From UVs to Metastases: Modeling Melanoma Initiation and Progression in the Mouse

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Cutaneous malignant melanoma is highly invasive and capable of metastasizing to distant sites where it is typically resistant to available therapy. While striving to prevent or eradicate melanoma, researchers have two significant advantages not shared by those working on many other cancers. The main environmental etiological agent, UV radiation, is known and melanocytic lesions are excisable for molecular analysis from most stages. Yet knowledge about how UV initiates melanoma has been insufficient to achieve prevention, and the understanding of metastatic mechanisms has been inadequate to reduce mortality. Here, we review the value of melanoma mouse models, focusing on these critical early and late stages.

Journal of Investigative Dermatology (2008) **128,** 2381–2391; doi:10.1038/jid.2008.177

INTRODUCTION

According to the latest Surveillance Epidemiology and End Results report (Espey *et al.*, 2007), cutaneous melanoma is one of the three remaining major malignancies that exhibit significantly positive rates of increase (annual percent change) in the United States (Surveillance Epidemiology and End Results Incidence Trends by Race/Ethnicity, 1995–2004, Table I-23, http://seer.cancer.gov/csr/1975_2004/sections.html). It is estimated that more than 60,000 new cases of malignant melanoma will be diagnosed in 2008. Worryingly, the prevalence of melanoma is rapidly increasing in women under the age of 40, among whom it has now surpassed breast cancer to become the most prevalent malignancy (US Prevalence Counts for 2004, Surveillance Epidemiology and End Results, Table I-22). This is grim news among the highly favorable statistics showing that many types of cancers are on the decline, or at least leveling off. It should sound an alarm and provide the impetus for the melanoma research community to seek novel and innovative approaches to close the gaps in our understanding of the biology of this disease.

Melanoma is thought to develop in a classical stepwise manner (Figure 1) (Miller and Mihm, 2006), starting with a benign nevus consisting of a clonal population of melanocytes that have aberrantly proliferated to a hyperplastic lesion that does not progress due to cellular senescence (Mooi and Peeper, 2006; Gray-Schopfer *et al.*, 2006). Once senescence is overcome, the nevus exhibits dysplasia and can subsequently progress to a superficial spreading stage (radial growth phase (RGP)) that is confined to the epidermis and has low invasive potential. Finally, RGP cells acquire the ability to invade the dermis (vertical growth phase (VGP)) and metastasize (Miller and Mihm, 2006). Sadly, once distant metastases are evident, patient prognosis is dismal and the median survival is ~6 months. It is noteworthy that clinical data also suggest that not all melanomas arise in such stepwise fashion, and metastatic disease can arise in patients without overt primary melanoma.

The major environmental etiologic agent for melanoma is known to be the UV spectrum of solar radiation, which should provide a huge advantage over the majority of other cancers and inform preventive strategies. The facts that the major risk factor for melanoma has long been appreciated and its different disease states, from benign nevus to metastatic primary melanoma, are readily visible and excisable make melanoma highly amenable to molecular analysis. However, despite these advantages, the mechanisms by which sunlight initiates melanoma and melanoma cells achieve the capacity to metastasize are not adequately understood. This has been due, in part, to limitations in tissue acquisition, because human melanocytic lesions are often archived for later analysis, and in the availability of relevant experimental animal models of human melanoma. Here, we review the current and improving status of mouse models of human melanoma, focusing on how appropriate models can provide key insights concerning these critical early and late stages of melanomagenesis.

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Abbreviations: CPD, cyclobutane pyrimidine dimer; CT, computed tomography; GEM, genetically engineered mouse; HGF/SF, hepatocyte growth factor/scatter factor; MAPK, mitogen-activated protein kinase; MRI, magnetic resonance imaging; PET, positron emission tomography; RGP, radial growth phase; VGP, vertical growth phase; UVR, ultraviolet radiation

Received 14 March 2008; revised 7 May 2008; accepted 10 May 2008

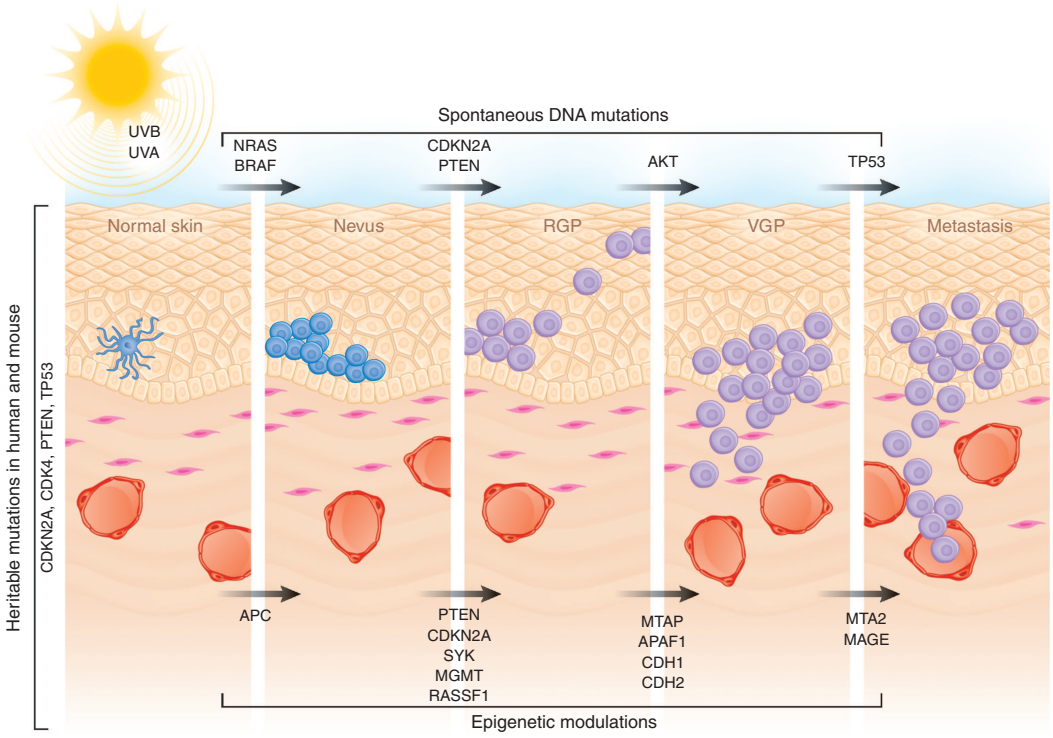

Figure 1. Proposed molecular alterations associated with the initiation and progression of melanoma. This illustration depicts human melanoma progression, which is imitated in some mouse models (for example, HGF/SF-transgenic mouse), and molecular alterations that can occur at different stages. Aberrant proliferation of normal melanocytes, presumably in response to UV radiation, results in the formation of benign or dysplastic nevi. Radial growth phase (RGP) melanoma exhibits the ability to grow intraepidermally, followed by invasion of the dermis in the vertical growth phase (VGP), and culminating with metastasis. Note that only about half of the melanomas are known to arise from nevi, and progression can occur without going through all the stages depicted. Several melanoma susceptibility genes have been identified in kindreds and have been validated in mouse models. It should be noted that while heritable *CDKN2A* and *CDK4* mutations are often associated with melanoma, individuals with germline *PTEN* and *TP53* mutations (Cowden and Li-Fraumeni syndromes, respectively) develop melanoma rarely. Spontaneous DNA mutations have been observed in several genes and are postulated to be involved at different stages of melanoma progression. Recent studies have also provided evidence for the presence of several genes whose expression is altered in melanoma by epigenetic modulations (for example, DNA methylation and/or histone modification). We propose that epigenetic events play an important role in the intricate gene–environment interplay associated with the initiation and progression of melanoma. Although more than 50 genes are now known to be epigenetically modulated in melanoma, we speculate on the importance of a few cancer-related genes. We anticipate that UV-induced GEM models will be of great value in working out details of melanoma progression (see text for details).

GENE–ENVIRONMENT INTERPLAY IN MELANOMA INITIATION

Extensive epidemiological evidence implicates intermittent intense exposure to UVR, especially during childhood, as a major risk factor in the etiology of melanoma (Maddodi and Setaluri, 2008). Melanoma risk also has a significant genetic component, not only with respect to family history of melanoma (for example, kindreds carrying mutations at the *CDKN2A* locus, or in *CDK4*), but also the physical characteristics of fair skin that easily freckles and/or sunburns without tanning and the presence of blond or red hair, which significantly increase the risk of melanoma. Although this phenotypic variation in hair and skin is likely a multigenic trait (Sulem *et al.*, 2007), the presence of polymorphic variants of the *MC1R* (melanocortin 1 receptor) gene plays the most important role (Lin and Fisher, 2007). Exactly how these physical characteristics are related to increased susceptibility to melanoma has yet to be fully resolved. Clearly, the presence, amount, and type of pigment and its protective role against genotoxic effects of UVR are important factors in the initiation of melanomagenesis; however, photobiology is much more complex. It has been shown that melanin has both photoprotective and photosensitizing effects. Melanin and the process of melanogenesis have been shown to be photosensitizers because they generate genotoxic reactive oxygen species (ROS) (Maddodi and Setaluri, 2008). This conflicting scenario clearly indicates that, in addition to the pigmentation phenotype, there are other as yet unknown background genetic elements that contribute to the UVR-induced transformation of melanocytes.

Ultraviolet radiation is the most ubiquitous environmental carcinogen. The UVR spectrum of sunlight is divided into three regions: UVA (320–400 nm wavelength), UVB (280–320 nm), and UVC (200–280 nm). UVC is biologically irrelevant, as it is almost completely absorbed by the atmospheric ozone layer. Both UVA and UVB reach the earth's surface and have deleterious effects on nucleic acids and proteins. UVB is considered to be more carcinogenic than UVA, as it directly causes two types of DNA lesions: cyclobutane

pyrimidine dimers (CPDs), formed between adjacent thymine (T) or cytosine (C) residues, and 6-pyrimidine 4-pyrimidone photoproducts (6-4PP) (Matsumura and Ananthaswamy, 2002). The CPDs are more abundant, more carcinogenic, and less efficiently repaired. These UVB-induced lesions give rise to DNA mutations hallmarked by CT→T and CC→TT transitions, the so-called ''UVB signature mutations.'' In contrast, UVA mutates DNA indirectly via absorption by non-DNA endogenous sensitizers, which generate ROS and lead to DNA damage (Lund and Timmins, 2007). Although there is experimental evidence that UVB is melanomagenic, whether UVA also initiates and/or promotes melanomagenesis is controversial.

The interplay of gene and environment is considered as a highly important phenomenon underlying initiation of melanomagenesis. UVR initiates an acute stress response, inducing a cascade of genes and pathways involved in cellular proliferation, apoptosis, DNA repair, and cellular survival, in which p53 plays a major coordinating role (Latonen and Laiho, 2005). Direct DNA damage by UVR is believed to be the major stimulus for the p53-dependent stress response culminating in a rapid onset of DNA repair mechanisms (Latonen and Laiho, 2005), which efficiently remove the CPDs and 6-4PPs within 48–72 hours post irradiation (Young *et al.*, 1996). Although several studies have examined the immediate genomic response of UVR-induced stress in several skin cell types (that is, melanocytes, keratinocytes, and fibroblasts (Blumenberg, 2006)), the long-term persistent response beyond the initial 48 hours has not been investigated. In human skin, UV irradiated *in vivo* persistent changes in the expression of growth factors, such as stem cell factor and endothelin-1, and their receptors, which are critical players in melanocyte biology, have been reported lasting from days to weeks after exposure (Hachiya *et al.*, 2004). Little is known, however, about the long-term UVR-induced changes in the melanocyte transcriptome, changes that could significantly contribute to melanoma susceptibility.

There is a clear association between UVR-induced DNA damage and skin cancer. This is exemplified by a drastically increased risk of skin cancer in xeroderma pigmentosum, a syndrome characterized by severe defects in the nucleotide excision repair genes. In nonmelanoma skin cancer (for example, squamous cell carcinoma and basal cell carcinoma), UVB signature DNA mutations have been found in several genes and are considered to play an essential role. The most well-characterized example is the tumor suppressor p53, which exhibits UVB signature mutations in a large majority of nonmelanoma skin cancer and appears to be an initiating event (Melnikova and Ananthaswamy, 2005). In contrast, definitive evidence for UVB signature mutations in melanoma genes has been surprisingly lacking. *CDKN2A*, which encodes two different tumor suppressor proteins, $p16^{INK4A}$ and $p14^{ARF}$, harbors mutations in melanoma at dipyrimidine sites, but many of these genetic alterations are not UVB signature mutations. Furthermore, C→T transition mutations in *CDKN2A* are similarly observed in cancers of internal organs protected from sun exposure, calling into question a central role for these mutations in UVR-induced melanomagenesis (Hocker and Tsao, 2007). *BRAF*, a part of the RAS signaling pathway and critical for the cellular response to growth signals, has been shown to carry mutations in a majority of melanomas, but these are not UVB signature mutations (Davies *et al.*, 2002; Hocker and Tsao, 2007). Similarly, mutations in *NRAS* are found in melanomas but do not carry UVB signatures (Hocker and Tsao, 2007). Although a small percentage of melanomas have UVB signature mutations in *TP53*, such mutations are considered as late events during the progression of melanoma to a higher grade (Hussein *et al.*, 2003). This lack of definitive evidence for UVB-induced mutations has led to the suggestion that UVA-induced oxidative lesions in DNA, including *BRAF* and *NRAS*, may be responsible for melanomagenesis (Lund and Timmins, 2007). However, this hypothesis is weakened by the studies on the hepatocyte growth factor/scatter factor (HGF/SF) transgenic albino mouse model, which have shown that UVB, but not UVA, is the melanomagenic waveband (De Fabo *et al.*, 2004). Additional studies will be required to resolve this important question.

It should be noted that although *in vitro* studies of the effects of UVR on melanocytes have yielded important information, the results obtained must be viewed with caution because cultured melanocytes are outside their morphological and physiological microenvironment and lack the necessary cellular interactions with other cell types (for example, keratinocytes) to elicit the proper responses to extracellular stimuli. This problem is exemplified by a study that showed that cultured and *in vivo* epidermal keratinocytes exhibit profoundly different responses to UVB (Enk *et al.*, 2006). Clearly, in-depth study of the biology of any cell lineage is best performed while the cells reside in their natural morphological and physiological microenvironment. Unfortunately, purification of melanocytes from whole mice after exposure to UVR is a significant challenge because the melanocytes make up only about 1% of the cells in the mammalian skin. Development of a genetically engineered mouse (GEM) in which labeled melanocytes could be exposed to UVR *in situ* in the whole animal and then isolated for detailed molecular analyses would be of great value in the study of the biological effects of UVR on melanocytes.

Mouse models of UVR-induced melanoma

Animal models of melanoma have been instrumental in gaining the current level of understanding of the initiation, progression, and metastasis of melanoma. Although several animals have been described, including guinea pig, opossum, and *Xiphophorus* fish, mouse models have provided the most significant and most recent advancements in melanoma research. We will therefore focus here only on the mouse models that have been utilized to study the biology of UVR-induced melanomagenesis. For a more comprehensive review of mouse models of melanoma, the readers are referred to an excellent review by Larue and Beermann (2007).

Mouse skin is different from human skin in some significant ways. In human skin, melanocytes reside in the basal layer of the epidermis, where they make dendritic connections with the surrounding keratinocytes. Melanosomes are transferred from melanocyte to keratinocytes, providing skin pigmentation and protection from UVR. In mouse skin, melanocytes are located predominantly at the base of the hair follicles embedded in the dermis and are only found in the epidermis of hairless areas, such as ears, tail, and paws. It follows that mouse melanoma will be qualitatively different from human melanoma. Indeed, wild-type mice are not prone to UVR-induced melanomagenesis, perhaps due to the fact that their melanocytes are located deeper in the dermis where they are relatively well sheltered from the penetrative reach of UVB. In some UVR-inducible melanoma models, such as the HGF/SF transgenic mouse, melanocytes are aberrantly located in the epidermis and at the dermoepidermal junction, resulting in more ''humanized'' skin (Noonan *et al.*, 2001).

The grafting of bona fide human skin onto severe combined immunodeficient mice, although technically arduous, offers both the relevance of human skin and the experimental flexibility of the mouse. Using this approach, Berking *et al.* (2004) showed that the combination of UVB and expression of three growth factors, basic fibroblast growth factor, stem cell factor, and endothelin-3, rapidly produced melanomas. It was observed that neonatal skin produced more aggressively invasive melanomas, whereas adult skin grafts yielded only *in situ* melanomas. These experiments demonstrated for the first time that a combination of physiological factors and environmental carcinogens could result in the transformation of human melanocytes.

Over the last decade, the INK4A/ARF and mitogen-activated protein kinase (MAPK) pathways have emerged as leading players in the etiology of human melanoma (Bennett, 2008), mainly due to the consistent detection of mutations in *CDKN2A*, *NRAS*, and *BRAF* (Figure 1). Gratifyingly, success with most GEM models of human melanoma has been achieved through deregulation of these exact pathways. Klein-Szanto *et al.* (1994) reported the first UVR-induced melanoma mouse model in which the melanocyte-specific tyrosinase promoter was used to drive expression of the SV40 T-antigen, which inactivates both p53 and pRb and is functionally equivalent to the loss of the *CDNK2A* locus. An activating mutation in *HRAS* ($HRAS^{V12G}$) gives rise to melanoma when combined with UVR, 7,12-dimethylbenz[a]anthracene and/or secondary alterations in the p53 and/or pRb pathways through mutations in *Ink4a/Arf* (Broome Powell *et al.*, 1999; Chin *et al.*, 1999; Kannan *et al.*, 2003). In their HRAS-transgenic mice, Kannan *et al.* (2003) observed amplification of *Cdk6* in UVR-initiated melanomas only, which was distinctly exclusive of *Ink4a* loss. Further, UVR had no effect on melanomagenesis in $HRAS^{V12G}$, *Ink4a*−/− mice, which suggested that the pRb pathway might be the primary target of UVR.

Hepatocyte growth factor/scatter factor is a growth factor that regulates melanocyte proliferation and survival through binding to the receptor tyrosine kinase MET, activating downstream MAPK and PI3K pathways. Untreated HGF/SF-transgenic mice exhibit a low penetrance of melanoma after a long latency. Chronic exposure of adult HGF/SF-transgenic mice to UVR did nothing to change this tendency. In contrast, a single erythemal (burning) UVR dose to neonatal HGF/SF mice greatly exacerbated melanomagenesis with much reduced latency (Noonan *et al.*, 2001). This effect was significantly enhanced by Ink4a/Arf deficiency (Recio *et al.*, 2002). Importantly, the histopathology of the melanomas produced in this UVR-induced model can closely resemble human melanoma, perhaps because of the humanization of mouse skin by HGF/SF (noted above), which makes this model well suited for gene–environmental interaction studies. Indeed, in this albino mouse model, UVB, but not UVA, was shown to be the melanomagenic waveband (De Fabo *et al.*, 2004).

$Cdk4^{R24C}$-transgenic mice give rise to melanoma when challenged with 7,12-dimethylbenz[a]anthracene/TPA carcinogenesis, accompanied by mutations in *RAS* and enhanced phosphorylation of extracellular signal-regulated kinase, further solidifying the notion that activation of the MAPK pathway and suppression of Ink4a/Arf pathways are two critical genetic events in melanomagenesis (Sotillo *et al.*, 2001). Interestingly, the $Cdk4^{R24C}$-transgenic mice did not develop melanoma when irradiated with UVB. This fact and the lack of UVB signature mutations in both *NRAS* and *BRAF* suggest that the MAPK pathway components are not the primary targets of UVB. Moreover, the synergy observed between Ink4a/Arf deficiency, MAPK pathway activation, and UVR raises the possibility that the primary targets of UVR are outside the p53 and pRb pathways. A concerted effort will be needed to identify the true UVR targets to better understand the effects of this important environmental carcinogen on melanocytes and their transformation.

UVR and melanoma epigenetics

Over the years, the hunt for melanoma-initiating genes and deregulated pathways has remained focused on UVR-induced DNA damage and the resultant mutations in DNA sequence. However, these efforts have so far failed to establish a clear association between UVR-induced DNA damage and melanoma initiation. It is, therefore, plausible to suggest that DNA mutations alone may not fully account for UVR-induced melanomagenesis. Inevitably, it may be valuable to view this problem from a new perspective and explore other mechanisms by which UVR mediates melanocyte transformation. The relatively recent discovery of the involvement of epigenetic events in oncogenesis provides one such alternative mechanism that warrants consideration.

''Epigenetics'' refers to heritable gene expression changes without alterations of DNA sequence. There are two major types of epigenetic events: modifications of the DNA itself and changes in the chromatin packaging proteins, the histones (Egger *et al.*,

2004). DNA methylation occurs exclusively on the carbon-5 of cytosine in a CpG dinucleotide and is mediated by DNA methyltransferases. CpG-dense regions, or CpG islands, tend to locate to the promoters of about 50% of genes, and hypermethylation of CpG islands is often associated with silencing of gene expression. In contrast, hypomethylation of the normally methylated promoters gives rise to aberrant expression of genes. Post-translational covalent modifications of the histones (H2A, H2B, H3, and H4), most importantly acetylation and methylation, at their amino-terminal tails are also important gene-expression regulators. Acetylation of histone tails allows transcription factors to gain access to DNA promoter and enhancer regions and results in transcriptional activation. Conversely, histone deacetylation leads to a tight repackaging of the nucleosomal unit and hinders binding of transcription factors leading to gene silencing. Site-specific histone H3 N-terminal tail methylation at specific lysine residues also have been associated with transcriptional regulation (Ruthenburg *et al.*, 2007). These modifications are thought to bring about transient as well as heritable structural and expressional genetic changes.

It has been shown that DNA methylation and histone modifications cooperate with each other to determine access to DNA and thus play a key role in the control of gene expression, DNA replication and repair, and mitosis (Esteller, 2007). Epigenetic modifications are well described in cancer and are considered to be major contributors to the cancer phenotype (Esteller, 2007). Inactivation of tumor suppressor genes and aberrant activation of oncogenes associated with the cancer phenotype have been attributed to promoter hypermethylation and hypomethylation, respectively. It has been proposed that early epigenetic changes increase the probability that genetic alterations will result in cancer and thus play a major role in cancer etiology (Feinberg, 2004).

Epigenetic modulation of gene expression in melanoma has recently attracted keen attention. A number of genes are dysregulated in melanoma by DNA methylation, as well as by modifications in histones and associated machinery (Hoon *et al.*, 2004; Gallagher *et al.*, 2005; Muthusamy *et al.*, 2006; Rothhammer and Bosserhoff, 2007) (Figure 1). More than fifty genes are currently known to be silenced by DNA methylation in melanomas (Rothhammer and Bosserhoff, 2007). Among these are some of the tumor suppressor genes long known to be associated with melanoma, including *CDKN2A*, *PTEN*, *APC*, and *APAF1* (de Snoo and Hayward, 2005; Muthusamy *et al.*, 2006). Aberrant expression of the normally silent *MAGE* (melanoma antigen gene) in melanoma has also been attributed to promote hypomethylation (van Doorn *et al.*, 2005). Moreover, several genes involved in histone modifications have been shown to be dysregulated in melanoma, such as those encoding the histone deacetylases HDAC1, HDAC2, HDAC3, and Sirtuin 1 (Bandyopadhyay *et al.*, 2004; Wang *et al.*, 2005). The precise mechanisms underlying these modulations have yet to be revealed.

It is not currently known if UVR can initiate melanomagenesis through an epigenetic mechanism. In some nonmelanoma cancers, silencing of genes by hypermethylation has been described (Belinsky, 2005), as in early and late experimental and human lung cancer lesions. Similarly, such a silencing mechanism has been correlated with aflatoxin adducts in hepatocellular carcinoma (Zhang *et al.*, 2003). Persistent epigenetic changes in response to ionizing radiation have been demonstrated in breast cancer (Barcellos-Hoff and Brooks, 2001; Tsutsumida *et al.*, 2004) and in experimental chemically induced cancers (Tsutsumida *et al.*, 2004; Yamashita *et al.*, 2004). It can, therefore, be envisaged that UVR influences epigenetic modulation of gene expression in melanocytes as well, and that at least a fraction of these modulations persist long-term post-irradiation and contribute significantly to melanomagenesis.

Perhaps a link can be found between epigenetic modulation of DNA and UVR-induced melanomagenesis from studies showing that a significant percentage of UVR-initiated CPDs, and the consequent mutations, form preferentially at 5-methylcytosines within the 5′-CC^{m}G and 5′-TC^{m}G trinucleotide sequences (Pfeifer *et al.*, 2005). This is effected by the preferential deamination of the 5-methylcytosine within CPDs, which is postulated to be an important mechanism of UVB-induced mutagenesis (Pfeifer *et al.*, 2005). Moreover, it has been shown that UVR induces global chromatin relaxation, dependent on histone acetylation by p53-dependent recruitment of the histone acetyltransferase p300 (Rubbi and Milner, 2003). This chromatin modification is an essential precursor for nucleotide excision repair of UVR-induced DNA damage because it allows greater accessibility to DNA. Aberrant persistence of such a relaxed chromatin state at either a global or local level could have substantial effects on gene transcription and, ultimately, on melanomagenesis. It is anticipated that GEM models will be designed to help experimentally test hypotheses relating UVR-induced epigenetic alterations to melanoma.

MOUSE MODELS OF MELANOMA PROGRESSION AND METASTASIS

Melanoma patients face two strikingly distinct fates: those diagnosed with earlier stages can be completely cured by outpatient surgery, whereas those harboring disseminated, metastatic disease usually die within 6 months, irrespective of treatment with currently available chemotherapy, radiotherapy, and/or immunotherapy. Therefore, understanding the mechanisms that confer a metastatic phenotype upon melanoma cells and that allow them to evade therapy is of paramount importance to researchers and clinicians hoping to combat this devastating malignancy. Lessons from the clinic come slowly and with a heavy toll. Relevant experimental animal models of metastatic melanoma provide a vital platform for molecular discovery, target validation, and the preclinical testing or screening of antimelanoma therapies. Below, we broadly review the available mouse models for studying metastatic melanoma and speculate on where the field is heading.

Mouse models of metastatic melanoma have a relatively long and rich history in cancer research. In a now classic set of 1977 experiments, Fidler and Kripke (1977) successfully generated individual sublines of B16 mouse melanoma and subsequently tested their capacity to form foci in the lungs by intravenous injection into syngeneic C57BL/6 mice. The differential capacity to colonize the lung among these sublines demonstrated the heterogeneous nature of tumor cell populations. In 1980, Hart and Fidler (1980) intravenously injected B16 melanoma cells into syngeneic C56BL/6 mice whose skin or muscle had been implanted with tissues from various organs. B16 tumors developed preferentially in grafts of ovarian and lung tissue in skin as well as mouse lung organ, rather than in renal tissue grafts or at the site of surgical trauma, indicating that tissue selectivity at distant sites determines the pattern of tumor metastasis (Hart and Fidler, 1980). These studies revived Paget's 1889 ''seed and soil'' hypothesis and laid the foundation for modern cancer metastasis research (Langley and Fidler, 2007).

Since then, the repertoire of mouse models for studying melanoma progression and developing therapies has been greatly expanded through use of human xenograft and GEM models (Talmadge *et al.*, 2007). These models can now be categorized into four major types: (1) human melanoma cells grafted onto immunocompromised mice; (2) murine melanoma, either from autochthonous murine or GEM tumors, transplanted into immunocompetent syngeneic mice; (3) GEM melanoma with a natural history more similar to human disease; (4) murine melanoma transplanted into GEM hosts (Figure 2). Each type of model has its advantages and limitations in the study of melanoma progression. For example, human melanoma cells are overtly more relevant to human disease than mouse melanoma cells, but their requirement for transplantation into immunocompromised host mice lacking normal immune function and microenvironment interactions represents a serious limitation. Mouse melanoma transplanted into syngeneic wild-type mice provides an excellent match between tumor and environment; however, mice are not human. GEM model studies can be lengthy and expensive, but are more relevant to the natural development of human disease and can be used to determine the contribution of a candidate gene to melanoma progression through genetic addition or subtraction. Once a candidate gene involved in melanoma progression/metastasis has been selected, models employing transplantation of appropriately engineered human or murine melanoma cells are useful to validate and dissect its function. Finally, the transplantation of murine melanoma cells into various lines of GEM provides the means to experimentally manipulate the tumor environment by genetically engineering the host (Figure 2).

Mouse models for identifying genes involved in melanoma progression

Mouse models can greatly facilitate metastasis gene discovery. For example, as an extension of the Fidler experiments, a cancer cell line can first be subjected to limiting dilution cloning to generate subline populations of single cell-derived progeny. When transplanted into mice, these sublines show distinct organ-specific metastatic potential. Subsequent gene expression profiling of these sublines, often in concert with primary human tumor data, can be used to mine for genes involved in organ-specific metastasis (Gupta *et al.*, 2005). Alternatively, a cancer cell line can first be subjected to multiple transplantation cycles to select for the ability to metastasize to a specific organ, and then sublines can be established (Cranmer *et al.*, 2005). In one elegant study, variants of melanoma derived from the inducible *HRAS*-transgenic mouse were selected for their metastatic propensity following transplantation. A comparative oncogenomics approach was then used to identify and validate highly relevant metastasis genes in human melanoma, including the scaffold protein NEDD9 (Kim *et al.*, 2006). It is anticipated that this approach will ultimately provide new insight into the nature of drug resistance in metastatic disease.

Genetically engineered mouse models have provided surprising information about genes contributing to melanoma progression. As a case in point, unexpected differences in the consequences of expression of members of the *RAS* family on melanoma metastasis were recently revealed. One of the earliest successful GEM models of melanoma was driven by targeting expression of activated *HRAS* to melanocytes, but metastatic lesions were very rare even in association with inactivating mutations in *Ink4a/Arf* or *Tp53* (Chin *et al.*, 2006). In contrast, a more recent combination of melanocyte-specific expression of activated *NRAS* and *Ink4a/Arf* deficiency induced the development of aggressive melanoma in which 30% of the melanomas metastasized to lymph nodes and distant organs such as the lungs and liver (Ackermann *et al.*, 2005). NRAS could certainly have been predicted to have a preferential early role in melanomagenesis based on the fact that up to 25% of human melanomas harbor activating mutations in *NRAS*, whereas *HRAS* alterations have only occasionally been detected in melanoma. However, these animal models provided new insight with respect to melanoma progression.

Those genes that have been implicated in melanoma progression/metastasis can be validated through transplantation of genetically engineered melanoma cells in which expression of the gene in question is either enhanced or diminished. For example, in the study alluded to above, knockdown of NEDD9 expression in melanoma cells using RNAi technology negatively impacted their ability to proliferate, invade, and metastasize (Kim *et al.*, 2006). Similarly, Gupta *et al.* (2005) discovered that *SLUG*, a *SNAIL* superfamily gene, could help in mediating the metastatic potential of transformed, immortalized human melanocytes and validated its role using SLUG knockdown experiments. However, it should be noted that when attempting to study metastasis-specific genes, one must be careful in distinguishing between those genes that might indirectly influence melanoma metastasis (for example, by generally

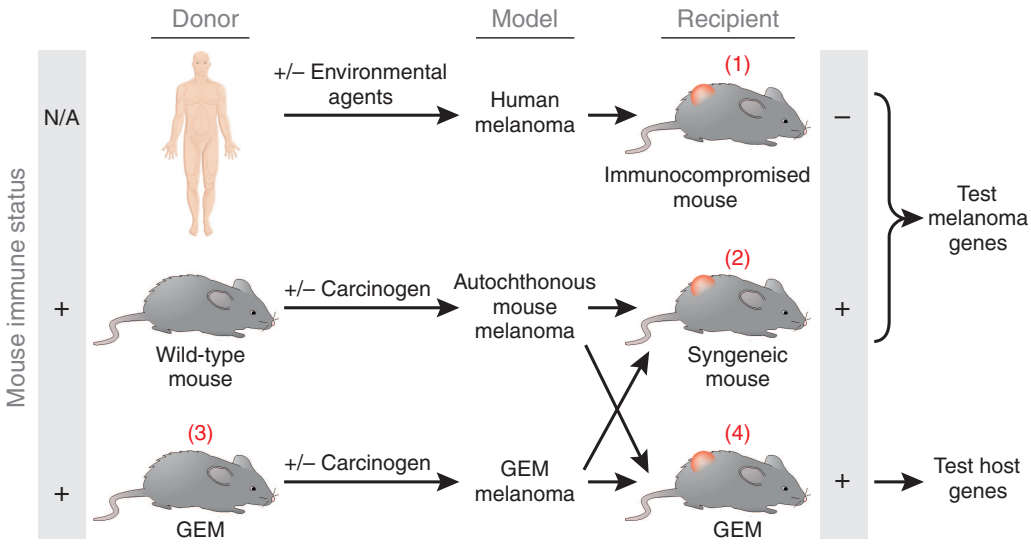

Figure 2. Types of mouse models used for studying melanoma progression and metastasis. Models can be categorized into four basic types: (1) tumor cells derived from human melanoma, implanted into immunocompromised mice, which are deficient in normal immune function; (2) tumor cells derived from either autochthonous or genetically engineered mouse (GEM) melanoma, implanted onto syngeneic wild-type mice, which possess normal immune system and stroma; (3) melanoma arising from GEM either spontaneously or via induction by a carcinogen such as UVR; (4) tumor cells derived from either autochthonous or GEM melanomas, implanted into a GEM model, where the tumor microenvironment in the host can be manipulated by genetic engineering. Examples of applications for these models can be found in the text.

stimulating tumor growth and/or preventing apoptosis) and those that affect only metastasis and not primary tumor development. For example, ectopic expression of activated NRAS would almost certainly affect melanoma cell proliferation. In contrast, knockdown of MMP-1 had no effect on growth of primary human melanoma xenografts in nude mice, but significantly decreased the metastatic potential from the primary site, reducing collagenase and angiogenic activity (Blackburn *et al.*, 2007). Perhaps this is best illustrated by the actions of the metastasis suppressor genes. *KISS1*, originally identified in melanoma cells, helps in maintaining metastatic dormancy, although having no effect on tumorigenicity or invasiveness (Nash and Welch, 2006).

Genetically engineered mouse models also provide novel opportunities to assess the role of host genes on the metastatic process. Here, mouse melanoma cells, engineered or not, can be transplanted into various appropriately modified strains of host GEM. For example, to assess the role of nonautonomous, heterotypic receptor tyrosine kinase signaling in melanoma metastasis, mice broadly expressing an *HGF/SF* transgene were used as hosts for mouse melanoma cells expressing the HGF/SF receptor MET. These studies demonstrated that metastatic potential under nonautocrine signaling conditions (for example, where MET-expressing tumor cells are transplanted into transgenic hosts producing HGF/SF) was equivalent to autocrine conditions (for example, where tumor cells expressing both HGF/SF and MET are transplanted into wild-type hosts) and demonstrated that host cells secreting potent growth factors can have a profound effect on metastatic colonization (Yu and Merlino, 2002).

Mouse models of melanoma invasion into local tissue

As noted above, skin melanocytes are scattered throughout the basal layer of the human epidermis; in contrast, most mouse melanocytes are located in hair follicles embedded within the dermis. The transition from RGP to VGP, a hallmark of human melanoma progression and an important determinant of clinical outcome, is marked by the invasion of epidermal melanoma cells through the basement membrane and into the dermis (Figure 1). These factors raise concerns about the use of mouse models to study human melanoma progression. Several approaches have been employed to overcome these limitations. GEM models have been generated, in which melanocytes are retained within the mouse epidermis and at the dermoepidermal junction through expression of either stem cell factor (Kunisada *et al.*, 1998) or HGF/SF (Otsuka *et al.*, 1998). In *HGF/SF* mice, lesions resembling RGP melanoma and invasive melanoma are readily observed following neonatal UV radiation (Noonan *et al.*, 2001).

Another valuable approach employs human skin reconstructs composed of a stratified, terminally differentiated epidermal cells and dermal fibroblasts embedded in collagen (Satyamoorthy *et al.*, 1999). When human melanoma cells were incorporated into such reconstructs and grafted onto immunocompromised mice, they retained the difference between RGP and VGP melanoma, providing an appropriate model for addressing questions about local invasion of melanoma. Using this reconstruction model, Meier *et al.* (2000) compared the behaviors of normal melanocytes, RGP, VGP, and metastatic melanoma cells: RGP melanoma cells grew but could not penetrate the basement membrane of the epidermis; VGP primary melanoma cells formed clusters at the dermo-epithelial junction and invaded into

the dermis, but formed an irregular basal membrane; whereas metastatic melanoma cells rapidly proliferated and aggressively invaded deep into the dermis. Interestingly, basic fibroblast growth factor-transduced RGP melanoma cells gained an invasive phenotype characteristic of VGP cells (Meier *et al*., 2000).

To determine which mutations are required for melanocyte transformation and melanoma invasion, Chudnovsky *et al*. (2005) engineered human melanocytes to express specific genes implicated in human melanoma. These cells were incorporated into the epidermal compartment of human skin reconstructs and then grafted onto immunocompromised mice. They found that a combination of mutant *NRAS*, mutant *CDK4*, a dominant-negative p53, and hTERT were required to generate invasive neoplasia. To dissect the RAS pathway in the invasive melanoma, various downstream effectors were used to replace activated NRAS in the gene transfer combination mentioned above. Replacement of RAS with activated PI3K still generated invasive melanocytic hyperplasia, whereas mutant *BRAF* did not. These data demonstrate that the grafting of human melanoma/skin reconstructs onto mice provides a relevant humanized model to study local invasion of melanoma and the genes that regulate it.

Mouse models of immune response in metastasis and tumor dormancy

Syngeneic transplantation models have been employed in the study of the immune response during melanoma metastasis. A common approach is to immunize mice by implanting an autochthonous tumor, remove the tumor after an appropriate growth period, and then challenge the mice by injecting melanoma cells into another subcutaneous site. The incidence of subcutaneous and metastatic tumors is then used to assess protection. Notably, Donawho *et al*. (2001) used this approach to show that such immunization was able to protect mice from subcutaneous tumor challenge, but insufficient to prevent spontaneous metastasis melanoma. The underlying mechanisms can be elaborated in this model by genetically engineering or depleting immune cells.

Immunocompromised mice provide a unique advantage in the study of human immune response to melanoma. Mice that have very low residual immune activity, such as severe combined immunodeficient mice, allow transplantation of both human melanoma cells and immune cells. This provides a platform to test the interaction between specific human immune cells and melanoma cells in the metastatic process. As an example, Sabzevari and Reisfeld (1993) reported that injection of human cytotoxic T cells suppressed lung metastasis of human melanoma subcutaneously implanted into severe combined immunodeficient mice.

The frequent recurrence of tumors many years after treatment is a well-recognized but poorly understood characteristic of cancer. This most important clinical phenomenon is enabled by the capacity of residual tumor cells to remain dormant for prolonged periods before recurrence. Recent evidence suggests that the immune system helps to sustain tumor dormancy and thus delay cancer progression. As described above, immune cells of mice immunized with subcutaneously implanted tumors can target and kill most tumor cells in the challenge injection. However, one study has shown that some residual cells still persist and that the immune system keeps them dormant (Rabinovsky *et al*., 2007). Virus-activated dendritic cells were found to effectively prolong the dormancy of highly metastatic B16F10 melanoma and enhance survival in a mouse model by strongly enhancing CD8+ T-cell response (Shibata *et al*., 2006). How immunosurveillance prevents melanoma cells from expansion during dormancy and how these cells ultimately escape dormancy and recur remain critical, yet unanswered, questions.

Preclinical mouse models of melanoma treatment

Preclinical models can be used to assess the efficacy of arising anticancer therapies, to test that molecularly targeted drugs actually hit their targets, and as a discovery tool in the development of new clinical agents, biomarkers, and assays. It would therefore be advantageous that such a model mimics the entire spectrum of human melanoma progression and be able to predict the response of human melanoma to a wide variety of therapeutic agents and approaches. In reality, it is clear that no single animal model is suited for all things. Therefore, many of the types of mouse models discussed above, which focus on a particular stage of progression or on a specific mechanism of melanoma metastasis, may turn out to be useful for preclinical studies.

For the development of antimelanoma immunotherapy, the subcutaneous or intravenous transplantation of B16 mouse melanoma cells into syngeneic mice remains a standard in the field. The B16 models have been used to test many different types of vaccines and immune cell transfer protocols. For example, mice have been immunized with a recombinant adenovirus encoding human dopachrome tautomerase (DCT, formally known as TRP-2) and then challenged with either intravenous injection or subcutaneous implantation of B16 cells. The vaccine protected all of the mice from pulmonary tumor formation in the former group, and half of the mice from subcutaneous tumor formation in the latter group (Kochenderfer and Gress, 2007).

Inoculation of murine melanoma cells into syngeneic mice and xenografts of human melanoma cells into immunocompromised mice are both employed for the assessment of the efficacy of antimelanoma small molecules and monoclonal antibody drugs. It should be noted that because of the differences in how drugs are processed or metabolized in mice (pharmacokinetics), the maximum tolerated dose of a given drug might be much higher in mice than in any patient. Therefore, a positive response to a relatively high dose of a drug in a preclinical mouse setting may not be achievable in melanoma patients. In an attempt to avoid bringing unusable drugs into clinical trials, more ''humanized'' pharmacokinetic protocols are being

considered to more closely reflect the actual maximum tolerated dose of drugs used in human patients (Jansen *et al.*, 1999; Ramirez-Montagut *et al.*, 2006; Donawho *et al.*, 2007).

The recent identification of tumor-initiating cells, so-called cancer stem cells, has brought exciting new insights into cancer biology, and along with it new candidate targets into preclinical modeling. This specific population of cells is thought to be capable of indefinite self-renewal and differentiation, driving tumor formation. Moreover, like normal stem cells, tumor-initiating cells could possess the ability to resist drug treatment and survive in a state of relative quiescence over a dormant period (Lobo *et al.*, 2007). In an attempt to identify tumor-initiating cells in melanoma, Schatton *et al.* (2008) has proposed that melanoma-initiating cells are enriched in a cellular population expressing the transporter molecule ABCB5. As proof of their stem-like behavior, Schatton *et al.* (2008) showed that ABCB5+ human melanoma cells could preferentially form tumors after serial transplantation and limiting dilution experiments in immunocompromised mice. They further demonstrated that these putative melanoma stem cells were sensitive to treatment with a monoclonal antibody directed against ABCB5 after transplantation into immunocompromised mice. This study fuels hope that tumor-initiating cells and/or their markers can serve as effective therapeutic targets. It is anticipated that preclinical mouse models will be used to further validate these new targets and to test those therapies designed to attack them.

The incorporation of bioimaging technology such as optical imaging (fluorescence and bioluminescence), radiological imaging (micro-CT, micro-PET, high-resolution MRI), and ultrasound into preclinical models will greatly accelerate the screening process by monitoring disease progression *in vivo* and providing information on tissue anatomy, histological changes associated with treatment, drug distribution, tumor metabolism, and molecular targeting (Brindle, 2008). Moreover, mouse models are being designed to fully exploit bioimaging, allowing metastatic cells and their interactions with the microenvironment to be precisely tracked. This point is well illustrated by Kaplan *et al.* (2005) in their study of the premetastatic niche. By transplanting green fluorescent protein-labeled bone marrow-derived hematopoietic progenitor cells and red fluorescent protein-labeled tumor cells into lethally irradiated mice, they showed that hematopoietic progenitor cells home to tumor-specific sites and form cell clusters, creating a permissive niche before tumor cell arrival. This study reveals that hematopoietic progenitor cells are intimately involved in the metastatic process, a finding that carries significant potential clinical implications.

CONCLUSION

In this review, we have tried to argue for the need to focus melanoma research on mechanisms by which UVR initiates melanoma, to improve prevention strategies, and by which metastatic melanoma evolves and evades therapeutic eradication, to improve intervention strategies. In addition, we have attempted to illustrate how these efforts are being significantly facilitated through the creation and use of a variety of mouse models. However, the obstacles, like the stakes, continue to be formidable.

While melanoma incidence continues to increase at an alarmingly accelerating rate, there exists a major gap in our current knowledge with regard to how solar or artificial UVR initiates melanoma, considerably hampering our ability to accurately determine the risk of melanoma from various UVR sources. Increased use of tanning salons has been linked to increased risk for melanoma in multiple studies, but direct cause and effect remains elusive and controversial, confusing public health policy and legislation regarding tanning sunbed use. Sunscreen use is consistently recommended for protection from sunlight, but there is considerable controversy over its effectiveness against melanoma (Lund and Timmins, 2007). And because of the ambiguities associated with remembering and/or recording sun exposure history, the absence of molecular biomarkers of such exposure severely hinders attempts to accurately assess melanoma risk and thus prevent its development.

Despite the problems with understanding the initiation of early-stage melanoma, it is readily curable. In contrast, the prognosis of patients with metastatic melanoma could not be worse. Advanced melanoma is notorious for its resistance to all forms of available therapy, highlighting the need for radically new approaches and drugs, the development of which has been obstructed by the lack of relevant experimental and preclinical animal models. Currently, preclinical analysis of candidate antimelanoma therapies relies almost exclusively on models in which human melanoma cells previously adapted to cell culture are subcutaneously transplanted into inadequate immunocompromised host mice, with the readout for treatment success being tumor growth at the primary site. This overly simplistic representation of advanced melanoma has not been particularly useful for identifying new drugs or predicting their efficacy.

We believe that creative and judicial incorporation of genetically engineered melanoma cells and animal models will be instrumental to both understanding and preventing melanoma initiation and progression. Mouse models now provide outstanding opportunities for filling long-standing gaps in knowledge and resolving controversies regarding the different UVR wavelengths (De Fabo, 2006), childhood sun exposure, pigmentation, sunscreen protection, and tanning salon risk. Built on a foundation of continual improvements fueled by fresh molecular and mechanistic insights, mouse models are also now among the best tools for identifying novel and successful strategies for prevention, early detection, and treatment of melanoma. There is, therefore, reason to be optimistic about the clinical prospects for melanoma patients as new basic revelations from human and mouse models are translated into efficacious antimelanoma therapy.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank members of the Merlino lab and Drs Frances Noonan and Edward De Fabo (George Washington University Medical Center, Washington, DC) for invaluable discussions and advice. We apologize to the authors of many papers that have significantly contributed toward the current state of the melanoma field but could not be acknowledged here due to space constraints.

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