Cancer stem cells versus phenotype-switching in melanoma
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Summary
Tumours comprise multiple phenotypically distinct subpopulations of cells, some of which are proposed to possess stem cell-like properties, being able to self-renew, seed and maintain tumours, and provide a reservoir of therapeutically resistant cells. Here, we use melanoma as a model to explore the validity of the cancer stem cell hypothesis in the light of accumulating evidence that melanoma progression may instead be driven by phenotype-switching triggered by genetic lesions that impose an increased sensitivity to changes in the tumour microenvironment. Although at any given moment cells within a tumour may exhibit differentiated, proliferative or invasive phenotypes, an ability to switch phenotypes implies that most cells will have the potential to adopt a stem cell-like identity. Insights into the molecular events underpinning phenotype-switching in melanoma highlight the close relationship between signalling pathways that generate, maintain and activate melanocyte stem cells as well as the inverse correlation between proliferation and invasive potentials. An understanding of phenotype-switching in melanoma, and in particular the signalling events that regulate the expression of the microphthalmia-associated transcription factor Mitf, points to new therapeutic opportunities aimed at eradicating therapeutically resistant stem cell-like melanoma cells.

Tumour heterogeneity and cancer stem cells
Cancers arise via the acquisition of mutations that suppress senescence and promote cell division. Subsequently, some cancer cells acquire properties of invasiveness and disseminate to seed new tumours. After several decades of research, almost half of the patients diagnosed with cancer still succumb to its metastatic spread, and surgical intervention remains the most effective therapy despite its limited impact on metastatic disease. The failure of many current approaches to provide successful cancer treatment reflects an underlying problem that is only poorly understood at the molecular level: cancer cell population heterogeneity. Increasing evidence suggests that tumours comprise multiple subpopulations of cancer cells, some of which may be resistant to therapy. Superimposed on this is the notion, originating from studies of acute myeloid leukaemia (Lapidot et al., 1994), that a proportion of cancer cells play a role akin to normal stem cells in tissue homoeostasis, being proposed to self-renew be necessary for tumour maintenance and be responsible for the initiation of new tumours which themselves grow to be comprised of their own heterogeneous repertoires of cancer cell subpopulations (Clarke et al., 2006). However, the term ‘cancer stem cell’ can be misleading. First, unlike normal stem cells, cancer stem cells will have mutations in key signalling pathways that mean their capacity to be regulated like their normal counterparts will be compromised. As discussed later, this may lead to a phenomenon of ‘phenotype instability’ in which cancer cells can switch their phenotypes in response to microenvironmental cues in ways that their normal counterparts would not. Second, normal stem cells are usually regenerated each time they divide by a process of asymmetric cell division. In melanoma for example, it is not yet clear whether asymmetric division
occurs, because phenotype instability can also generate heterogeneous populations. Thus, a more accurate term may be ‘stem cell-like’, implying that a cell shares a number of properties with normal stem cells, but may possess substantially different potential.

The challenges to cancer therapy posed by tumour heterogeneity have long been recognised (Fidler, 1978), but the presence of multiple cancer cell subpopulations, some of which may possess stem cell-like properties, raises a number of important questions. How many cancer cell subpopulations may be identified in tumours and what are their biological properties in terms of differentiation, proliferation and invasion? What is the relative contribution to tumour cell heterogeneity of extrinsic effects such as the microenvironment, or intrinsic properties such as epigenetic patterning? What are the molecular mechanisms underpinning the generation of each subpopulation? Once established, are subpopulation phenotypes static or dynamic, and if dynamic, is phenotype-switching subordinate to a hierarchical relationship resembling that between normal stem cells and their differentiated progeny? Indeed, do any subpopulations within a tumour possess stem-like attributes? Finally, what therapeutic opportunities arise from understanding the biology of cancer subpopulations? Only once these questions are addressed will it be possible to overcome the barrier presented by tumour heterogeneity and deliver an effective anticancer therapy.

In discussing these issues, this review will focus on melanoma, because recent advances in our knowledge of the molecular mechanisms underlying melanoma evolution present an opportunity to explore the cancer stem cell hypothesis, and the origins and consequences of tumour cell heterogeneity. Moreover, there are substantial similarities between melanoma and normal melanocyte development, in terms of the signalling pathways involved and the observed parallels between melanoma metastasis and melanoblast migration from the neural crest. As such, dissecting the molecular switches operating during development to generate or activate melanocyte stem cells can help provide a framework for understanding the origins of melanoma heterogeneity and the biological properties of melanoma subpopulations.

Melanocytes and melanocyte stem cells

Melanocytes derive from pluripotent neural crest cells as non-pigmented melanoblasts (Thomas and Erickson, 2008). Migration via the dorso-lateral pathway leads melanoblasts to populate the basal layer of the epidermis and hair follicles, as well as a number of other sites including the inner ear and choroids; migration via a ventral pathway can also generate epidermal melanocytes via a Schwann cell precursor intermediate (Adameyko et al., 2009). Melanoblasts are differentiated from other neural crest-derived populations by the expression of the tightly regulated microphthalmia-associated transcription factor (Mitf), which is critical for both melanoblast survival and differentiation of the retinal pigment epithelium (Bharti et al., 2006; Hodgkinson et al., 1993; Hughes et al., 1994). Consequently, Mitf-null mice exhibit a white coat colour arising from the lack of melanocytes and a small (microphthalmic) eye phenotype.

Melanoblasts that reach the epidermis differentiate into pigment-producing melanocytes. By contrast, those that arrive at the hair follicle are directed either to become Mitf-positive differentiated melanocytes located in the bulb or to adopt an Mitf-negative stem cell fate and reside in the bulge (Nishikawa and Osawa, 2007). While details of this fate choice remain sketchy, an early event in becoming a stem cell is down-regulation of the transcription factor Sox10 (Osawa et al., 2005), an activator of the Mitf promoter, and TGF-β-mediated down-regulation of Mitf expression (Nishimura et al., 2010). Melanocyte stem cells also exhibit elevated Notch signalling that is required to suppress differentiation of stem cells and melanoblasts (Kumano et al., 2008; Moriyama et al., 2006; Nishikawa and Osawa, 2007; Osawa et al., 2005; Schouwey et al., 2007, 2010) and inhibit the activity of the stress-activated kinase p38 via the activation of a p38 phosphatase (Kondoh et al., 2007). Melanocyte stem cells also suppress Wnt signalling and exhibit elevated expression of TGF-β signalling-related genes (Osawa et al., 2005). It may be that this profile is imposed upon melanoblasts by an extrinsic signal within the niche, but it is not clear whether the niche is required to maintain it, because once a stem cell identity is established, it can be maintained in a niche-independent fashion under simple culture conditions (Nishikawa and Osawa, 2007).

In contrast to melanocyte stem cells, differentiated melanocytes manufacture the pigment melanin in specialised organelles termed melanosomes. In the epidermis, melanosomes are transferred via dendritic processes from melanocytes to nearby keratinocytes where they shield nuclei from UV irradiation (Miyamura et al., 2007). In the hair follicle, melanocytes transfer melanosomes to the growing hair shaft to generate pigmented hair. The expression of the genes necessary for melanosomal biogenesis, melanin synthesis, melanosome transport and dendritogenesis is controlled coordinately by Mitf (Cheli et al., 2010; Steingrimsson et al., 2004; Vance and Goding, 2004).

For each hair cycle, the development of a new hair follicle necessitates the activation of melanocyte stem cells and, via a transit amplifying population, the genesis of differentiated melanocytes in the bulb. Loss of melanocyte stem cells or their failure to activate can give rise to hair greying (Nishimura et al., 2005). As Wnt/β-catenin signalling is a key factor in the generation of a new hair follicle (Gat et al., 1998; Huelsken et al., 2001; Lo Celso et al., 2004; Silva-Vargas et al., 2005), it
Melanoma

Melanoma is among the most aggressive cancers, as life-threatening metastases can occur very early during primary tumour development (Chin et al., 2006). While surgery may be curative if the removed primary is thin, metastatic melanoma is highly refractive to treatment by radio-, chemo-, or immunotherapy and has a median survival of <9 months from diagnosis. Why melanoma is so dangerous a disease, even compared to other cancers, is not at all understood.

Epidemiological evidence points strongly to the primary cause of melanoma being DNA damage elicited by solar UV irradiation correlating with a history of childhood sunburn (Bennett, 2008b). Accordingly, a number of genetic lesions have been reported to contribute to melanoma (Bennett, 2008a). These include constitutive activation of the MAP kinase pathway, for example via activating mutations in BRAF that are found in around 60% of primary melanomas (Davies et al., 2002). As activation of BRAF, or NRAS, alone induces senescence in melanocytes, melanoma progression must be accompanied by compensating events, for example inactivation of the CDKN2A locus encoding p16INK4a via genetic lesions (Bennett, 2008a), epigenetic silencing (Richards and Medrano, 2009; Rothhammer and Bosserhoff, 2007), or repression of p16INK4a expression through activation of Wnt/β-catenin signalling (Delmas et al., 2007). Consistent with senescence representing a major barrier to melanoma initiation is the observation that benign nevi, as well as carrying frequent activating mutation of BRAF or NRAS, include a mass of senescent melanocytes (Gray-Schopfer et al., 2006; Michaloglou et al., 2005). If the initial senescence barrier is overcome, melanomas can progress to a radial growth phase. For the majority of melanomas that do not arise from a pre-existing nevus, senescence bypass via bi-allelic loss of p16INK4a would occur prior to activation of BRAF/NRAS. By contrast, nevi may be generated by mono-allelic loss of p16INK4a prior to BRAF/NRAS mutation, with subsequent inactivation of the second p16INK4a allele leading to melanoma. Thus, the order in which mutations occur will determine whether melanoma arises de novo or from a pre-existing nevus.

It is possible that melanomas can arise by transformation of either a melanocyte stem cell, which unlike many other stem cells may be mono-potent, or a differentiated melanocyte. In mice, co-expression of activated NRAS together with stabilised β-catenin in the melanocyte lineage (Delmas et al., 2007) leads to melanocytic lesions originating from within the hair follicle bulge where melanocyte stem cells are localised, rather than the bulb where differentiated melanocytes are found. However, although the cell of origin for human melanoma has yet to be determined, several lines of evidence suggest that hair follicle stem cells are not likely to be the target. For example, melanoma in situ/early melanoma nearly always involve the junction of epidermis and dermis and tends to spare the hair follicles, very little UV light penetrates to the follicular bulge, and there are no hair follicles at the sites of origin for most acral and mucosal melanomas. Although these observations do not exclude the possibility that oncogenic transformation occurs in as yet uncharacterised inter-follicular melanocyte stem cells at the dermal–epidermal boundary, it is possible that melanoma originates from differentiated melanocytes by a process of de-differentiation arising from ‘phenotype-switching’ as discussed later, especially because differentiated melanocytes can be induced to proliferate in culture.

Melanoma stem cells and tumour initiation

For solid tumours, the cancer stem cell paradigm (Clarke et al., 2006) has become increasingly complex and a matter for some debate (Rosen and Jordan, 2009). Putative cancer stem cells are proposed to share a number of properties with normal stem cells including self-renewal, and just as normal stem cells are necessary for tissue homoeostasis, cancer stem cells are proposed to be essential for tumour maintenance. Yet the property of self-renewal alone will not distinguish cancer stem cells from any other cell within the tumour that retains the potential to divide and survive. This has led to ‘tumour-initiating cells’ as a term that is gaining currency and which is frequently used as a loose alternative to ‘cancer stem cell’. However, as discussed later, while tumour-initiating cells have the capacity to seed new tumours in transplantation assays, they do not always recapitulate the heterogeneity of the original tumour (Heid et al., 2010) and so the term ‘tumour-initiating cell’ is not the same as ‘cancer stem cell’.
Although the cancer stem cell theory does not imply that cancer stem cells are necessarily rare (Gupta et al., 2009; Kennedy et al., 2007), early experiments appeared to confirm that, like normal stem cells, melanoma stem cells would represent a very minor population within the tumour (Fang et al., 2005; Schatton and Frank, 2008; Schatton et al., 2008; Zabierowski and Herlyn, 2008). For example, the ABCB5 transporter as detected by the UG3C2-2D12 monoclonal antibody (Frank et al., 2003) is heterogeneously expressed in primary and metastatic melanomas, but not in nevi, and was identified as a marker for cells initiating melanomas, because ABCB5+ cells derived from human melanomas form tumours comprising both ABCB5+ and ABCB5− populations following serial transplantation into NOD/SCID mice, while ABCB5− cells formed tumours much less efficiently. It was calculated that the numbers of melanoma-initiating cells in this study were less than one per million and that while purification with the anti-ABCB5 antibody used led to around a 10-fold enrichment of melanoma-initiating cells, not all ABCB5+ cells were able to initiate tumour formation, though anti-ABCB5 antibody did inhibit tumour growth. However, the frequency of melanoma-initiating cells in the ABCB5+ population in this study could be an underestimate as concerns have been raised over the use of fluorescent antibodies to sort cells prior to implanting into mice to assay for tumour-initiating potential; the Fc portion of antibodies used in fluorescence-activated cell sorting (FACS) for obtaining discrete populations can severely reduce in vivo survival of xenografted cells (Taussig et al., 2008).

The notion that stem cells would represent a rare population within the tumour was challenged by the observation that of single, unselected melanoma cells derived either from xenografts or directly from patients embedded in matrigel and injected into severely immunocompromised NOD/SCID IL2Rγ−/− mice at least 25% initiate tumorigenesis (Quintana et al., 2008). One conclusion from these studies is that if the ability to seed new tumours following implantation is a particular characteristic of stem cells, then either stem cells are not rare or the stem cells reported in solid tumours that can initiate new tumours may simply be the cancer cells that are better able to survive the transplantation procedure. In part, the increased proportion of tumour-initiating cells in the Quintana et al., study might reflect the use of a more severely immunocompromised mouse in combination with the embedding of transplanted cells in matrigel, a complex proteinaceous extract secreted by a mouse sarcoma cell line that appears to allow human cells to grow faster in mice. Interestingly, no tumours were formed even if 10,000 primary human melanocytes were transplanted (Quintana et al., 2008). Moreover, in the Quintana et al. (2008) study, no correlation between tumour-forming potential and any of 50 diverse markers, including CD271/p75/Ngfr, could be identified. In contrast, Boiko et al. (2010) found that sorting of CD271+ (Ngfr/p75) melanoma cells directly from tumours revealed they preferentially formed tumours in severely immunocompromised mice compared to their CD271− counterparts (70% versus 7%), and the CD271+ population generated heterogeneous tumours comprising both CD271+ and CD271− cells. Intriguingly, there was an increase in transplantation efficiency of xenopassed CD271− cells, rising from 7% when taken directly from the human tumour to 23% of xenopassed cells. Moreover, despite the apparent enrichment of tumour-initiating cells in the CD271+ population and that most CD271− cells did not express markers of differentiation such as TYR and MART, a substantial minority of CD271− cells co-expressed these differentiation markers that are not expressed in melanocyte stem cells and that might be expected to mark non-stem cells.

Following these studies, it is clear that outcomes can be affected by technical issues such as the severity of the immune defect of the mice used, the processing of the cells taken from the human tumour and the precise site of injection. In this respect, it is also important to ask why some cells did not form tumours. Perhaps some succumbed to the mechanical forces of transplantation, or apoptosed, or senesced owing to lack of survival/proliferation signals. For example, although CD271 was used as a marker to positively select for cells (Boiko et al., 2010), it cannot be ruled out that during the 3 h used for tumour dissociation, a proportion of cells lost expression of functional receptors, including CD271, required for in vivo proliferation such that they remained quiescent on transplantation. Recovery of receptor expression would delay the acquisition of proliferative potential leading to a loss or delay in tumour formation in the CD271− population. In this scenario, while it is clear that CD271+ and CD271− cells co-exist in tumours, processing of the tumour to generate single cells could in principle bias tumour formation towards cells that retain expression of functional receptors such as CD271. Future studies will need to ensure that absence of any particular marker on non-tumour-forming populations does not reflect a generalised loss of pro-proliferative receptors. Moreover, in direct contrast, (Held et al., 2010) isolated individual melanoma cells from three different genetically engineered mouse melanoma models bearing combinations of the CD34 and CD271/Ngfr/p75 markers. Although CD34 has yet to be identified as a marker for melanocyte stem cells, it does mark keratinocyte stem cells in the bulge (Blanpain et al., 2004) and has been identified in a small subset of melanomas (See Breza and Magro, 2005 for example), as well as a number of non-melanoma cells such as fibroblasts, haematopoietic and epithelial progenitor cells. Strikingly in this study, all single CD34+CD271− cells formed tumours, while CD34−CD271− cells formed tumours 57% of the time, and no tumours were formed using CD271+ cells derived from four of five tumours even if up to 1000 cells were injected. Moreover, all
CD34+CD271- clones failed to exhibit heterogeneity in the tumours generated, whereas heterogeneity was detected in tumours derived from the CD34+CD271+ population. Apart from the obvious discordance with the results from Boiko et al. on the usefulness of CD271/NGfr/p75 as a marker for melanoma stem cells, two key conclusions can be drawn from the Held et al. study: that tumour-initiating cells derived from the same original tumour have different capacities to recapitulate heterogeneity and that tumour initiation and ‘self-renewal’ equating to proliferation can be uncoupled from the self-renewal linked to the generation of heterogeneity. Moreover, when single cells were sorted into 96-well plates, expanded and analysed by analytical flow cytometry at each passage for three passages, several of the marker subsets changed both following sorting and with each passage (Held et al., 2010), supporting the concept of phenotypic-switching outlined below. These types of single-cell experiments are particularly important to establish the extent of phenotypic variability when only one marker (i.e. two subsets) is being analysed.

In the studies highlighted previously, the key criteria in the assays for melanoma stem cells are their ability to form tumours when serially transplanted into immunocompromised mice and their capacity to generate heterogeneity. However, an important distinction should be made between seeding a tumour by artificial implantation in immunocompromised animals versus the natural process whereby cells from the primary tumour invade the surrounding tissue to form local or distant metastases in which heterogeneity may arise. In patients, only the subpopulation of cells within the tumour that are invasive can be natural tumour-initiating cells that generate the heterogeneity characteristic of metastases. It is therefore the properties of invasive melanoma cells in human cancer that should be examined for stem-like characteristics.

**Phenotype-switching and melanoma metastasis**

A key event in melanoma progression occurs when cells undergo a transition from radial to vertical growth phase associated with a heightened potential for metastasis and evasion from senescence mechanisms (Gray-Schopfer et al., 2006; Vance et al., 2005). At this point in melanoma progression, cellular heterogeneity is already established. The molecular events that drive melanoma metastasis are likely to be diverse, but may be represented by one of two mechanisms (Figure 1), each with substantially different implications.

In the genetic model, the acquisition of pro-metastasis mutations represents an irreversible event where metastases will be genetically distinct from cells in the original primary tumour. While care has to be taken in distinguishing mutations that drive metastasis from those that are bystanders, several studies have identified mutations in genes that are clearly associated with metastatic behaviour in melanoma. For example, interspecies comparative oncogenomics identified NEDD9 as a gene whose genetic amplification is associated with melanoma metastasis (Kim et al., 2006). However, if metastasis is indeed driven primarily by the acquisition of irreversible genetic changes, then it is surprising that a consistent gene expression signature that reflects this has yet to be identified.

An alternative model that is gaining recognition and has significant implications for our understanding of melanoma stem-like behaviour predicts that metastasis and phenotypic heterogeneity is driven by specific gene expression programmes that are imposed by the cellular microenvironment rather than by the accumulation of genetic events. In this model, changing microenvironmental conditions, to which melanoma cells contribute, regulate melanoma cell switching back-and-forth between phenotypes circumscribed by either the potential for proliferative tumorigenesis or a melanoblast-like migratory capacity. Thus, genetic lesions yielding constitutive activation of melanoma-associated signal transduction pathways, coupled to microenvironment-mediated interchanges, would in principle be sufficient to drive melanoma progression. Moreover, phenotype-switching implies that metastasis can be triggered as soon as melanoma cells encounter appropriate microenvironmental signals and would account for the propensity for melanoma to metastasise early because the acquisition and selection of specific pro-metastasis mutations is unnecessary. A further implication of phenotype-switching is that once cells have migrated away from the primary tumour, they will only continue to

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**Figure 1.** Pathways towards metastasis. In the genetic model, acquisition of mutations that promote invasiveness leads to cells migrating away from the primary tumour before arriving in a new site where they divide to form a new metastasis. As the genetic lesions are irreversible, metastases will be genetically distinct from the primary tumour. By contrast, an alternative model suggests that metastases arise because of microenvironment-driven changes to a cell’s phenotype, leading to some cells acquiring a slow proliferating, stem cell-like phenotype with invasive potential. Given the appropriate signals, these cells will migrate to find a new niche where a different microenvironment will lead to either a resumption of proliferation and formation of a new metastasis or dormancy. The molecular mechanisms that mediate phenotype-switching in melanoma are described in the text.

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migrate or revert to a proliferative phenotype if they encounter an appropriate microenvironment. It has been observed in a mouse model that invasive phenotype cells escape melanoma allografts in large numbers (Pinner et al., 2009). It is possible that nearly all of these succumb to senescence or apoptosis and only a fraction survives to switch back to the proliferative state. Alternatively, it may be that the frequency of the switch from invasive to proliferative is very low, giving the appearance of dormancy over long periods. Another possibility is that surviving invasive cells may encounter a niche where an absence of pro-migratory and proliferative signals means they remain dormant, perhaps for many years, but all the while retaining their potential to switch back to a proliferative state. The presence of dormant melanoma cells, discussed by Ossowski and Aguirre-Ghiso (2010), implies that although all melanoma cells in an individual would possess the same activating mutations in BRAF or NRAS, some cells must override the pro-proliferation signals engendered to enter quiescence and that the emergence from dormancy would arise from the reinstatement of the pro-proliferative signal. Such a series of events would be more readily explained by the effects of a changing microenvironment imposing a reversible phenotype-switch than a series of genetic events that first suppress and then reactivate pro-proliferation signalling. However, as we discuss in the following paragraphs, while there is accumulating evidence to suggest that phenotype-switching plays a key role in melanoma, it should not be viewed as being mutually exclusive to the impact of clonal evolution from a genetically diverse pool.

Support for the phenotype-switching model comes from several lines of evidence. In development, melanoblasts exhibit an intrinsic migratory capacity that is switched on and off by extracellular cues, leading to a precise and coordinated programme of proliferation and migration away from the neural crest along specific pathways within the embryo (Thomas and Erickson, 2008). In addition, in melanoma, E-cadherin expression is lost during the initial acquisition of invasive properties and then frequently re-expressed in metastases (Danen et al., 1996), again suggesting that gene expression patterns associated with metastasis are reversible. The observation that microenvironmental conditions contributed by metastatic melanoma cells secreting extracellular matrix-modifying components can reprogramme normal melanocytes to a reversible invasive phenotype (Sefor et al., 2005) also lends weight to the argument for phenotype-switching playing a significant role in melanoma progression.

Further evidence for phenotype-switching comes from gene expression profiling of 86 melanoma cell lines in culture that identified two major expression signatures (Hoek et al., 2006). One is associated with weakly invasive, but rapidly proliferative cells whose growth can be inhibited by TGF-β. By contrast, the second comes from melanoma cells that divide slowly, yet exhibit a high migratory/invasive potential and resist TGF-β-mediated growth inhibition. The invasive cohort also exhibited increased expression of CD271/Ngfr/p75. Significantly, although the gene expression profiles of these cell lines were fixed in culture, when transplanted in vivo, each class generated heterogeneous tumours containing cells with both kinds of expression profile (Hoek et al., 2008), highlighting the potential for phenotype-switching in vivo. Inspection of genes differentially expressed between cell lines with different profiles revealed the central finding that invasive phenotype cells exhibited low levels of Mitf expression, while proliferative phenotype cells expressed high levels of Mitf (Hoek et al., 2006).

These observations were reinforced by examining in melanoma the expression of the Brn-2 (POU3f2, N-Oct3) transcription factor (Cook and Sturm, 2008), which is up-regulated by both MAP kinase and β-catenin signalling (Goodall et al., 2004a,b). Brn-2 is expressed in a mutually exclusive subpopulation of melanoma cells to those expressing Mitf, consistent with its capacity to down-regulate Mitf expression and promote invasive-ness in vitro (Goodall et al., 2008). Importantly, intravitral imaging in real time in vivo of a subcutaneous tumour formed by melanoma cells engineered to harbour a Brn-2 promoter-GFP-reporter (Pinner et al., 2009) revealed a striking heterogeneity of expression: motile cells in the primary tumour tended to express higher levels of Brn-2-GFP and low pigmentation, a surrogate marker for Mitf expression, which was maintained when cells entered the bloodstream, but low Brn-2-GFP cells predominated in the resulting lung metastases. Thus, Brn-2 promoter activity reflected the invasive potential of the cells that were able to switch: low and high GFP in the primary tumour, high GFP in the bloodstream, and again a mix of high and low GFP in the resulting lung metastases. Taken together these experiments provide substantial experimental evidence to support the notion that phenotype-switching occurs in vivo and that one of the key drivers in the switch is the regulation of Mitf expression.

Mitf and phenotype-switching

Mitf was initially identified as a survival factor in melanocyte development (Hodgkinson et al., 1993; Hughes et al., 1994) and was subsequently implicated in differentiation through the activation of pigmentation genes (Steingrimsson et al., 2004). Its role in melanoma was initially confusing. Some reports suggested that Mitf could inhibit proliferation via up-regulation of the CDKN1A and CDKN2A genes encoding the cyclin-dependent kinase inhibitors p21CIP1 and p16INK4a (Carreira et al., 2005; Loercher et al., 2005) and ectopic expression of Mitf could counteract BRAF-mediated stimulation of melanocyte proliferation (Wellbrock and
pressing both p27Kip1 expression and senescence and inhibition of p53 and senescence (Giuliano et al., 2010). Thus, cycle arrest (Carreira et al., 2006), while long-term depletion of Mitf in melanoma cell lines leads to decreased expression of the diaphanous-related formin Dia1, and thereby indirectly to a p27Kip1-dependent cell cycle arrest (Carreira et al., 2006), while long-term depletion triggers a DNA-damage response, up-regulation of p53 and senescence (Giuliano et al., 2010). Thus, paradoxically, Mitf can promote cell division by suppressing both p27Kip1 expression and senescence and inhibit proliferation via up-regulation of p21Cip1 and p16INK4a (though the latter is frequently mutated or silenced in melanoma). Significantly, Mitf-depleted melanoma cells are also highly invasive (Carreira et al., 2006), and in vivo it is also likely that in those cells that go on to initiate metastases, low levels of Mitf are balanced by enhanced anti-senescence signalling.

To account for the pro- and anti-proliferative roles for Mitf, Carreira et al. (2006) proposed a rheostat model (Figure 2A) that suggests a minimum of three distinct phenotypes of melanoma cell will exist within a tumour: differentiated, proliferative, and invasive. Differentiated cells are non-proliferative and non-invasive and express Mitf and its downstream differentiation-associated genes such as MART1 and Tyrosinase (Steingrimsson et al., 2004), leading to the pigment characteristic of most melanomas. Proliferative cells would also be Mitf-positive but would express Mitf-target genes implicated in proliferation and survival such as CDK2 and BCL2 (Du et al., 2004; McGill et al., 2002). The difference between Mitf-positive proliferating and Mitf-positive differentiated cells is likely to reflect post-translational modifications directing Mitf to different sets of target genes (Cheli et al., 2010). However, how Mitf deciphers the output from the signalling pathways that lead to its phosphorylation, acetylation, ubiquitylation and sumoylation to drive expression of different sets of target genes (differentiation versus proliferative) is not well understood. By contrast, invasive cells would proliferate slowly and like melanocyte stem cells would not express Mitf or its differentiation-associated target genes involved in pigmentation.

**Sources of melanoma heterogeneity: signalling the phenotype-switch**

The evidence suggests that phenotype-switching occurs in vivo in response to microenvironment-driven changes in Mitf expression, and as a consequence, the generation of a heterogeneous population of melanoma cells with different biological properties. But what is the origin of the heterogeneity? Any biological system may be subject to a varying degree of noise in key signalling pathways that may lead to heritable changes in gene expression through epigenetic mechanisms (Brock et al., 2009; Turner, 2009). To prevent such noise triggering an inappropriate cellular response, signalling systems may be buffered so that only once a critical signalling threshold is crossed will cells respond to yield a specific biological output such as a switch in phenotype. One way to achieve an effective buffer is to require a cooperative input from two distinct signalling pathways to trigger a response (Figure 2B). In normal cells, such signal cooperation would be unlikely to occur by chance fluctuations in signalling output. However, in cancer cells, genetic lesions leading to the constitutive activation of one signalling pathway that plays a key role in buffering the output from a second pathway may in effect cause cells to become hypersensitive to changes in their microenvironment leading to what we may term ‘phenotype instability’. Thus, phenotype-switching in cancer cells may in part reflect a lowering of the threshold necessary to trigger a change in cell identity in response to external signals originating within the tumour microenvironment that may vary substantially from location to location. Cells may encounter a multitude of variable environmental signals that include nutrient availability and oxygenation, as well as cell–cell signalling between cancer cells and from non-cancer cells such as fibroblasts, or infiltrating cells from the immune system. A changing microenvironment may also be experienced in response to therapies, for example DNA-damaging agents or regulators of epigenetic inheritance, such as the deacetylase inhibitors that are currently in clinical trials.

Although at the molecular level key factors that drive melanoma phenotype-switching are likely to impact on...
Mitf expression, at the cellular level heterogeneity can be generated by cell division-dependent and independent mechanisms (Figure 3). The activation of a stem cell is generally thought to generate two daughter cells by asymmetric cell division, though this is yet to be proven in the melanocyte lineage. One daughter ultimately differentiates after a rapid proliferation phase, while the other returns to quiescence and regenerates the stem cell. Frequently, asymmetric division is controlled by Notch signalling, and in melanocytes, Notch is necessary for the maintenance of melanocyte stem cells and Notch activity is frequently activated in melanoma (for review see (Pinnix and Herlyn, 2007)). However, while asymmetric cell division may operate in development, it is not clear whether cell division is required for phenotype-switching in melanoma. In principle, simply down-regulating Mitf in a cell will lead to a p27-dependent cell cycle arrest and adoption of an invasive stem cell-like phenotype. The forward and reverse reaction of each step between the three different phenotypes (differentiated, proliferating and stem cell-like) may vary depending on a combination of microenvironmental cues combined with altered thresholds imposed by genetic alterations and will lead to tumours with different characteristics.

Intriguingly, there are substantial parallels between the pathways regulating melanocyte stem cell quiescence and those that have been implicated in the generation of the slow proliferating, invasive melanoma phenotype. Of these, TGF-β signalling is attracting increasing attention. Many human tumours secrete substantial quantities of TGF-β that can promote invasiveness, dissemination and tumour growth. The autocrine and paracrine roles of TGF-β in melanoma progression, particularly in promoting invasiveness and anchorage independence, have recently been reviewed (Javelaud et al., 2008). As mentioned earlier, TGF-β signalling down-regulates Mitf expression and appears to be critical for the generation and maintenance of melanocyte stem cells (Nishimura et al., 2010), and consistent with this, TGF-β target genes are up-regulated in melanocyte stem cells (Osawa et al., 2005). In melanoma, cell lines with a more invasive, low Mitf gene expression profile also exhibit hallmarks of high levels of TGF-β signalling (Hoek et al., 2006), and increased expression of TGFB2, TGFB3 and TGFB1 was observed in ABCB5+ compared to ABCB5- cells (Schatton et al., 2010). Moreover, a role for TGF-β in melanoma dissemination in vivo was recently highlighted by intravital imaging of xenograft tumours (Pinner et al., 2009), and in breast

Figure 3. Phenotype-switching, self-renewal and generation of heterogeneity. Activation of a stem cell (I) leads to cell division in which one daughter cell proliferates and eventually generates differentiated progeny, while the other becomes quiescent and regenerates the stem cell. In principle, activation of a stem cell, for example by transient up-regulation of Mitf, could give rise to two stem cell daughters (II). By contrast, stem cells could be renewed by an indirect mechanism (III) in which some proliferating cells are driven by microenvironmental signals to adopt a stem cell-like identity. Whether some or all such mechanisms operate in melanoma to generate stem cell-like cells is not clear. By contrast, phenotype-switching in response to a changing microenvironment (IV) may generate different phenotypes without a need for cell division. For example, simply down-regulating Mitf in a cell will lead to a p27-dependent cell cycle arrest and adoption of an invasive stem cell-like phenotype. The forward and reverse reaction of each step between the three different phenotypes (differentiated, proliferating and stem cell-like) may vary depending on a combination of microenvironmental cues combined with altered thresholds imposed by genetic alterations and will lead to tumours with different characteristics.
cancer cell lines, similar studies using intravital imaging of cells containing TGF-β reporters have also indicated that transient up-regulation of TGF-β signalling is characteristic of a switch to invasiveness (Giampieri et al., 2009). These data together imply that TGF-β, most likely acting in part via its ability to down-regulate Mtf, may play a key role in the generation of melanoma stem-like cells with high invasive potential. However, whether a melanoma cell exposed to TGF-β realises its potential for invasion is likely to depend on it also activating the RHOF-ROCK pathway that is well known to promote melanoma invasiveness in vitro (Carreira et al., 2006; Sahai and Marshall, 2003; Wilkinson et al., 2005). The conditions that activate TGF-β signalling and drive the switch to invasiveness likely include hypoxia (Coppie, 2010; Noda et al., 2010) and wound-healing processes (Brinckmann et al., 2010).

While it is evident that TGF-β signalling may be a key trigger in the switch from a proliferative to an invasive phenotype, other pathways are also likely to be involved. In particular, activation of the p38 stress-signalling pathway can up-regulate Mtf expression and trigger activation of melanocyte stem cells out of synchrony with the hair cycle (Saha et al., 2009) as well as modulate the ability of Mtf to regulate transcription (Mansky et al., 2002). In melanoma increased p38 signalling would arise via UV irradiation of the skin, but also via any inflammatory response. Superimposing the up-regulation of p38 signalling on the Mtf rheostat would indicate that cells with a low Mtf, invasive profile would switch to a proliferative phenotype, while proliferating cells might be driven to differentiation.

The possible role of p38 in promoting the invasive stem-like cell to proliferation switch in melanoma can potentially be counteracted by activation of the Notch signalling pathway. Notch signalling is required for the maintenance of normal melanocyte stem cells (Aubin-Houzelstein et al., 2008; Kumano et al., 2008; Moriyama et al., 2006; Schouwey et al., 2007) with inactivation of the pathway leading to hair greying. Significantly, the effects of Notch inactivation may be reversed through inactivation of p38 signalling pathway (Nishikawa and Osawa, 2007). Notch signalling is constitutively activated in many melanomas and confers a transformed phenotype on melanocytes (Pinnix and Herlyn, 2007; Pinnix et al., 2009), but can also stimulate expression of the p38-phosphatase MKP-1 (Kondoh et al., 2007), thereby potentially suppressing the anti-stem cell-like effects of p38 signalling. Although there appears to be a significant antagonism between Notch and p38 signalling, Notch also impacts on other melanoma-associated signalling molecules. In particular, Notch is both upstream and downstream of the serine-threonine protein kinase Akt, a key survival factor downstream from PI3K that can promote switching to an invasive phenotype (Govindarajan et al., 2007; Liu et al., 2006) [reviewed in (Madhunapantula and Robertson, 2009)] and is regulated by hypoxia (Bedogni et al., 2008).

The parallels between the signalling pathways de-regulated in melanoma and those that control the activation or quiescence of melanocyte stem cells are further highlighted by the fact that the β-catenin, which is central to the activation of normal melanocyte stem cells, is constitutively nuclear in many melanomas (Rubinfeld et al., 1997), activates the Mtf promoter (Takeda et al., 2000) and directly interacts with the Mtf protein (Schepsky et al., 2006). However, as β-catenin also activates the expression of Brn-2, a regulator of the Mtf promoter, the consequence of β-catenin for Mtf expression will depend on whether Brn-2 is a repressor (Goodall et al., 2008; Kobi et al., 2010) leading to a transient up-regulation of Mtf, or an activator (Wellbrock et al., 2008) that enables a further potentiation of Mtf expression. The signals that facilitate the ability of Brn-2 to switch from a repressor to an activator may be a key determinant of melanoma biology, though their identities are currently unknown.

These data implicate TGF-β, Notch, p38 and β-catenin, key factors regulating normal melanocyte stem cell quiescence, as major modulators of phenotype-switching in melanoma. For each phenotype-switch, there will be both a forward and reverse reaction (Figure 3) with the balance between the different signalling pathways determining to a large extent whether or not Mtf is expressed and consequently whether melanoma cells proliferate or adopt an invasive, slow proliferating and potentially therapeutically resistant phenotype. In the switch from melanocyte stem cells to differentiation via a transit amplifying population, there is a clear hierarchy: differentiated cells do not normally generate stem cells. By contrast, in melanoma, the reversibility of any phenotype-switch may be determined by the nature of the signalling pathways that are de-regulated via genetic lesions, which in turn affect the thresholds required for cells to switch phenotypes, coupled with the impact of the cellular microenvironment. If the combination favours the proliferative phenotype over the invasive, a tumour may grow rapidly, but for a given size, it will seed fewer metastases than a tumour whose cells are not subject to a proliferative phenotype bias.

Much of the debate as to whether melanoma stem cells exist and their frequency within a tumour turns on what is meant by ‘tumour-initiating cells’ or ‘cancer stem cell’ and the distinction between a cell’s phenotype at a given moment and its potential to adopt a different identity. Candidates for melanoma stem-like cells include the high Brn-2, low Mtf melanoma subpopulations that is invasive and poorly proliferative and resemble melanocyte stem cells (Pinner et al., 2009), as well as ABCB5+ (Schatton et al., 2008), and CD34+CD271+ (Held and Bosenberg, 2010) cells. Yet if phenotype-switching is reversible, then perhaps the greater majority of melanoma cells have the potential to adopt a
‘stem cell-like’ phenotype, accounting for the high proportion of cells able to seed tumours in severely immunocompromised mice (Quintana et al., 2008). On the other hand, if, as for normal melanocyte stem cells, there is a hierarchy of phenotype-switching, then at least some cells within a tumour may bear stem cell characteristics for prolonged periods. In this respect, a hierarchy has been observed in some studies. For example, Schatton et al. (2008) used differentially fluorescently tagged melanoma cells to show that cells isolated using the anti-ABCB5 antibody and mixed with ABCB5− cells could generate tumours containing both ABCB5+ and ABCB5− cells, while the ABCB5− cells did not generate ABCB5+ cells. Held et al. (2010) demonstrated that CD34+/CD271+/Ngfr/p75− cells formed tumours that appeared to be restricted to CD271− cells, whereas CD34+/CD271+/Ngfr/p75− cells initiate tumours containing both CD271+ and CD271− populations. Moreover, FACS sorting and tail vein injection of high and low Brn-2 promoter-GFP-expressing melanoma cells isolated from mouse tumours revealed that while the high GFP cells generated tumours with a mix of high and low GFP, the low GFP cells generated tumours with an overwhelming majority of cells having a low GFP profile (Pinner et al., 2009). Thus, in these experimental contexts, there may exist a hierarchy of phenotype-switching in a proportion of melanoma cells. However, further experiments are needed to determine whether this apparent hierarchy truly exists. For example, to obtain detectable tumours, at least a proportion of the low Brn-2 population, or other stem cell candidates, must switch to a proliferating phenotype, thereby generating a mixed population, while no such selective bias would be present on the low Brn-2 proliferating population. Nevertheless, taken together, the results may suggest that either the threshold for invasive stem-like cells to switch to a proliferative state is lower than the threshold for the reverse event or that within a tumour the microenvironmental trigger for the switch from proliferative to stem cell-like phenotype is less common than the signals that drive the stem cells to divide. Thus, the hierarchy implicit within the stem cell model may simply reflect a combination of the frequency of key signals and the thresholds required to switch phenotypes.

In agreement with this, recent experiments form the Herlyn lab have identified a low-frequency subpopulation of slow-cycling melanoma cells within cell lines and tumours that express high levels of the histone H3 lysine 4 demethylase JARID1b (Roesch et al., 2010). Although the JARID1b-positive cells can self-renew and give rise to rapidly proliferating progeny, the capacity of melanoma cells to initiate new tumours in immunocompromised mice is independent of JARID1b expression. This is reminiscent of the results from Held et al. (2010), discussed earlier, where sorting for CD34 and CD271 uncoupled tumour-initiating capacity from the ability to generate a heterogeneous population. Significantly, like Brn-2, JARID1b expression is dynamic and does not follow a rigid hierarchical model, supporting the notion that most melanoma cells will have the potential to adopt a transient stem cell fate in response to appropriate signals. Moreover, because a histone H4 tri-methylation mark is a characteristic of active transcription, the increased expression of the JARID1b demethylase in a subset of slow-cycling stem-like melanoma cells suggests that they may be relatively transcriptionally inert. This idea is compatible with the fact that melanocyte and other adult stem cells appear negative for phosphorylation of Ser-2 in the RNA polymerase II C-terminal domain, another marker of active transcription (Freter, R., Osawa, M., and Nishikawa, S.-I. (2010). Adult stem cells exhibit global suppression of RNA Polymerase II Ser-2 phosphorylation (Freter et al. 2010). Indeed, if melanoma stem cells resemble their normal counterparts and are also transcriptionally inert, then one might anticipate that their defining characteristic may well be the absence of any specific stem cell markers, a notion reminiscent of the CD34+/CD271− population with stem-like properties detected in mouse melanomas (Held et al., 2010).

### Therapeutic implications and future directions

For anti-melanoma strategies to be successful, it is imperative that any non-dividing, stem cell-like cells be eliminated because they drive metastatic disease, may initiate new tumours even after many years of apparent dormancy and may provide a reservoir of therapeutically resistant cells. As such, the presence of multiple subpopulations of melanoma cells each with distinct biological properties poses a particular challenge to anti-melanoma therapies that are primarily targeted at dividing cells (chemotherapy) or those expressing differentiation markers (immunotherapy). Increasing success is being achieved via a new generation of inhibitors designed to target specifically activated BRAF (PLX 4032) or kit (Imatinib). However, while PLX 4032 can apparently clear overt melanoma in some patients, subsequent relapse may indicate that cells acquire resistance via acquisition of additional genetic or epigenetic events (Heidorn et al., 2010; Poulakis et al., 2010). Whether resistant cells appear at random by selection from a genetically diverse pool or whether, for example, dormant Mitf-negative or CD34+/CD271− stem cell-like cells represent an epigenetically resistant reservoir from which resistant proliferating cells may emerge remains to be investigated.

The generation of diverse melanoma subpopulations clearly represents a moving target for melanoma therapy and is reflected in the fact that different metastases within a patient may exhibit variable sensitivities to therapy, something that would not necessarily be predicted by a simple cancer stem cell model in which all metastases might be expected to exhibit the same
heterogeneity. Variable resistance to therapy could however be achieved either by genetic variation or by phenotype-switching generating different proportions of each melanoma subpopulation in response to different local microenvironments. The fact that a small subpopulation of JARID1a-expressing non-small-cell lung cancer cells in culture exhibit reversible drug tolerance (Sharma et al., 2010) tends also to support the notion that variations in drug resistance may be induced by microenvironmentally driven switches in phenotype and that phenotype-switching may be a mechanism common to many cancers.

The development of an effective anti-melanoma strategy therefore requires us to monitor and manage the subpopulations, which means that we must identify and validate robust markers that distinguish each population and reflect their differing biological properties. To date, however, there is little consensus on which marker(s) best defines a melanoma stem cell-like population. The ABCB5 marker is largely based upon the use of a single anti-ABCB5 monoclonal, and it is likely that technical reasons (Shackelton, 2010) underpin the discrepancies concerning the correlation (Boiko et al., 2010) or not (Held and Bosenberg, 2010; Quintana et al., 2008) of CD271/Ngrf/p75 with cells with stem cell characteristics. The potential of Brn-2 as a marker for stem cells in human tumours has yet to be fully explored, and further in depth comparisons between JARID1b and other markers are required before any consensus may be reached. Possibly, melanoma cells with stem-like properties will be best characterised by the absence of most markers, including Mitf. Alternatively, melanoma stem-like cells may express a specific marker but that marker may vary depending on the genetic and epigenetic events that drive tumour formation in the first place. That is, there is no reason to suppose that all melanoma stem-like cells in all melanomas will express a single marker common to that subpopulation. In this respect, the development of new epigenetic markers based on chromatin architecture may represent an important step forward because chromatin states may differ in cells with different biological properties independent of whether a gene is expressed.

While no consensus has yet been reached on the identity of a robust marker for melanoma stem cells, it is nevertheless clear that melanomas contain cells with the stem-like property of being able to initiate tumours that recapitulate heterogeneity. Indeed, the existence of such cells can be inferred from the fact that metastases are usually heterogeneous and have presumably been seeded by single cells escaping from the primary tumour. In other words, as predicted by the Mitf rheostat model, invasive cells will have stem-like properties. Importantly, most cells within the tumour, irrespective of their current identity, may have the potential to adopt a stem cell-like invasive phenotype given appropriate microenvironmental cues. Self-renewal and heterogeneity may be generated either by asymmetric cell division or by phenotype instability. Moreover, although phenotype-switching is likely to be reversible, the thresholds for any microenvironment-driven phenotype-switch may be higher when switching from a more differentiated or proliferative phenotype than when switching from a stem cell-like phenotype. A deep understanding of the molecular mechanisms underlying phenotype-switching in vivo, and in particular of how Mitf is regulated, may enable the manipulation of its expression and activity and consequently represent a major approach towards eradicating those populations of melanoma cells that provide a reservoir of therapeutically resistant cells with high metastatic potential. However, given our current understanding of the mechanisms underpinning phenotype-switching in melanoma, care also needs to be taken to ensure that therapies do not promote the generation of invasive stem-like cells. For example, HDAC histone deacetylase inhibitors being tested for efficacy in melanoma down-regulate Mitf expression (Yokoyama et al., 2008) and therefore may trigger an increase in the invasive stem-like subpopulation of melanoma cells. Despite these issues, anti-melanoma strategies designed to take into account heterogeneity and phenotype-switching are more likely to succeed than those that do not.

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