-expressed specifically in cells that follow the strict definitions of being a stem cell. In addition, since stem-cell niches have been identified for a number of stem cells, cells within these niches may be stem cell candidates. Frequently, a cell is identified as a stem cell since it is in a niche and expressing putative stem cell markers; however, several other supportive cells also are found in the niche, and therefore anatomic location cannot be a sole standard for determining a cell's status as a stem cell.

Melanocyte stem cells

Melanocyte stem cells are a source of transient amplifying cells and differentiated melanocytes. Melanocyte stem cell biology is a newly developing field. Not much is known of how melanocyte stem cells function within their niche, how external signals regulate their ability to remain quiescent or to divide, or how plastic this population is. In addition, the microanatomic location of melanocyte stem cells in various organisms, such as the m ouse, fly, zebrafish, and humans, is only now being explored in detail (39, 40).

To date, melanocyte stem cells have been studied most extensively in the mouse; while significant findings also exist in other organisms, this review will mostly focus on murine systems. An advantage to studying the murine melanocyte is that growth and apoptosis are paired to the growth cycle of the hair follicle. The hair follicle is a skin appendage composed of epidermal keratinocytes and follicular cells, mesenchymal cells, as well as the pigment-producing melanocytes. The hair follicle undergoes cyclic expansion and regression in response to normal growth or as a stress response due to hair removal. The phases of this follicular cycle are anagen (follicular growth and expansion), catagen (regression of the hair follicle) and telogen (resting follicle) (41). The anatomy and timing of the phases, as well as the location and differentiation of the melanocytes within the back-hairs of the C57B6 mouse strain are well characterized (42–47). The fate of the melanocytes within the follicle is tightly linked to the phases of the hair follicle, with expansion of the melanocyte pool and differentiation during anagen, and reduction of melanocytes through apoptosis in catagen.

In a major advance for melanocyte stem cell biology, a transgenic mouse model has been utilized, (2)in which the expression of a beta-galactosidase reporter gene was driven by a segment of the dopachrome tautomerase (Dct) gene promoter (Dct-LacZ) (48). Dct is an enzyme with a role in melanin synthesis, and is an early marker of melanoblasts. Endogenous Dct and the Dct-LacZ transgene are expressed within the matrix of the hair bulb and in the bulge region of the hair follicle. The bulge region, first characterized histologically many years ago as a thickened area in the upper portion of the follicle, was found to harbor slow-growing and BrdU-retaining cells (21, 49, 50). In this study (2), the anatomic location of the melanocyte stem cell niche was confirmed to be in the bulge region, also defined as the lower permanent portion (LPP), of the hair follicle (51). The Dct-LacZ expressing cells within the LPP region have stem cell traits of self-renewal and

multipotency. To deplete dividing and pigment-producing melanocytes, an antibody targeting CD117 (Kit receptor) was applied to neonatal mouse skin (52, 53). Loss of these cells in the neonates led to completely unpigmented coat hairs in the C57B6 mouse strain; however, quiescent unpigmented melanocyte precursors remained, and after the first hair cycle there was a partial recovery of pigmentation; the hair follicles showing repigmentation were those that retained expression of the Dct-LacZ transgene (2). In addition, the LPP of vibrissal hair follicles, containing Dct-LacZ expressing cells, were transplanted and shown to be able to provide pigmented progeny cells. These melanocyte stem cells were slow-cycling cells that retained BrdU. This label retention was co-expressed with Dct-LacZ expressing cells. Dct was expressed in these melanocyte precursor cells, but this gene was also expressed in transient amplifying and differentiated cells, so it is not an exclusive melanocyte stem cell marker. While these two other Dct expressing cell populations were located in the hair matrix, the Dct positive cells in the LPP were unpigmented and the data strongly support that they were melanocyte stem cells. This study demonstrated that the Dct-LacZ expressing cells of the LPP have stem cell characteristics of self-renewal and multipotency, either *in situ* or after transplantation, and that these cells are relatively quiescent unless there is a need for melanocyte stem cell expansion.

The melanocyte stem cell niche

A niche for melanocyte stem cells has been discovered in the LPP of the hair follicle (2). This microenvironment regulates the stem cells through direct contact with adjacent cells, via scaffold proteins of the extracellular matrix, and through secreted signaling proteins. The niche may maintain the stem cell population avoiding loss of the stem cell pools, as well as promoting quiescence in order to prevent overgrowth of cells. Supportive cells within the niche, as well as the stem cells themselves, may express adhesion and extracellular matrix molecules including integrins or cadherins to support cells within the tissue-specific stem cell niche (54). For some stem cell niches (mostly known in specific Drosophila niches (55)), the only scaffold for the stem cell niche is the basement membrane, and supporting cells are absent. For the limited number of mammalian niches discovered, however, there usually are some additional support cells and extracellular matrix proteins.

In addition to the melanocyte stem cells, the hair follicle LPP is also home to at least one other stem cell population, the epithelial stem cells (30, 32). This epithelial stem cell is a multipotent stem cell that can give rise to a number of cell types, including keratinocytes and follicular cells. Because these two stem cell populations reside anatomically in the same neighborhood, it is plausible that there would be at least some interaction between these cell types. While keratinocytes tightly regulate cutaneous melanocytes in their pigment production, growth, and apoptosis, it is logical that the keratinocyte precursors would have a similar role. Indeed, this is supported by recent findings involving cells of the follicular LPP niche. In an elegant study utilizing transgenic mice, cross talk between these two stem cell populations within the niche has been demonstrated (56). The investigators activated or blocked canonical Wnt signaling in either epithelial or melanocyte stem cells through expression of a constitutively active beta-catenin, or via Cre-silencing of the beta-catenin locus. Intrinsically, when beta-catenin activity was either increased or decreased within melanocyte stem cells, hair pigmentation was lost. Forced activation induced differentiation at the expense of melanocyte stem cell renewal, phenotypically parallel to prior studies in which stem cells were depleted (3). Loss of beta-catenin also led to hair graying, albeit by a different mechanism. The LPP melanocytes were not depleted; these cells were similar to controls, except that they did not yield daughter cells. This report, as well as earlier studies (57), support that canonical Wnt signaling and beta-catenin become activated in the melanocyte stem cells progeny as they begin to proliferate and differentiate. Stabilization of beta-catenin in the epithelial stem cells caused an extrinsic effect on the melanoycte stem

cells. This led to an enlargement of the bulge region in general, and an increase in proliferation of the melanocyte pool; melanocyte expansion was mediated by the epithelial stem cells through an endothelin-dependent signaling mechanism. This supports that the initiation of melanocyte stem cell proliferation is dictated at least in part by signals directed by epithelial cells.

These results add to the growing body of evidence that Wnt signaling is important in regulating the melanocyte stem cell within its niche (58). Some of this work may be indirectly applied to the melanocyte stem cell, and is primarily focused on melanoblast development or the effects on melanocytes in general. For example, two Wnt factors, Wnt1 and 3a, are necessary for melanoblast development in the embryo (59, 60). Although these experiments are only indirectly related to the melanocyte stem cell, they suggest that there is a differential response to Wnt signaling by the melanocyte stem cell and the differentiating melanocyte, and that this can be modulated through overall levels of signals that activate or inhibit the Wnt pathway. The importance of repressing Wnt signaling for the melanocyte stem cell is supported by the presence of several Wnt inhibitors in the niche, including DKK3, Sfrp1, and Dab2 (30, 61). The melanocyte stem cells themselves also express several Wnt inhibitors themselves, such as DKK4, Sfrp1, Dab2 and Wif1 (51, 62). These experiments support a model in which inhibition of Wnt signaling promotes the melanocyte stem cell phenotype, whereas activation leads to melanocyte differentiation.

While an active canonical Wnt signaling pathway plays a role in proliferation and differentiation of the melanocyte stem cells within the niche, other factors, such as transforming growth factor-beta (TGF-beta), regulate melanocyte stem cell quiescence and maintenance of an undifferentiated state (63). TGF-beta is a signaling molecule that can regulate a number of cellular responses, such as cell growth and survival (64). In the hair follicle, TGF-beta proteins are expressed as the hair follicle regresses during catagen and promote apoptosis of the epithelial-derived components of the follicle (65, 66). In addition, the bulge cells of the hair follicle express nuclear phospho-Smad2 (indicating active TGF-beta signaling) (61). This exposure to TGF-beta results in cell-cycle arrest and immaturity in melanocytes, both *in vitro* and *in vivo* (63). Changes in TGF-beta expression are seen in normal physiological hair growth and follicular cycling as well as in response to UVR, and key transcription factors such as MITF and PAX3, as well as melanin-associated enzymes such as tyrosinase are down-regulated in response to TGF-beta (63, 67). TGF-beta signaling is a major mechanism for the maintenance of the melanocyte stem cell, as well as the production of pigment-producing melanocyte progeny.

Disruption of the Notch canonical signaling pathway also disrupts the ability of melanocyte stem cells to self-renew. There are four Notch receptors, which interact with specific ligands (Jagged 1 and 2, Delta-like 1,3,4) through cell-cell interactions (68). Once activated, the Notch intracellular domain (NID) is cleaved by gamma secretase. The now-freed NID can disassociate from the membrane and translocate into the nucleus. The NID interacts with the transcription factor called RBP-Jκ in mice (an ortholog to CBF1 in humans) and activates Notch down-stream genes. Inhibition of the Notch signaling cascade, through a gamma secretase inhibitor (GSI) mediated block of Notch cleavage, leads to hair graying. Unlike the use of Kit inhibitor antibodies, this graying is not reversible, suggesting a permanent effect on the melanocyte stem cell population (69). GSI treatment leads to apoptosis of the melanocytes and depletion of the melanocyte stem cell pool (69, 70). This effect is mediated through Notch receptors 1 and 2, but not 3 or 4. Notch1 and Notch2 compensate for each other; while deletion of one receptor in the melanocyte population leads to a partial pigmentation defect, the double Notch1/2 mutant has a fully gray coat (71). The phenotype of the Notch1/2 mutant is similar to that of mice with a melanocyte-targeted deletion of the RBP-Jκ gene.

Examination of the skin in both of these murine models demonstrated that Dct expressing cells in the LPP had been depleted. The phenotypes for the GSI treatment, Notch1/2 deletion, and RBP-Jκ were most likely due to the same pathway, since the addition of Notch intracellular protein in the Notch1/2 melanocyte-deleted mice rescued the graying phenotype and protected the melanocyte stem cell pool (72). This raises many interesting questions about how the Notch pathway is regulated in the stem cell niche, and what are the ligand-expressing cells that mediate the signal. Perhaps, again, the epithelial stem cells will be shown to have a role in Notch pathway regulated self-renewal melanocyte stem cells.

The epithelial stem cells within the follicular niche also regulate themselves and the melanocyte stem cells through the production of extracellular matrix protein. The epithelial stem cells highly express the hemidesmosomal transmembrane collagen called collagen XVII (also called COL17A1, BP180 or BPAG2) (73). This protein attaches to the underlying basement membrane, and deficiency of this protein leads to a reduction of cellular anchorage, hair graying and loss, and follicular atrophy (74–79). In Col17a1-deficient transgenic mice, the hair loses pigmentation prior to the occurrence of extensive hair loss (77). Collagen XVII provides the stem cell niche architecture within the LPP for normal maintenance of both the epithelial and melanocyte stem cells (73). Dysregulation of collagen XVII leads to premature differentiation of melanocytes within the niche, possibly due to direct loss of proper microenvironmental structure, and/or loss of signaling from the epithelial stem cells. This work provides insight into the architecture of the follicular stem cell niche, and may provide the foundation for creating artificial niches for growing stem cells *ex vivo*.

A picture of how melanocyte stem cells function and are maintained is starting to come into focus. They are sustained and regulated in a distinct location of the LPP of the hair follicle, and are influenced by other cell types such as the epithelial stem cells, extracellular matrix proteins, and a number of secreted factors that promote or restrict multipotency or self-renewal (Figure 2). Disruption of these regulatory signals lead to errors in the ability of the stem cell to maintain their stem cell pools or provide pigmented progeny.

Melanoma stem cells

Cancer stem cell definitions and models for tumor development and progression

To standardize the definition of the cancer stem cell, in 2006 the American Association of Cancer Research stated, "The 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 (80).” This statement defines cancer stem cells as having the essential properties of stem cells: self-renewal and the ability to give rise to progeny that can grow and differentiate. This definition implies that the cancer stem cells possess a hierarchical nature; however, it is not clear whether all tumors pursue a hierarchically organized pathway, in which the stem cell trait is stable, or rather follow a stochastic model, where all tumor cells are equivalent but their fates are governed by environmental factors (81)(Figure 1).

Tumors are heterogeneous, undergo uncontrolled proliferation and exhibit some self-renewal properties. Two major models, both of which incorporate versions of a cell with stem cell-like qualities, could explain how tumors grow and progress (17, 81). In the stochastic model, all the tumor cells are equivalent but their fates are governed by intrinsic signals as well as environmental factors. Not only do all the cancer cell progeny have the potential to behave like a cancer stem cell but they also retain plasticity to go from a non-stem cell to a stem cell-like precursor. In the hierarchical model, the cancer stem cells are biologically distinct, can renew themselves, and give rise to various progeny cells that lack the ability to self-renew. In this model, the cancer stem cell phenotype is a stable

characteristic. The hierarchical model is often considered the “cancer stem cell model” for sustained tumor propagation, whereas the stochastic model does not posit a separate and specific stem cell population but rather assumes the existence of many cells that may act as cancer stem cells. The stochastic model is not considered to include cancer stem cells as narrowly defined, because it lacks some characteristics that are also linked to cancer stem cells, namely rarity and quiescence or dormancy. The latter feature allows cancer stem cells to resist standard cancer therapies, leading to clinical recurrence of tumors after an initial response to treatment regimens; however, under the broad definitions provided by the AACR workshop, none of these attributes define the cancer stem cell. While the stochastic model of tumor propagation may not be considered to include cancer stem cells in a narrow sense, alternatively, it may be argued that all the cells of the tumor according to this model have the ability to act as a cancer stem cell.

At present, methods for testing and determining cancer stem cells are limited. The assay utilized most widely is to xenotransplant the cells into an immunodeficient mouse model. As with the methods described for stem cell biology, this method will reveal whether the transplanted cells possess the ability both to self-renew and to generate progeny *in vivo*. Again, the major drawback to this method is that it removes the cells from their natural environment. This method is often further complicated by the use of various murine strains and multiple techniques for isolating the cell population to be tested and transplanted. At present, there is little direct evidence of a separate stem cell pool within unmanipulated solid tumors.

Melanoma cancer stem cell markers and evidence for the hierarchical model of tumor growth

A major focus of melanoma stem cell research is the identification of markers for the cancer stem cell population. Note that markers should not be used as a sole criterion for identifying cancer stem cells. Many of the markers utilized in cancer stem cell research have the advantage of being cell-surface proteins that allow cells to be separated by fluorescence-activated cell sorting, and these factors may not have any role in cancer stem cell biology. An additional complication is that expression of these markers may not be stable, and cells that are either positive or negative for a particular marker may yield both positive and negative daughter cells (82–84). The markers may not be unique to the cancer stem cells, being expressed in other cell types as well. Despite these caveats, some important insights have been obtained through the use of these markers and the identification of new potential markers may provide future therapeutic targets.

The first characterization of potential cancer stem cells in a solid tumor was accomplished in breast cancer, and these cells expressed CD44 but were CD24-negative (85). This finding was followed by similar reports in other solid cancers, such as brain and colon cancer (86–89). All these studies utilized a xenograft transplantation mouse model for testing their hypotheses, and they demonstrated that the tumors contained a rare subpopulation that was hierarchically organized. The resulting tumors after transplantation resembled the original tumors both morphologically and in heterogeneity.

CD20, a cell surface marker normally found on B-cells, has been associated with melanoma cells having stem cell characteristics (90). The investigators isolated cell populations from established cell lines that grew in non-adherent spheroids in stem cell culture medium. The cells derived from these spheroids were multipotent and able to differentiate into multiple neural crest-like progenitor cells, and also were self-renewing both in culture and in xenograft transplants in SCID mice. The spheroid cells were enriched with cells that expressed CD20 and demonstrated a much higher degree of multipotency in comparison to the CD20-negative cell population. In patient samples, CD20 expression was found in a very

limited population (less than 2% of the tumor) in 4/5 primary tumor tissues (91). T-cells, genetically engineered to recognize and have specificity for CD20, were transplanted into immunodeficient NIHIII mice harboring these CD20+ melanoma tumors (91). The T-cells were able to eradicate the tumors in the mouse tumor model, although the CD20 antigen was present on the surface of only a small minority of tumor cells. In addition, the use of the anti-CD20 antibody rituximab produced a lasting remission in a patient whose melanoma had failed to respond to immunotherapeutics and dacarbazine treatments (92). Rituximab produced complete regression of all but one of the melanoma metastases, and the remaining tumor showed a partial and stable regression. Rituximab has also been utilized in a small study in nine patients without evident residual disease after standard therapies but are high risk for melanoma recurrence; 5/9 of the patients were tumor-free for over 42 months (93). CD20 needs more investigation as a putative cancer stem cell marker in melanoma and as a potential therapeutic target. However, these recent studies are promising, and may provide a paradigm shift in cancer therapeutics, in which targeting only a small minority population within the tumor could be effective in reducing the entire tumor mass.

CD133 (Prominin-1), a cell surface protein of unknown function first found on the surface of immature hematopoietic and neural stem cells (94, 95), has also been linked to cancer stem cells in a wide variety of different tumor types including melanoma (88, 89, 96, 97). CD133-expressing cells from primary melanoma tumor tissue had enhanced ability to form tumors in NOD/SCID mice (98). In addition, an established melanoma cell line (WM115), with the ability to form multiple cellular phenotypes, highly expressed CD133 and was able to form spheroids in stem-cell medium. More rigorous tests linking CD133 expression to self-renewal and multipotency need to be performed in melanoma. In addition, other studies have found that both CD133-expressing and non-expressing tumor cells can initiate tumors (83); however, CD133 expression has been linked to an increased disease progression, while inhibition of CD133 leads to a decrease in tumor cell growth and metastatic progress, suggesting some role in melanoma advancement (99, 100).

Members of the ATP-binding cassette (ABC) transporter family of proteins have also been reported to be expressed in melanoma stem cells. These transmembrane proteins hydrolyze ATP to perform functions such as the transportation of various substrates (101). Four of the ABC transporter proteins have known roles in multidrug resistance to cancer therapeutics, and two of these, ABCG2 and ABCB5, have been identified in potential melanoma stem cells. ABCG2 (also known as breast cancer resistance protein, BCRP) provides a mechanism of resistance against a wide variety of drugs, including the tyrosine kinase inhibitors imatinib and gefitinib, antibiotics, and HMG-CoA inhibitors (102). While ABCG2 is expressed in a number of epithelial tissues, it has not been detected in cutaneous melanocytes or melanoma cells in primary tissues (103). In WM115 cells, ABCG2 is co-expressed with CD133, although both are down-regulated in cell spheroids (98). At present, the data are too preliminary to strongly link ABCG2 to melanoma stem cells.

A 2008 report provides strong support for ABCB5 expression in a melanoma stem cell population (104). ABCB5 shares similarity with other B family ABC transporter proteins ABCB1 (Multi-Drug Resistant protein 1, MDR1) and ABCB4 (105). ABCB5 is co-expressed in an overlapping but not identical pattern with CD133 in G3361 melanoma cells and primary melanoma tissue samples, and in a subpopulation of CD271/VEGFR-expressing cells (106, 107). ABCB5 also mediates doxorubicin chemoresistance in melanoma cell lines (106). In addition, ABCB5 expression was found in melanoma cells that had qualities of cancer stem cells (104).

Here, both melanoma stem cell qualities of self-renewal and multipotency were demonstrated through serial dilutions in an NOD/SCID immunodeficient mouse

xenotransplant model and *in vivo* lineage tracking. Primary melanoma tissues from patients were sorted for expression of ABCB5; only those cells that expressed this factor were able to yield both ABCB5-positive and negative cells, and regenerated tumors with a similarly heterogeneous phenotype after multiple rounds of serial dilutions. For the lineage tracing studies, the melanoma cell line G3361 was stably transfected with constructs expressing either DsRed or yellow-green EYFP proteins. Each cell line was sorted, and ABCB5-positive cells were isolated from G3361-DsRed cells, and ABCB5-negative cells were isolated from G3361-EYFP cells. The yellow-green cells demonstrated weak growth in Balb/c nude mice and gave rise only to ABCB5-non-expressing daughter cells, while the DsRed cells were able to generate new tumors and cells that were either ABCB5-positive or negative. In addition, the stem cell qualities of the ABCB5-expressing population were suppressed if the expression of ABCB5 was directly inhibited with a specific monoclonal antibody.

Despite these strong findings, other groups havefound tumor initiation to be ABCB5-independent, and could generate melanomas with the use of ABCB5 non-expressing cells (82). Clinical data support a role for ABCB5, in that expression of ABCB5 is linked to disease progression. Expression of ABCB5 is generally low in pigmented melanocytic nevus tissue, but is found in a subpopulation of thick primary melanomas and metastases (104, 108, 109). Furthermore, induction of terminal differentiation is correlated with decreasing levels of ABCB5 (110). An intriguing potential mechanism for melanoma promotion by ABCB5 has been proposed through an immune-invasive capacity of the ABCB5-expressing population through suppression of T-cell activation (111). The ABCB5-positive population displayed lower levels of MHC class I receptors and melanoma-associated antigens as well as co-stimulatory molecules B7.2 and PD-1 in comparison to the ABCB5-non-expressing melanoma cells. This finding may prove to be beneficial in melanoma therapeutics, including ongoing clinical trials targeting the PD-1 receptor (112).

CD271 expression has also been described in melanoma stem cells. CD271, also known as low-affinity nerve growth factor receptor (NGFR) or p75NTR, has been identified as a marker of neural crest cells (113). CD271 expression has been linked to perineural invasion and migration in melanomas (114). A subpopulation of primary melanomas may be expressed as CD271, comprising approximately 2.5% to 41% of the total tumor cell population (115). The CD271-expressing population was able to establish tumors from 70% of the transplanted cells in a $Rag^{-/-}\gamma c^{-/-}$ mouse model, which is deficient in T-, B-, and Natural Killer (NK) cells. In comparison, CD271-non-expressing tumor cells were able to establish tumors from only approximately 7% of cells transplanted into this highly tolerant mouse model. In addition, the ability of the CD271-positive population to act as a cancer stem cell was tested in a model more physiologically relevant to human disease, where melanoma cells were seeded in normal human skin or bone tissue that was then grafted on both the $Rag^{-/-}\gamma c^{-/}$ and NOD/LTSscid/IL2Rγ^{null} immunodeficient mouse models. The resultant tumors regenerated the heterogeneity of the parental tumors.

Other investigators have found a similar phenomenon when CD271-positive cells were transplanted into NOD/SCID mice (116). Alternatively, other studies discovered that both CD271+ and CD271- melanoma cells were equally competent to produce tumors after transplantation into NOD/LTSscid/IL2Rγ^{null} mice (82, 116). In addition, the expression of CD271 is negatively correlated with the ability of mouse melanoma cells to form tumors in Nu/Nu mice (117).

Evidence that melanoma cells follow a stochastic model for tumor propagation

While there is ample evidence for the hierarchical model for tumor propagation in melanoma, there is also strong support for a stochastic pattern in melanoma or, perhaps

stated more eloquently, a model whereby melanoma is dynamically regulated (84). Two seminal publications demonstrate that the ability to self-renew and provide new cells to the population is a common trait in melanoma cells (82, 83). The investigators demonstrated that more than over 25% of the cells from the tumors were able to generate new tumors in xenotransplantation assays (83). The cells could generate tumors whether or not they expressed various putative melanoma stem cell markers, including CD133/Prominin-1, ABCB5, and CD271/NGFR (82, 83).

Additional studies provide further evidence that melanomas are dynamically regulated. The histone demethylase JARID1B is expressed in a melanoma cell subpopulation that promotes continuous tumor growth, self-renewal, and multipotency, but unlike other markers its expression does not follow a hierarchical model of cancer stem cells (84). JARID1B is a member of the jumonji/ARID1 histone 3 K4 (H3K4) demethylases, and through its enzymatic function it can reawaken previously silenced genes. JARID1B is expressed at low levels or not expressed in many normal tissues, but is abundant in regenerative tissues and in melanocytic nevi (118). In melanoma, however, JARID1B-positive cells constitute only a small subset (5–10%) of the tumor cells (118). Cells expressing JARID1B exhibited self-renewal and multipotency *in vitro* (84).

When the cells were sorted for JARID1B expression and xenotransplanted in NOD/LTSscid/IL2Rγ^{null} mice, there was no difference between the JARID1B+ and JARID1B− groups in the ability to regenerate tumors. The expression of JARID1B was able to arise from populations either that expressed this factor or did not. The connection between JARID1B and cancer stem cells is illustrated in experiments where the expression was inhibited; while there was an initial increase in cellular growth after blocking JARID1B expression, there was an eventual exhaustion of the population both *in vitro* and *in vivo*. While expression of JARID1B does not follow a hierarchical model for cancer stem cells, its expression is needed for self-renewal and the subsequent ability to generate daughter cells. These studies support that JARID1B is a cancer stem cell associated gene.

Melanoma – hierarchical or stochastic tumor propagation?

The studies presented here offer, at least on the surface, contradictory evidence to support either the hierarchical or stochastic model for melanoma propagation. Because these reports demonstrate high standards of scientific methodology, the findings are hard to dismiss as misinterpreted or incorrect. One likely explanation for these differences is the variety of methods used to determine the ability of cells to self-renew and propagate. One such variable is the choice of mouse strain for *in vivo* transplantation studies (Table 1). The use of xenotransplantation is presently the gold standard for determining cancer stem cells, but the natural environment for the tumors is not maintained and there are variances in the levels of immune cells present. While all of the studies incorporate immunocompromised mouse models, different strains were utilized. In addition, many of the studies utilized severe combined immunodeficient (SCID) mice, or mice with both a non-obese diabetic (NOD) and SCID background, the studies demonstrating a stochastic tumor propagation utilized even more profoundly immunodeficient NOD/LTSscid/IL2Rγ^{null} mice. These mice lack natural killer cell activity through the deletion of IL2Rγ in addition to their NOD/SCID background (119). In addition, some of the studies utilized Matrigel to aid in the transplantation, a substance that may also promote the establishment and growth of tumors (120). There were subtle but significant differences in methods for purifying cells, in terms of enzymes used (for example, trypsin and collagenase), concentrations of enzymes, and the duration of exposure of the cells to these enzymes.

It may be argued that the perceived ability of some of the cells to function as cancer stem cells may simply reflect the capability of the cells to survive the isolation methods (although

some of the studies controlled against this possibility). In addition, the differences may reflect how tolerant and fertile the environment is for the transplanted cells. The main question remains, what should be the criteria for defining a cancer stem cell? If the test is purely the ability of the cells to act as a tumor-initiating cell, then the use of the most tolerant of environments is most efficient. If, on the other hand, the test is to measure the ability of the cells to self-renew and efficiently propagate progeny cells in a more native environment for tumors, then the model systems utilized are limited.

Melanoma often engages the immune system, and it develops in an environment containing stromal and immune cells (121). Accordingly, the present model systems for xenotransplantation have several shortcomings. New models need to be developed, employing techniques presently utilized for melanocyte stem cell research. Fortunately, several transgenic models for melanoma already exist, including many that resemble human disease in their progression and metastasis (122, 123).

Conclusions

Our understanding of how melanomas arise and progress has advanced greatly, and with this knowledge new therapeutic strategies are being developed. These findings have also raised many new questions, and a more complicated picture is emerging. As more insight is gained into how melanoma cells grow and thrive, it is hoped that new drugs will be created to target the cells that promote the tumor, and spare normal tissues, thereby efficiently targeting the tumor without the bystander side-effects that often complicate present-day drug options.

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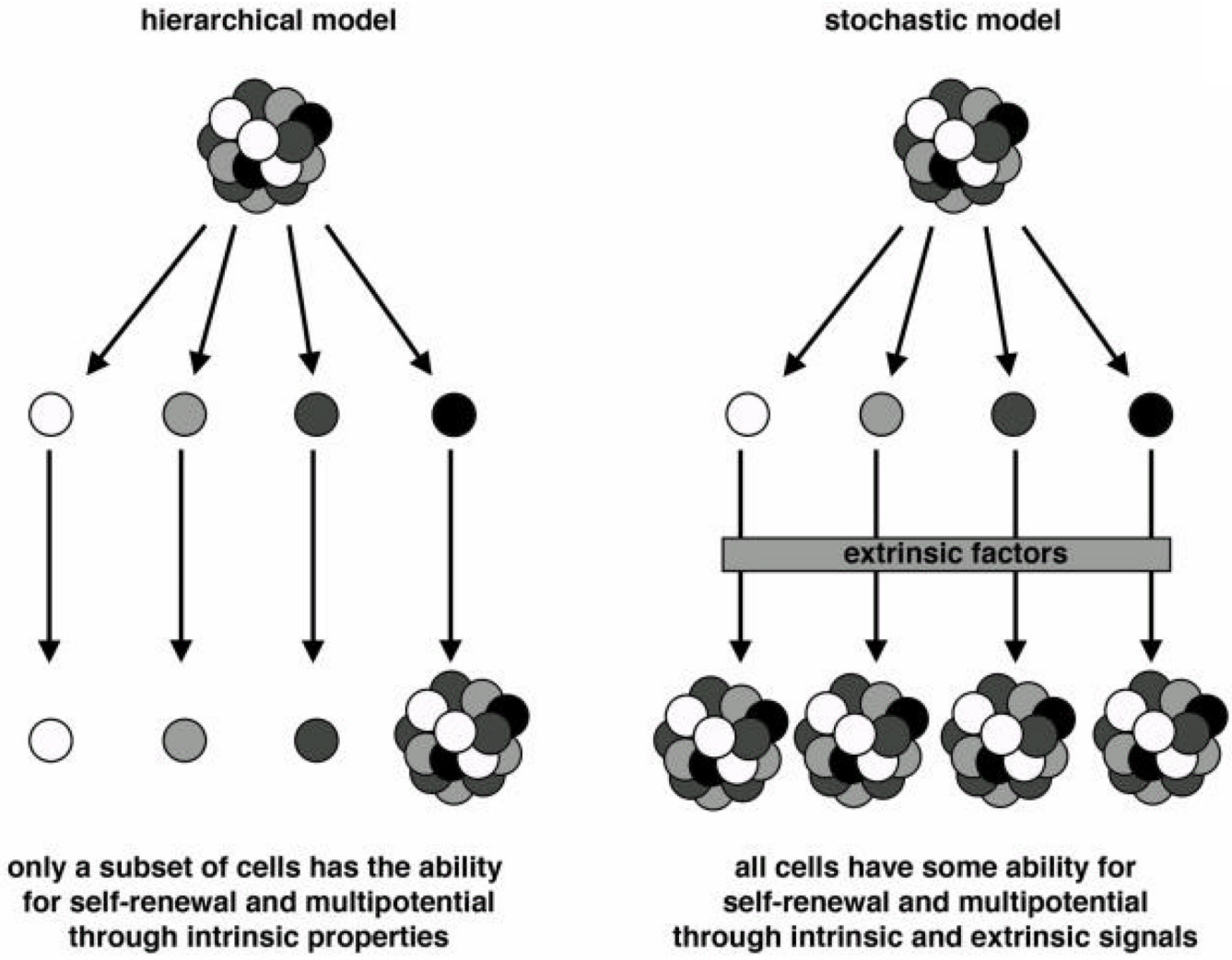

Figure 1. Models for stem cell growth and progression

For normal tissues as well as tumors, cells may grow in an asymmetrical (hierarchical) or a symmetrical pattern as a method for providing progeny cells for tissue remodeling or tumor growth (17, 81). In the hierarchical model, there is a defined subset of cells that are able to self-renew and to give rise to daughter cells. This quality is unidirectional and if this subset of cells, or the stem cell pool, is depleted, the tissue will be unable to replenish itself. In the stochastic model, all the cells in theory have some ability to behave as a stem cell in terms of self-renewal and multipotential. Extrinsic factors such as extracellular proteins, growth factors, and other cell types regulate the cell in terms of decisions for maintenance, differentiation, or cellular death.

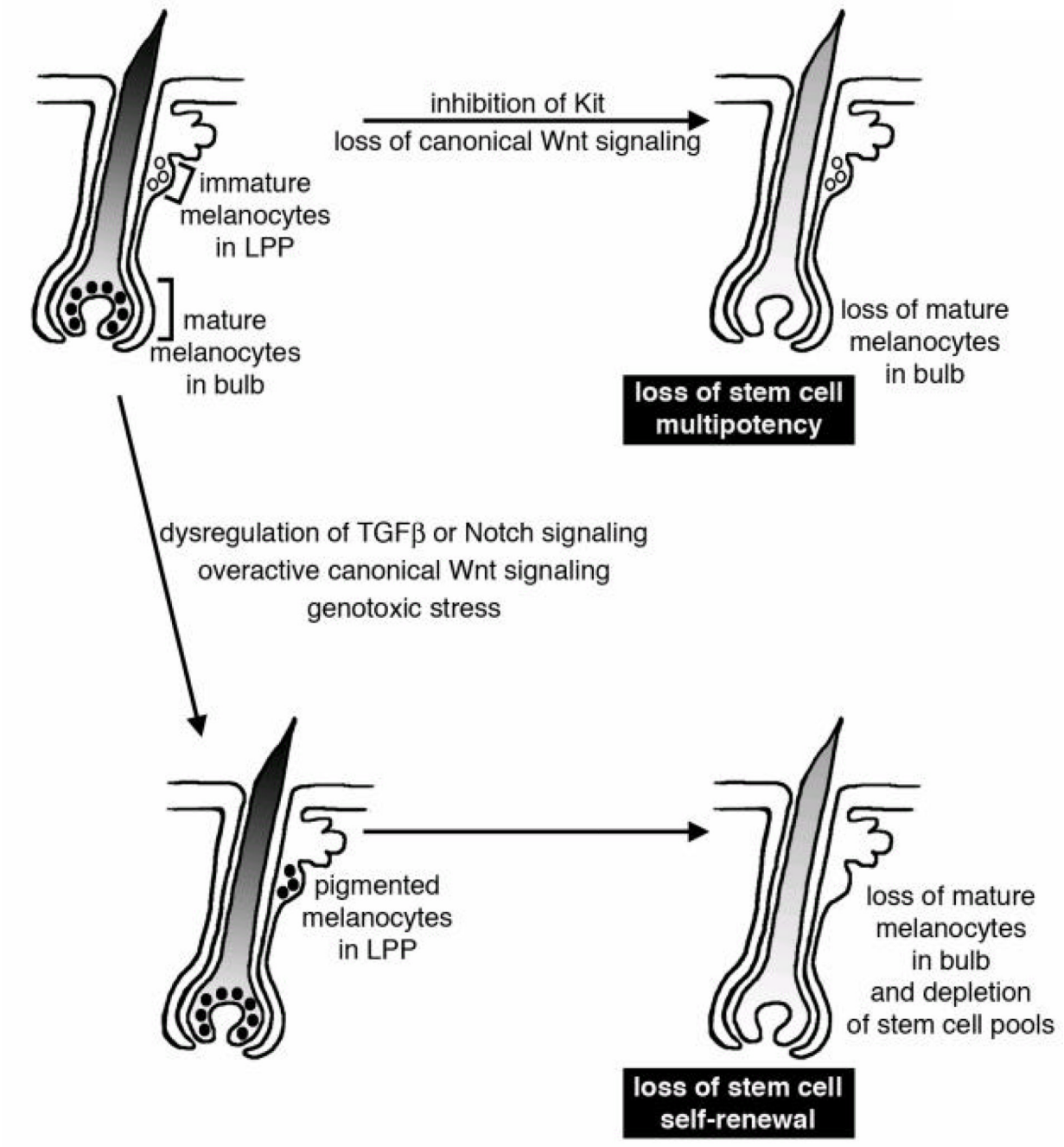

Figure 2. Mechanisms for pigmentation loss and hair graying through extrinsic signals

In a hair follicle (shown as a schematic for an anagen follicle) immature melanocytes are located in a stem cell niche within the bulge region of the hair follicle, or in a region defined as the lower permanent portion (LPP) (51). Loss of stem cell multipotency occurs when the stem cells fail to differentiate, either permanently or transiently. While immature melanocytes are still found in the LPP, there is a loss of pigmented melanocytes in the bulbar region of the hair follicle. Loss of stem cell multipotency may be caused by inhibiting Kit receptor signaling (52, 53) or through loss of canonical Wnt signaling (56). Loss of stem cell self-renewal occurs when the stem cells prematurely differentiate in the LPP and the stem cell pool is depleted. An error in self-renewal first leads to pigmented melanocytes in

the LPP, followed by exhaustion of the stem cells and subsequently by a loss of differentiated melanocytes. A loss of melanocyte stem cell self-renewal may be induced by overactive canonical Wnt signaling (56), dysregulation of TGFβ or Notch signaling (63, 69–72), or through genotoxic stress (124).

Table 1

Studies supporting stable markers in melanoma stem cells.

	SCID or NOD/SCID (mice with severe impairment of T- and B-cells)	NOD/LTSscid/IL2Rγ^{null} (mice with severe impairment of T- and B-cells and an absence of NK-cells)	Rag$^{-/-}$γc$^{-/}$ (mice with an absence of T-, B-, and NK-cells)	"Humanized" mouse models
CD20	Yes (90)	NT	NT	NT
CD133/Prominin-1	Yes (98)	No (83)	NT	NT
ABCG2	NT	NT	NT	NT
ABCB5	Yes (104)	No (82)	NT	NT
CD271/NGFR/p75NTR	Yes (116)	No (82, 116)	Yes (115)	Yes (115)
JARID1B	NT	No (84)	NT	NT

NT=not tested