REVIEW

Malignant melanoma: genetics and therapeutics in the genomic era

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Cell for cell, probably no human cancer is as aggressive as melanoma. It is among a handful of cancers whose dimensions are reported in millimeters. Tumor thickness approaching 4 mm presents a high risk of metastasis, and a diagnosis of metastatic melanoma carries with it an abysmal median survival of 6–9 mo. What features of this malignancy account for such aggressive behavior? Is it the migratory history of its cell of origin or the programmed adaptation of its differentiated progeny to environmental stress, particularly ultraviolet radiation? While the answers to these questions are far from complete, major strides have been made in our understanding of the cellular, molecular, and genetic underpinnings of melanoma. More importantly, these discoveries carry profound implications for the development of therapies focused directly at the molecular engines driving melanoma, suggesting that we may have reached the brink of an unprecedented opportunity to translate basic science into clinical advances. In this review, we attempt to summarize our current understanding of the genetics and biology of this disease, drawing from expanding genomic information and lessons from development and genetically engineered mouse models. In addition, we look forward toward how these new insights will impact on therapeutic options for metastatic melanoma in the near future.

The disease

Melanomas most often arise within epidermal melanocytes of the skin, although they can also derive from noncutaneous melanocytes such as those lining the choroidal layer of the eye, GI and GU mucosal surfaces, or the meninges. Melanoma is also among the more common causes of “metastatic cancer of unknown primary,” which may reflect either a propensity to arise in unex-

pected sites along the neural crest migratory route, or rapid growth of poorly differentiated lesions arising from indolent or unrecognized cutaneous primary lesions. Clinical staging for primary cutaneous melanoma employs measurements of thickness (in millimeters), presence of ulceration, penetration through cutaneous layers, mitotic rate, evidence of “in transit” metastasis, tumor spread to draining lymph nodes, and evidence of distant metastasis. Management issues in melanoma can be classified in terms of prevention, diagnosis, local disease management, and treatment of metastatic disease.

In view of the epidemiological and emerging experimental evidence linking melanoma incidence to UV exposure and skin phototype, prevention and screening strategies represent key areas for reduction of disease incidence and severity. For most cutaneous melanomas in the so-called radial growth phase (e.g., thin melanomas), surgical removal affords curative treatment. In contrast, a significant fraction of patients diagnosed with intermediate-thickness (2–4 mm) cutaneous melanoma eventually succumb to recurrence at regional or distant sites.

Critical biological questions facing the melanoma research community include: (1) What genetic and environmental factors contribute to and/or modulate risk of melanoma development in man? (2) What biological or molecular features (biomarkers) in early lesions can predict high risk of subsequent metastasis? (3) What genetic events underlie its propensity for metastasis and treatment resistance (phenotype)? (4) Which genetic alterations responsible for development and progression of melanoma are also essential for maintenance of established disease? (5) Finally, what maintenance-essential biological or molecular pathways/networks might prove amenable to preventive and/or therapeutic intervention in man?

The genetics of melanoma

Many studies conducted over several decades on benign and malignant melanocytic lesions as well as melanoma

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cell lines have implicated numerous genes in melanoma development and progression. In this review, we focus on validated genetic events, which include predisposing or somatic structural alterations in melanoma specimens on the DNA level, such as translocation, amplification/deletion, and point mutations (Futreal et al. 2004).

CDKN2A, the familial melanoma locus

Physical characteristics such as light complexion, an inability to tan, red hair, and blue eyes correlate with increased risk for melanoma development (Gilchrest et al. 1999). Likewise, the presence of multiple pigmented lesions, including freckles and banal or clinically atypical moles, is associated with a quantitatively increased melanoma risk (Gandini et al. 2005a). However, one of the most significant risk factors for melanoma occurs in individuals with a strong family history of melanoma. Older case-control studies of patients with familial atypical mole-melanoma (FAMM) syndrome suggested an elevated risk of ~434- to 1000-fold over the general population (Greene et al. 1985). A more recent meta-analysis of family history found that the presence of at least one first-degree relative with melanoma increases the risk by 2.24-fold (Ford et al. 1995; Gandini et al. 2005b). Genetic studies of this heritable trait in large melanoma-prone families ultimately led to the initial identification of *CDKN2A* as the familial melanoma gene. Located at chromosome 9p21, LOH or mutation at this locus cosegregated with melanoma susceptibility in familial melanoma kindred (Hussussian et al. 1994; Kamb et al. 1994b), and 9p21 homozygous deletions centered on *CDKN2A* were frequently observed in cancer cell lines of different types (Kamb et al. 1994a; Nobori et al. 1994). The *CDKN2A* story is one illustrative example of how rare inherited cancer syndromes can provide key insights into the genes and pathways relevant to malignant transformation of specific cell lineages in both familial and sporadic settings.

The genomic complexity of the 9p21 locus fueled considerable debate on the relative importance of the several overlapping proteins encoded therein. For example, a significant proportion of the 9p21 deletions also included the neighboring *CDKN2B* gene (for review, see Haluska and Hodi 1998; Ruas and Peters 1998). To confirm a causal role of *CDKN2A* loss in 9p21-mediated melanoma, a gene targeting approach was used to delete exons 2 and 3 of the *Cdkn2a* gene in the mouse germline (Serrano et al. 1996). These mice succumbed to fibrosarcomas and lymphomas with high frequency (Serrano et al. 1996) and became highly prone to cutaneous melanomas with short latency when combined with an activated *H-RAS* mutation in their melanocytes (*Tyr-RAS*⁺) (Chin et al. 1997). Moreover, 100% of melanomas that arose in *Tyr-RAS*⁺; *Ink4a/Arf*^{+/-} animals exhibited loss of heterozygosity (LOH) in the remaining wild-type allele (Chin et al. 1997). Specifically, exon 2 sequences were eliminated in all cases, the significance of which relates to the peculiar genomic organization of this gene

that is unique in mammalian cells (human, mouse, opossum) (Sharpless et al. 2003; see below).

CDKN2A encodes two distinct proteins, INK4A and ARF, through utilization of alternative promoters and first exons (1 α for *INK4A* and 1 β for *ARF*) (for review, see Chin 2003). The shared second exons of the two transcripts are translated in different reading frames, thus encoding two proteins with no amino acid homology yet each possessing potent anticancer activities. In particular, INK4A (also known as p16^{INK4a})—the founding member of the INK4 (Inhibitor of cyclin-dependent kinase 4) family of proteins—inhibits the G1 cyclin-dependent kinases (CDKs) 4/6, which phosphorylate and inactivate the retinoblastoma protein (RB), thereby allowing for S-phase entry (Serrano et al. 1993). Loss of INK4A function therefore promotes RB inactivation through hyperphosphorylation, resulting in unconstrained cell cycle progression. ARF—the alternative reading frame product of the locus (Quelle et al. 1995) (also known as p14^{ARF} in humans, or p19^{ARF} in mice)—inhibits MDM2-mediated ubiquitination and subsequent degradation of p53 (Kamijo et al. 1998; Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998); thus, loss of ARF inactivates p53.

INK4A–CDK4/6–RB pathway In humans, germline intragenic mutations have been identified in familial melanoma kindred and melanoma-prone families that specifically inactivate INK4A while preserving ARF (e.g., exon 1 α mutations) (Hussussian et al. 1994; Kamb et al. 1994b; FitzGerald et al. 1996). These findings provided the smoking gun fingering this protein as a bona fide melanoma suppressor (for review, see Chin 2003). In addition to the 25%–40% of melanoma-prone families and 0.2%–2% of sporadic melanoma patients harboring *INK4A* mutations in the coding region (Aitken et al. 1999; Tsao et al. 2000), polymorphism in both 5' and 3' untranslated regions (UTRs) that alter translation or possibly regulate message stability of *INK4A* as well as promoter and splicing mutations of *INK4A* have since been identified in association with 9p21-linked melanoma-prone families (Liu et al. 1999; Kumar et al. 2001; for review, see Sharpless 2004). In line with this, mice with specific *Ink4a* inactivation did show increased susceptibility to carcinogen-induced and spontaneous melanoma (Krimpenfort et al. 2001; Sharpless et al. 2001); however, the phenotype observed was relatively weak. Consistent with this weak melanoma phenotype, it has recently been shown BRAF^{E600}-induced senescence in nevus melanocytes is in part INK4A-independent, suggesting presence of another melanoma suppressor(s) that must be inactivated for unconstrained melanoma development (Michaloglou et al. 2005).

Germline mutations of *CDK4*, an Rb-kinase that is inhibited by INK4A, have also been identified in melanoma-prone kindreds (Wolfel et al. 1995; Zuo et al. 1996; Soufir et al. 1998; Tsao et al. 1998; Molven et al. 2005). These mutations, which are a rare cause of familial melanoma, target a conserved arginine residue (Arg24) and render the mutant protein insensitive to inhibition

by the INK4 class of cell cycle inhibitors. Melanomas from patients harboring these germline *CDK4* mutations do not demonstrate somatic *INK4A* inactivation, suggesting that INK4A inactivation and CDK4 activation are mutually exclusive. Accordingly, the mutant *Cdk4* Arg24Cys (R24C) “knocked-in” mouse mirrored the human situation, in that it developed increased susceptibility to melanomas after carcinogen treatment, while lacking evidence of somatic *Ink4a* inactivation (Sotillo et al. 2001). Moreover, this R24C knockin mouse readily cooperates with activated HRAS in spontaneous and UV-induced melanoma genesis (Hacker et al. 2006). In line with these data in the mouse, somatic alteration of *CDK4*, in form of focal amplification, is observed in sporadic melanomas in human (Muthusamy et al. 2006).

Finally, although less commonly associated with familial melanoma than with hereditary retinoblastoma, patients with germline inactivation of the retinoblastoma gene (*RB1*) are predisposed to melanoma (Draper et al. 1986; Sanders et al. 1989; Eng et al. 1993; Fletcher et al. 2004). In patients cured of bilateral retinoblastoma, the estimates of increased lifetime risk of melanoma range from fourfold to 80-fold (Eng et al. 1993; Fletcher et al. 2004). These melanomas do not necessarily occur in skin associated with radiotherapy treatment ports of the retinoblastoma, and likewise the excess incidence of melanoma in these cohorts has not been reduced by the practice of decreasing radiotherapy for retinoblastoma. In contrast, the lifetime risk of sarcoma in retinoblastoma survivors has markedly decreased in the setting of less primary treatment radiotherapy, suggesting that the bulk of sarcoma risk in these patients is treatment-related. Therefore, the increased risk of melanoma in patients who survive bilateral retinoblastoma appears to result from the stochastic loss of the remaining wild-type *RB1* allele in these patients. These data provide further evidence linking the entire p16/CDK/RB axis to melanoma suppression in human.

ARF-MDM2-P53 pathway Inactivation of the p53 pathway appears to be a rite of passage for virtually all tumor cells. While most human solid tumors inactivate this tumor suppression pathway at the level of p53 itself (e.g., within the *TP53* coding region), melanoma provides a notable exception to this rule. Numerous surveys have found either rare or absent *TP53* point mutations or allelic loss in surgical specimens from primary and metastatic melanomas (for review, see Chin 2003). Moreover, *HMD2* amplification (encodes human ortholog of MDM2) occurs in only 3%–5% of human melanomas (Muthusamy et al. 2006), raising questions as to the pathogenetic relevance of the p53 pathway in melanoma. On the other hand, support for a role for p53 has been suggested by Mintz and colleagues, who showed that SV40 T antigen (which inactivates both RB and p53) generates a highly penetrant and aggressive melanoma phenotype (Bradl et al. 1991). More direct evidence for a role of p53 in melanoma suppression derived from *Tyr-RAS*⁺; *Trp53*^{+/-} mutant mice. These mice readily developed cutaneous melanomas characterized by LOH of the wild-

type *Trp53* allele and retention of *Arf* (Bardeesy et al. 2001), a mirror image to the molecular profile of the *Tyr-RAS*⁺; *Ink4a/Arf*^{-/-} melanoma model, which retained wild-type p53 in all cases (Chin et al. 1997). These mouse genetic studies highlighted both the relevance of the p53 pathway in melanoma suppression and the reciprocal pattern of Arf–p53 inactivation, thereby suggesting their functional relatedness. Ultimately, elucidation of the role of ARF as a negative regulator of p53 (Kamijo et al. 1998; Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998) offered a mechanistic explanation for the patterns of genetic mutations in human disease, where the lack of p53 mutation in melanoma reflected preferred pathway inactivation at the level of ARF via 9p21 deletion.

A corollary to the above observation is that ARF represents an independent target of genetic inactivation at the 9p21 locus. Although insertions and deletions in *ARF* exon 1 β without affecting INK4A or INK4B expression have been reported in melanoma cell lines (Kumar et al. 1998), the most convincing data on ARF as an independent melanoma suppressor came from two melanoma patients with germline ARF-specific mutations. One patient harbored a 14-kb deletion in exon 1 β sparing both *INK4A* and *INK4B* genes (Randerson-Moor et al. 2001), while the second patient had a 16-bp insertion in exon 1 β generating a frame-shifted ARF mutant defective in cell cycle arrest (Rizos et al. 2001). However, intact *INK4A* activity was not demonstrated in either case, precluding definitive assignment of INK4A-independent activity to ARF. In addition, in one case of a splice mutation of exon 1 β that was detected in a family of melanoma, inactivation of INK4A was acquired somatically in a melanoma from the family, pointing to the requirement for dual inactivation of these two genes (Hewitt et al. 2002). More recently, a cluster of five different germline mutations at the exon 1 β splice donor site was identified in melanoma pedigrees known to harbor no mutation in *INK4A* exons or in *CDK4* exon 2 (*INK4A*-binding site) (Harland et al. 2005). These splice site variants were predicted to impair ARF activity, although their causal roles have not been functionally demonstrated. On the other hand, there is some evidence that point mutagenesis of shared exon 2 still preferentially targets the *INK4A* transcript. Previously, alterations in the shared exon 2 sequences were considered inactivating events for both INK4A and ARF, a notion that is being challenged by a recent survey, which examined reported exon 2 point mutations (both germline and somatic) and found a higher proportion of synonymous changes and a lower percentage of nonsense mutations in the *ARF* reading frame when compared with the *INK4A* reading frame (Yang et al. 2005). In silico modeling of sequence variants that yield missense mutations in both reading frames further suggests that the impact of the codon change is more severe for INK4A than ARF (Yang et al. 2005).

Contrary to the equivocal situation in human, Arf is a potent tumor suppressor in vivo in the mouse. While *Ink4a* deficiency alone resulted in a modest tumor-prone phenotype (Krimpenfort et al. 2001; Sharpless et al. 2001,

2002), this *Ink4a*-dependent susceptibility was significantly enhanced in the setting of *Arf* haploinsufficiency (Krimpenfort et al. 2001), arguing for an independent contribution from *Arf* in *Ink4a/Arf*-mediated melanoma suppression. Additionally, when crossed onto the melanoma-prone *Tyr-RAS* transgenic allele, either *Ink4a* or *Arf* deficiency facilitated enhanced melanoma formation, although *Arf* loss was more efficient than *Ink4a* (Sharpless et al. 2003). Moreover, the resultant melanomas showed reciprocal p53 or RB pathway inactivation. In other words, Rb pathway lesions were observed in melanomas from *Tyr-RAS*⁺; *Arf*^{-/-} mice, and p53 pathway lesions were detected in *Tyr-RAS*⁺; *Ink4a*^{-/-} melanomas (Sharpless et al. 2003). This profile provides unequivocal genetic evidence that both products of the *Ink4a/Arf* locus served prominent and synergistic/independent roles in melanoma suppression in vivo, which corroborated observation in humans (Hewitt et al. 2002). Moreover, this shared function may underlie the need for coordinated regulation imparted in part by their intimate and evolutionarily conserved genomic arrangement (Sharpless and DePinho 2004). This argument also explains another interesting feature of melanoma: the high frequency of 9p21 deletion as opposed to other mechanisms of INK4A loss such as promoter methylation or point mutation. These latter mechanisms only target INK4A, and are more common in tumors such as non-small-cell lung cancer, where direct p53 inactivation occurs. In summary, while INK4A is the preferred target of inactivation in the INK4A-CDK4-RB pathway, extensive biochemical and genetic data suggest an independent role for the ARF-p53 axis in melanoma suppression, with ARF as its preferred target via 9p21 deletion.

Receptor tyrosine kinase (RTKs) activation

A large body of evidence has implicated hyperactive RTK signaling in the development and progression of melanoma. Although most were based on expression alterations, several RTKs map to known regions of recurrent DNA copy number gain or amplification. Moreover, considering the example of c-Kit (see below), it is expected that systematic resequencing efforts will identify activating mutations in these and other RTKs in melanomas.

Late-stage melanomas often exhibit epidermal growth factor receptor (EGFR) overexpression in association with increased copies of chromosome 7 (Koprowski et al. 1985; Bastian et al. 1998; Udart et al. 2001). Enforced activation of EGFR has been associated in metastatic progression in cell-based study (de Wit et al. 1992; Huang et al. 1996b). However, unlike glioblastomas or lung adenocarcinoma (Maher et al. 2001; Sihto et al. 2005), focal amplification and/or mutation of EGFR has not been reported in melanoma. The nonfocal nature of chromosome 7 gains in melanoma renders it impossible to assign EGFR as a target of such genomic alterations. In an inducible HRAS-driven mouse melanoma model (Chin et al. 1999), transcriptome analysis revealed the existence of a RAS-dependent EGFR signaling loop mediated

through up-regulation of EGF family ligands (e.g., amphiregulin and epiregulin) (Bardeesy et al. 2005). This EGFR signaling pathway provides important survival signals involving PI3-K-dependent activation of AKT, as sustained EGFR activity is able to prolong viability of established melanoma upon inactivation of RAS. Conversely, inactivation by dominant-negative EGFR abolishes tumorigenicity of RAS-driven melanoma cells, consistent with observations in other cell systems (fibroblasts, keratinocytes, and intestinal epithelial cells) that autocrine EGFR signaling is required for transformation by activated RAS (Dlugosz et al. 1997; Gangarosa et al. 1997; Sibilio et al. 2000). Thus, in addition to providing experimental evidence that EGFR activation is biologically relevant, the abovementioned study in the inducible model also points out the possibility that EGFR or its ligands may constitute alternative point(s) of therapeutic intervention in RAS-activated melanoma. It should be mentioned that the contribution of EGFR signaling to melanoma development and possibly progression is evolutionarily conserved, as activating mutations in the EGFR homolog, *Xmrk*, increase melanoma susceptibility in *Xiphophorus* fish (Wittbrodt et al. 1992; Winnemoeller et al. 2005; Meierjohann et al. 2006; for review, see Bardeesy et al. 2000). It therefore remains possible that similar activating mutations exist in human melanoma, although systematic resequencing of large cohorts of melanomas from different ethnic and/or molecular subclasses will be required to uncover such examples.

The RTK c-MET is normally expressed on epithelial cells and melanocytes (Bottaro et al. 1991) and is activated by binding of its ligand, Hepatocyte growth factor/scatter factor (HGF). Although MET is normally activated in a paracrine manner, autocrine activation of HGF-MET has been described in melanoma progression (Li et al. 2001; for review, see Vande Woude et al. 1997). Accordingly, increased c-MET expression has been observed in metastatic melanoma (Natali et al. 1993), and copy number gain of the c-MET locus at 7q33-qter seems to be a late event in melanoma progression (Wiltshire et al. 1995; Bastian et al. 1998). However, similar to EGFR above, neither focal MET amplifications nor activating MET point mutations have been detected in melanoma, although both have been observed in other human cancers (Jeffers et al. 1997; Schmidt et al. 1997; Kong-Beltran et al. 2006; Smolen et al. 2006). However, several lines of experimental and functional evidence support a causal role for MET signaling in human melanoma. For example, in explant models, it has been shown that elevated c-Met expression or Met RTK activity may correlate with metastasis (Rusciano et al. 1995). In genetically engineered models, constitutive and ubiquitous HGF expression establishes an autocrine loop with c-Met, leading to stepwise development and progression of cutaneous and metastatic melanomas, which cooperates with UVB and *Ink4a/Arf* deficiency (Otsuka et al. 1998; Noonan et al. 2001; Recio et al. 2002). Correspondingly, while enforced expression of c-Met in melanocytes provides only weak cancer-initiating activity, this mutation

drives the development of metastatic disease, and such tumor lesions show concomitant activation of HGF and establishment of HGF-Met signaling loop (L. Chin, unpubl.). Finally, c-MET was recently shown to be a direct transcriptional target of microphthalmia-associated transcription factor (MITF) (McGill et al. 2006), the melanocytic lineage transcription factor that can be activated by focal amplification in melanoma (see below).

The c-Kit gene encodes a RTK that serves as the receptor for Stem Cell Factor (SCF). Numerous immunohistochemical studies have linked progressive loss of c-KIT expression with the transition from benign to primary and metastatic melanomas (Montone et al. 1997; Shen et al. 2003; Isabel Zhu and Fitzpatrick 2006). Reconstitution of c-Kit in metastatic melanoma cells apparently conferred sensitivity to SCF-induced apoptosis in vitro (Huang et al. 1996a). Thus, at first glance, Kit does not fit the profile of a RTK targeted for activation in melanoma. However, a recurrent L576P mutation in c-Kit has recently been reported in melanoma. Among 153 cases examined, Holden and colleagues identified four metastatic melanomas with robust expression of c-KIT on IHC. High-resolution amplicon melting analyses followed by direct DNA sequencing revealed that three of them harbored a L576P mutation with selective loss of the normal allele (Willmore-Payne et al. 2005, 2006). L576P is a known GIST-associated mutation that maps to the 5' juxtamembrane domain where most activating KIT mutations cluster (Nakahara et al. 1998; Fukuda et al. 2001). Although likely representing an uncommon path to melanoma, the example of EGFR mutational status as a predictor for therapeutic responses in NSCLC (Lynch et al. 2004; Paez et al. 2004) suggests the possibility of identifying a melanoma patient subpopulation that will respond to imatinib based on c-Kit mutational status.

RAS and RAF, activators of mitogen-activated protein (MAP) kinase signaling

Considering the prominent roles of RTKs in transmitting extracellular signals to intracellular effectors, and the importance of homotypic and heterotypic cell-cell interactions in cancers, it is not surprising that almost all of the direct signaling components of RTKs have been implicated in human melanoma. One of the major signaling mediators of RTK is the MAP kinase pathway (ERK1/2), which has been most directly linked to its growth-promoting activities. Since self-sufficiency in growth signaling is a requisite capability acquired by all cancer cells (Hanahan and Weinberg 2000), hyperactive ERKs are common in many human cancers, including melanoma (Takata et al. 2005; Zhuang et al. 2005). Such a hyperactive state can theoretically be achieved by activating mutations of any signaling mediators upstream of ERKs, but there are clear tumor-type-specific patterns of mutational activation. RAS is perhaps the most frequently activated component of this signaling cascade in human cancer with a reported incidence of 15%–30% of all cases (Bos 1989), and in some cancer types, such as

KRAS in pancreas cancer, its mutation rate approaches 100% (Hezel et al. 2006). BRAF is also commonly targeted in human cancers with an overall occurrence of 7% (Davies et al. 2002), although it is notable that the mutation frequency approaches 70% in metastatic melanoma (Maldonado et al. 2003; Pollock et al. 2003; Uribe et al. 2003; Daniotti et al. 2004; Kumar et al. 2004; Shinozaki et al. 2004; Libra et al. 2005). However, while classically considered primarily mitogenic, ERK activation can also regulate differentiation, senescence, and survival. For instance, activated ERK protein kinases can phosphorylate enzymes that regulate metabolism, or cytoskeletal proteins that regulate cell shape and migration. In the nucleus, ERK can regulate gene expression by phosphorylating transcription factors such as ETS1/2 (Paumelle et al. 2002), or other protein kinases such as S6 Kinase (Crews et al. 1991). Therefore, genotype-phenotype correlation must take into account consequences other than growth promotion by activating mutations in components of ERK signaling.

The RAS family of proto-oncogenes: H-RAS, N-RAS, and K-RAS In contrast to other solid tumors, activating mutations of RAS proto-oncogenes are not detected with high frequency in melanoma, ranging from low to 10%–15% incidence, with the highest frequencies detected in the amelanotic nodular subtype (for review, see Chin et al. 1998). N-RAS is the most frequent RAS family member targeted in the melanocyte lineage, with activating mutations in as many as 56% of congenital nevi (Papp et al. 1999), 33% of primary and 26% of metastatic melanoma samples (Demunter et al. 2001). Activating N-RAS mutations have been correlated with nodular lesions and sun exposure (Jafari et al. 1995; van Elsas et al. 1996). Interestingly, N-RAS mutations are rarely found in dysplastic nevi (Albino et al. 1989; Jafari et al. 1995; Papp et al. 1999), which may imply their distinct evolutionary path to melanoma. H-RAS activation has occasionally been detected in melanoma, albeit more commonly associated with Spitz nevi, based on amplification of its genomic locus on 11p and oncogenic point mutations (Bastian et al. 2000). K-RAS mutations have not been described in human melanocytic lesions. These mutation patterns point to distinct biological activities of the different RAS family members in melanocyte biology. The phenotypic impact of activated H-RAS versus N-RAS transgenic mice has reinforced this view. Specifically, an activated H-RAS transgene, together with inactivating mutations in *Ink4a*, *Arf*, and/or *p53* promotes development of nonmetastatic melanomas (Chin et al. 1997; Bardeesy et al. 2001; Sharpless et al. 2003). In contrast, when targeted to the melanocytic compartment, an activated N-RAS transgene and *Ink4a/Arf* deficiency drive cutaneous melanomas with high penetrance and short latency, as well as metastatic spread to lymph nodes and other distal sites (e.g., lung and liver) in a third of the cases (Ackermann et al. 2005).

BRAF, a potent activator of ERK protein kinases Activating BRAF mutations are the most prevalent somatic

genetic event in human melanoma. Since its discovery through a genome-wide cancer resequencing effort (Davies et al. 2002), mutations in BRAF have been detected in a variety of tumor types, with the highest incidence in melanoma (ranging from 27% to 70%) (Maldonado et al. 2003; Pollock et al. 2003; Uribe et al. 2003; Daniotti et al. 2004; Kumar et al. 2004; Shinozaki et al. 2004; Libra et al. 2005), followed by papillary thyroid tumors, colorectal cancers, and ovarian cancers (Ciampi and Nikiforov 2005; Young et al. 2005). These point mutations clustered in specific regions of biochemical importance, with the predominant melanoma mutation being a single phosphomimetic substitution in the kinase activation domain (V600E), which confers constitutive activation (Garnett and Marais 2004). The biology and detailed characterization of the RAF family in human cancers and in melanoma have been discussed in detail in several other reviews (Garnett and Marais 2004; Gray-Schopfer et al. 2005).

BRAF does not appear to be an inherited cancer predisposition gene (Laud et al. 2003; Casula et al. 2004; Jackson et al. 2005; Kelemen et al. 2005; Xing 2005), as individuals with germline mutations develop instead cardio-facio-cutaneous syndrome, which is not associated with increased cancer risk (Niihori et al. 2006; Rodriguez-Viciana et al. 2006). The high prevalence of BRAF mutations in cutaneous melanoma and the known epidemiological link between UV and melanoma has prompted speculation that the BRAF^{V600E} mutation is induced by UV damage. Arguing against this supposition is the fact that the T → A transversion that converts the valine to glutamic acid at amino acid 600 (V600E) is not classically associated with UV-induced damage (Daya-Grosjean et al. 1995). However, the cutaneous distribution of melanomas harboring this BRAF^{V600E} mutation suggests a complex yet undefined relationship with UV exposure. Specifically, BRAF mutations are common (59%) in melanomas arising in skin with intermittent sun exposure, such as trunk and arms, compared with only 23% of the acral melanomas and 11% of mucosal melanomas harboring BRAF mutations (Maldonado et al. 2003; Edwards et al. 2004; Curtin et al. 2005), but are absent in uveal melanoma (Cohen et al. 2003; Cruz et al. 2003; Edmunds et al. 2003; Rimoldi et al. 2003; Weber et al. 2003). A clear understanding of the UV-BRAF link is further complicated by the observation that melanomas from chronically sun-exposed areas (defined by histopathological evidence of chronic sun damage) possess only an 11% frequency of BRAF^{V600E} mutation (Maldonado et al. 2003; Edwards et al. 2004; Curtin et al. 2005). Together, these contrasting observations highlight the uncertainties surrounding the molecular factors driving BRAF mutation, in particular, the role of sun exposure.

B-RAF mutations are also common in benign and dysplastic nevi (Pollock et al. 2003; Yazdi et al. 2003; Kumar et al. 2004; Saldanha et al. 2004), suggesting a role in the earliest stages of neoplasia. It is notable that nevi are considered growth-arrested and only rarely progress into melanoma, raising the possibility of BRAF^{V600E}-induced

checkpoint mechanisms operating to constrain malignant transformation. Indeed, a recent study showed that human congenital nevi are invariably positive for senescence-associated acidic β -galactosidase (SA- β -Gal), the classical senescence-associated marker (Michaloglou et al. 2005). In vitro, BRAF^{V600E} expression in human melanocytes has been shown to induce cell cycle arrest, with concomitant induction of both INK4A and SA- β -Gal, as well as methylation of Lys 9 of histone H3 on senescence-associated heterochromatic foci (SAHF). Thus, this study, along with others, provided evidence that oncogene-induced senescence (OIS) is an in vivo phenomenon functioning to constrain progression of early premalignant lesions (Sharpless and DePinho 2005). Intriguingly, expression of INK4A is not in 100% concordance with SA- β -Gal positivity, suggesting presence of a non-INK4A-dependent pathway in mediating BRAF-induced OIS (Michaloglou et al. 2005). It seems probable that the identification of this pathway would lead to the discovery of a tumor suppressor whose importance in melanoma rivals that of INK4A.

The notion that BRAF^{V600E} is not sufficient for transformation of melanocytes has also been demonstrated in other model systems. In zebrafish, it has been shown that BRAF activation leads only to development of benign nevi, while progression to frank melanoma requires cooperation of p53 deficiency (Patton et al. 2005). Similarly, BRAF^{V600E} mutation alone in TERT-immortalized RB-p53 mutant human melanocytes was found to produce only junctional nevi in the human/mouse skin graft, in contrast to activated NRAS or PI3K p110a mutants, which generated invasive melanoma lesions (Chudnovsky et al. 2005). While these biological outcomes indicate distinct roles for NRAS and BRAF activation in melanoma genesis, some functional overlap is suggested by the predominantly nonconcurrent occurrence of activated *NRAS* and *B-RAF* alleles in melanoma and other tumor types (Davies et al. 2002; Rajagopalan et al. 2002; Goel et al. 2006). The single and compound mutant mouse models will prove useful in dissecting the common and distinct roles of activated NRAS and BRAF signaling in vivo in the melanocytic lineage.

PTEN, negative regulator of phosphatidylinositol 3-kinase (PI3K)–AKT pathway

The PI3 kinase–AKT pathway is often hyperactive in melanoma. Integrins and growth factors (such as HGF and IGF-1) promote melanoma cell growth and survival via PI3 kinase/AKT activation in vivo (Meier et al. 2005; Robertson 2005). In addition, elevated phospho-AKT levels appear to correlate adversely with patient survival (Dai et al. 2005). Taken together, these observations suggest the strong biological importance of PI3 kinase signaling in this disease. However, unlike the MAP kinase pathway, genetic alterations specifically targeting components of this signaling cascade do not occur at high frequency in melanoma. Of those that do occur, the best known culprit is the PTEN tumor suppressor.

PTEN encodes a lipid and protein phosphatase that

resides on chromosome 10q, a region known to sustain LOH in many human cancers, including melanoma (Bastian 2003; Wu et al. 2003). This protein regulates extracellular growth signals that use the lipid phosphatidylinositol phosphate (PIP₃) as an intracellular second messenger. In the presence of growth factor signaling, the intracellular level of PIP₃ rises, leading to phosphorylation of AKT, which is known to promote cell cycle progression and inhibit apoptosis. PTEN regulates PIP₃ levels, and its inactivation results in accumulation of PIP₃, AKT hyperphosphorylation, and enhanced cell survival/proliferation. In melanoma, allelic loss or altered expression of PTEN comprises 20% and 40% of melanoma tumors, respectively (Pollock et al. 2002; Mikhail et al. 2005; Slipicevic et al. 2005; Goel et al. 2006), although somatic point mutations and homozygous deletions are rarely observed. Functionally, ectopic expression of PTEN in PTEN-deficient melanoma cells can abolish phospho-AKT activity, induce apoptosis, and suppress growth, tumorigenicity, and metastasis (Robertson et al. 1998; Stewart et al. 2002; Stahl et al. 2003; for review, see Robertson 2005). Correspondingly, germline or somatic inactivation of *Pten* in the mouse strongly promotes tumor phenotypes in multiple cell lineages (Di Cristofano et al. 1998; Stambolic et al. 1998; Podsypanina et al. 1999; Ma et al. 2005), including melanoma (You et al. 2002).

In line with the experimental evidence supporting a melanoma-suppressive role of PTEN, constitutive activation of AKT has been shown to be a potent oncogenic lesion for melanocyte transformation (Chudnovsky et al. 2005). In addition, DNA copy gain involving the AKT3 locus has recently been described in melanoma, and selective AKT3 activation may characterize 40%–60% of sporadic tumors (Stahl et al. 2004). However, the complexity of this signaling cascade has not been fully understood. Recent data have suggested that activation of different AKT isoforms may elicit distinct effects on cell proliferation and survival. For example, one report found that AKT3 correlated most strongly with melanoma tumor progression among the three AKT isoforms, as described above; and that targeted AKT3 depletion triggered apoptotic signaling (Stahl et al. 2004). On the other hand, AKT1 activation was found to inhibit the migration and invasion of certain cancer cell lines (Yoeli-Lerner et al. 2005), including MDA-MB435, a line previously believed to derive from breast cancer but subsequently shown through transcriptional and SNP array profiling studies to be a melanoma cell line (Ross et al. 2000; Garraway et al. 2005). Thus, although the PI3 kinase/AKT pathway clearly demonstrates enhanced activity in many melanomas, the extent to which this constitutes a critical melanoma dependency remains unresolved.

Lastly, although *PTEN* is a bona fide tumor suppressor of 10q24, the existence of additional melanoma suppressors has been inferred by the observation that many more human melanomas with 10q LOH exist than do those with PTEN-specific homozygous/hemizygous loss or mutation, and that reintroduction of *PTEN* into such

melanoma cells seems to have no growth-suppressive effect (Robertson et al. 1998). The *Myc* antagonist, *MXI1*, is a candidate for this, as *Myc* is amplified or overexpressed in *RAS*-induced *Trp53*-deficient melanomas in mouse (Bardeesy et al. 2001); however, a role for *Mxi1* in melanoma genesis in mice and humans has not been rigorously evaluated.

The genomics of melanoma

Beyond these characterized signature pathway alterations, genomic profiling of human melanoma has revealed a highly rearranged melanoma genome, attesting to underlying molecular heterogeneity of this disease. Mining of such heterogeneity represents an active area of investigation, as it is widely assumed that embedded within this complexity are clues to disease pathogenesis, clinical behavior, and possibly response to therapy. In addition, high-resolution characterization has revealed the existence of many recurrent nonrandom amplification and deletions with as yet no obvious or validated targets, potentially representing many yet-to-be-discovered melanoma-relevant genes. That these genomic events serve as productive entry points for discovery of melanoma genes is exemplified by discovery of *MITF* (see below). However, the high level of complexity inherent in the melanoma genome also brings into focus a critical need for biological systems capable of prioritizing in-depth validation efforts as illustrated in the case of *NEDD9* (see below).

Genomic heterogeneity of melanoma

The underlying molecular heterogeneity of human melanoma has long been apparent on clinical grounds. For example, melanomas arising at different sites of the body may exhibit markedly distinct biological and clinical behaviors. Lentigo maligna melanomas are indolent tumors that develop over decades on chronically sun-exposed area such as the face. In contrast, acral lentiginous melanomas, which develop on sun-protected regions, tend to be more aggressive. “Thick” and “thin” primary melanomas provide additional examples: Although thick lesions have a much higher risk for metastasis than do their thinner counterparts, there is a subset of thin cutaneous melanoma that metastasizes early (Gimotty et al. 2005), suggesting inherent differences within this subset.

Recent advances in genome-wide technologies enable systematic documentation of tumor heterogeneity at high resolution, opening doors to better stratification and, ultimately, improved management of melanoma patients. Transcription profiling studies have provided evidence for distinct molecular subclasses of melanoma (Bittner et al. 2000; Segal et al. 2003; Tschentscher et al. 2003; Onken et al. 2004; Haqq et al. 2005). At the genomic DNA level, the nonrandom nature of the chromosomal alterations characteristic of melanoma likely also dictates disease behavior; thus, patterns of alterations

detectable at either the DNA or RNA level may segregate melanoma tumors into subtypes with distinct clinical behaviors and possibly therapeutic responses. Indeed, a recent genome-wide CGH profiling and targeted resequencing study on 126 primary melanomas showed that distinctive patterns of genomic alterations, including *BRAF* and *NRAS* mutation frequencies, can be identified in melanoma arising in different anatomic sites and with varying UV exposure histories (acral, mucosal, or with and without chronic sun damage) (Curtin et al. 2005). Similar genetic and biological heterogeneity characterized metastatic melanomas. High-resolution CGH studies of >80 metastatic melanomas found that metastatic tumors can be separated into three distinct classes by unsupervised classification. Such subdivision does not correlate with sites of metastasis; rather, these classes are determined primarily by patterns of chromosomal aberrations. When intersected with clinical outcome, one of the three classes showed a more significant survival advantage than the other two (L. Chin, unpubl.), suggesting that these DNA-based classes of metastatic melanomas may prove biologically pertinent.

The melanoma gene atlas

Beyond the characteristics described above, the melanoma genome also possesses numerous recurrent non-random chromosomal rearrangements indicative of the existence of many additional genetic elements governing disease genesis and progression. Whole-genome screening technologies such as spectral karyotype analysis and array-CGH have identified many recurrent nonrandom chromosomal structural alterations, particularly in chromosomes 1, 6, 7, 9, 10, and 11 (Bastian et al. 1998; Curtin et al. 2005; Garraway et al. 2005); however, in most cases, no known or validated targets have been linked to these alterations. Thus, there exists significant opportunity to discover novel melanoma genes and translate such discoveries into meaningful clinical endpoints.

In a systematic high-resolution genomic analysis of melanocytic genomes, array-CGH profiles of 120 melanocytic lesions, including 32 melanoma cell lines, 10 benign melanocytic nevi, and 78 melanomas (primary

and metastatic) have revealed a level of genomic complexity not previously appreciated. In total, 435 distinct copy number aberrations (CNAs) were defined among the metastatic lesions, including 163 recurrent, high-amplitude events. These include all previously described large and focal events (e.g., 1q gain, 6p gain/6q loss, 7 gain, 9p loss, and 10 loss). Not surprisingly, compared with the metastatic tumors, the degree of genomic instability (as reflected by number of CNAs) evident in primary melanomas and benign nevi is significantly diminished (Fig. 1). These types of primary versus metastatic comparisons provide an effective biological filter for progression-related events, thereby enriching for genetic events that constitute likely drivers of metastasis. Such comparative approaches should provide robust future grounds for melanoma gene discovery, as primary melanomas are largely curable with surgical resection, while metastatic disease is near universally fatal.

MITF, a melanoma oncogene targeted by amplification

The promise of DNA-based structural alterations as the entry point for gene discovery has been illustrated by the recent identification of *MITF* as a melanoma oncogene. The discovery of *MITF* amplification in melanoma derived from an integrated analysis of genomic copy gains and losses, together with sample-matched mRNA expression data (Garraway et al. 2005). When clustering algorithms were applied to SNP array-derived chromosomal copy number data generated for the NCI-60 cancer cell line collection, some of these cell lines aggregated according to tissue of origin, including several melanoma cell lines. The bidimensionality of the hierarchical algorithm also enabled the identification of chromosomal alterations driving these lineage-restricted clustering patterns, and suggested that lineage-specific cancer genes might reside within the genomic regions implicated. For the melanoma cell lines, the common genomic alteration was a region of copy gain at chromosome 3p14-3p13. To facilitate the identification of an oncogene targeted by this amplification event, the NCI-60 collection was partitioned based on the presence or absence of copy gain at the relevant chromosome 3p locus (Garraway et al. 2005). This partitioning served as a

Figure 1. The genomes of benign and malignant melanocytic lesions. Genome-wide profiles of representative benign nevus, primary, and metastatic melanoma showing increasing genomic complexity.

two-class distinction that drove a supervised analysis of sample-matched gene expression data. Although the gene expression signature that emerged was dominated by melanocyte lineage genes (as expected given that only melanoma cell lines comprised the 3p-amplified class), *MITF* was the only gene showing significantly increased expression in association with the 3p-amplified melanoma cell lines that also mapped to the common region of 3p copy gain.

MITF amplification was subsequently detected in 10% of primary cutaneous and 15%–20% of metastatic melanomas. Although the majority of amplifications were low level (e.g., four to six copies per cell), high-level amplicons were also observed, including one sample that exhibited >100 copies per diploid genome. A Kaplan-Meier analysis performed on metastatic melanomas suggested that *MITF* amplification in this setting correlated with adverse 5-yr patient survival. Finally, ectopic *MITF* overexpression complemented *BRAF*^{V600E} in conferring soft agar colony growth to immortalized melanocytes engineered to express TERT, and to lack the pRB and p53 pathways. These functional studies thereby suggested a genetic context that might characterize a subset of human melanomas whose survival is dependent on *MITF* (Garraway et al. 2005). *MITF* also exemplifies a newly recognized “lineage survival” oncogenic mechanism (Garraway and Sellers 2006a,b), wherein tumor genetic alterations may target survival functions also operant in the relevant cellular lineages during development and differentiation. Thus, while the discovery of *MITF* amplification began as a systematic genomics-based survey of many human cancer types, it provides a striking convergence of melanoma oncogene discovery and melanocyte development.

NEDD9, a melanoma metastasis gene identified by cross-species comparison The power of genetically engineered mouse (GEM) models as biological filters for human cancer gene discovery was illustrated in a recent study that identified *NEDD9* as a novel melanoma metastasis gene (Kim et al. 2006). Since evolution preserves key molecular mechanisms governing biological processes, it is hypothesized that overlapping genomic al-

terations in mouse and human tumors reflect selection driven by similar genetic pressure, and are thus more likely to represent critical events in tumorigenesis. Exploiting the experimental merits of an inducible mouse model of melanoma (Chin et al. 1999), Kim et al. (2006) compared nonmetastatic parental and metastatic variant melanomas in the mouse by genome-wide high-resolution array-CGH and identified a recurrent focal amplification associated with acquisition of metastatic potential. This amplification in mouse is syntenic to human 6p24-25, a region that sustains copy number gain in 36% of human metastatic, but not primary, melanoma (Bastian et al. 1998; Namiki et al. 2005). Although the recurrence of this regional event speaks to its potential pathogenic and/or prognostic importance, chromosome 6p gain in human tumors (including metastatic melanoma) typically involves an expansive region spanning >35 Mb, rendering identification of target(s) difficult to impossible. The cross-species comparison quickly narrowed one region of interest on 6p to an 850-kb region encompassing only eight annotated genes (Fig. 2). Further integration with expression data pointed to *NEDD9* as the potential target. Accordingly, *NEDD9* protein expression was shown to be up-regulated in a progression-correlated manner in human melanoma (Kim et al. 2006).

Biologically, *NEDD9* amplification and overexpression in melanocytes and melanoma cells translated into enhanced invasion and metastasis as demonstrated in a series of loss-of-function and gain-of-function assays. Mechanistic studies further established that *NEDD9* localizes to dynamic focal contacts at the cell periphery and physically and functionally interacts with focal adhesion kinase in mediating cell invasion. Here, the clear genotype (*Nedd9* amplification and persistent *HRAS* expression)–phenotype (metastatic potential) link defined in the mouse highlighted another major advantage of gene discovery in the GEM model, as it logically guided the selection of functional assays (invasion and metastasis) and defined the genetic context (cooperation with activated *HRAS* or *BRAF*) upon which such assays should be conducted (Kim et al. 2006). Without such insight, oncogenic activity of *NEDD9* would not have been apparent.

Figure 2. *Nedd9* amplification in metastatic mouse melanoma maps to region of frequent gain in human metastatic melanoma. (Top) Genomewide array-CGH profile of one mouse metastatic melanoma with *Nedd9* amplification on chromosome 13. (Bottom) One metastatic human melanoma profile showing gain of chromosome 6p, spanning a large region. (Arrows) Mouse chromosome 13 amplicon maps by synteny to human chromosome 6p24-25.

In addition to illustrating how comparative oncogenomics has enabled the identification and facilitated the validation of NEDD9 as a melanoma metastasis gene, this study also demonstrated evolutionarily conserved genomic activation of a component of the focal adhesion complex during melanoma metastasis (Kim et al. 2006), raising the possibility that inhibition of this signaling complex would halt progression of primary melanoma to metastatic disease. Along this line, it is interesting that NEDD9 expression appears to be positively selected for even at the primary melanoma stage, suggesting the potential predictive value of NEDD9 expression for future metastasis in primary melanoma.

Melanoma maintenance genes as rational therapeutic targets

The concept of tumor maintenance stems from the observation that cancer is the phenotypic end-point of numerous genetic and epigenetic alterations; moreover, the genomic complexity of human melanoma suggests that full malignant transformation of melanocytes requires the cooperating effects of multiple genetic lesions. This requirement has been well-demonstrated in genetically engineered models (GEMs), even when transformation has been driven by a potent initiating mutation (such as RAS). In the melanocyte lineage, even if one or more gain-of-function mutations are engineered into GEM models, transformed phenotypes only emerge (1) after a period of latency and (2) often with incomplete penetrance. Each of these characteristics implies a need for acquisition/selection of secondary cooperating events. Consistent with this, candidate or genome-wide molecular analyses of GEM melanoma have often identified spontaneous genetic alterations acquired de novo. For instance, in the HRAS-driven GEM model of melanoma on an *Ink4a* or *Arf*-deficient background, reciprocal p53 (e.g., p53 mutation or *Arf* loss) or Rb (e.g., *Cdk6* amplification or *Ink4a* loss) pathway lesions, respectively, have been detected in the resultant melanomas, such that melanoma from either genetic backgrounds harbored concomitant inactivation of both p53 and Rb pathway (Kannan et al. 2003; O'Hagan et al. 2003; Sharpless et al. 2003). Along the same line, even on an *In4a/Arf* double-null or p53-deficient background, new and non-random genomic alterations can be detected by array-CGH profiling in HRAS-driven melanoma (Bardeesy et al. 2001; Hochedlinger et al. 2004). In addition to the genetic lesions mentioned here, biological processes that maintain and sustain an established tumor likely involve epigenetic dysregulation and intimate tumor–stromal interactions, the molecular basis of which remains poorly understood. Clearly, efficacious therapeutic targets must play requisite roles in maintenance of established melanomas.

Inducible onco-transgene models have been developed to assess the tumor maintenance role of activated oncogenes in GEM (Chin and DePinho 2000). Using the tetracycline regulatory system, an inducible HRAS-driven melanoma model (*Tyr-rtTA⁺ Tet-RAS⁺ Ink4a/Arf^{-/-}*) has

been generated whereby expression of the oncogenic RAS can be regulated in a tissue-specific and developmental stage-specific manner (Chin et al. 1999). In this system, despite the presence of irreversible genomic alterations in RAS-initiated melanomas, inactivation of RAS via doxycycline withdrawal in fully established melanomas led to clinical and histopathological regression, a remarkable finding that attests to the essential role of RAS activation in tumor maintenance. In addition to addressing the role of an oncogene in maintenance, inducible GEMs provide an in vivo system to examine the heterotypic interaction of tumor and stromal cells. For example, it has been shown that RAS-mediated melanoma regression is associated with dramatic activation of apoptosis in both tumor and host-derived endothelial cells, a phenotypic outcome not necessarily anticipated based on the intimate link of RAS activation to proliferation via MAP kinase activation in vitro (Chin et al. 1999). Real-time imaging during tumor regression by MR and bioluminescence provided additional evidence that loss of vascular function in regressing melanomas upon RAS inactivation represented an active response to loss of RAS activity upon doxycycline withdrawal, rather than a passive bystander effect of tumor cell deaths (Tang et al. 2005). Together, these experiments underscore the importance of GEMs to interrogate the tumor maintenance requirement of putative melanoma genes prior to their designation as a rational therapeutic target.

The biology of melanoma—lessons from development

It is a commonly accepted premise in cancer biology that signature phenotypic features of a malignancy may be traceable to the cell and developmental biological characteristics of its lineage origins. Melanoma is no exception. Multiple key tumor biological hallmarks mirror the distinctive features of the melanocytic lineage. Indeed, a defining aspect of melanoma is a strong propensity for metastasis, reflecting well the developmentally encoded dispersion of melanocytes from the neural crest to cutaneous sites. Thus, it stands to reason that valuable information may derive from the careful scrutiny of melanoma biology through the lens of melanocyte lineage development.

Melanocyte stem cells

Melanocyte progenitor cells migrate over considerable distances, along a predictable route upon their exit from the neural crest. They traverse discrete stages of differentiation recognizable in terms of marker expression, ultimately populating the basal epidermis as well as the hair follicle in human. In certain furry mammals such as the rodents, the epidermal population may be transient, a feature likely arising from a lack of SCF expression by basal keratinocytes since persistent epidermal melanocytes have been observed in transgenic mice, which ubiquitously express the cytokine HGF (Takayama et al.

1996) or keratinocyte-targeted SCF (Kunisada et al. 1998). Within hair follicles, melanocytes reside in two locations: at the bulb (where they function as differentiated cells to provide pigment to the growing hair matrix) and as stem cells within the bulge region, located at the base of the permanent portion of the follicle (Nishimura et al. 2002).

As demonstrated by Nishimura et al. (2002), follicular bulge melanocytes satisfy the criteria of stemness, including properties of immaturity, slow cycling, self-maintenance, and the capacity to repopulate the hair follicle at the anagen phase. At the beginning of anagen, signals within the stem cell niche trigger an emergence from quiescence that results in two distinct melanocytic progeny. The differentiated population migrates to the base of the follicle (hair bulb), where melanin synthesis and transport to adjacent keratinocytes occur, resulting in melanization of newly formed hairs. Later in the hair follicle cycle, the deep (transient) portion of the follicle undergoes a regressive (catagen) phase characterized by apoptosis of the deep structures. Following a resting (telogen) phase of variable duration, a new anagen phase is triggered.

The existence of melanoma stem cells has also been investigated recently. Herlyn and colleagues identified a CD20-enriched subpopulation of cells from melanoma lines or metastases that grew as nonadherent spheroids when cultured in human ES-like medium. Moreover, these CD20-enriched cells could be induced to differentiate toward multiple lineages, including melanocyte, adipocyte, osteoblast, and chondrocyte, suggesting the presence of such a subpopulation with a stem cell phenotype (Fang et al. 2005). Separately, Frank and colleagues identified a subpopulation of melanoma cells with expression of a drug efflux transporter ABCB5 and showed that this subpopulation was enriched for the CD133+ (stem cell marker-enhanced) fraction (Frank et al. 2005). Interestingly, ABCB5 expression was associated with resistance to adriamycin treatment, and correspondingly, ABCB5 blockade resulted in adriamycin accumulation and enhanced drug sensitivity. While it remains unclear how melanoma stem cells might be related to melanocyte stem cells, these studies suggest novel strategies for identification and targeting of clinically critical tumor subpopulations that exhibit stem-like features in melanoma.

Pigmentation

Considerable information on genetic and signaling pathways important for melanocyte biology has come from analysis of hair pigmentation dynamics. These studies are aided by the accumulation of easily recognizable coat color mutations in multiple animal species, as well as the generally benign nature of altered melanocyte function and therefore survival for the host organism. Examination of hair graying represents a good example of such analysis. The geographic separation between follicular melanocyte stem cells (bulge region) relative to their differentiated progeny (hair bulb) permitted relatively

simple quantitative analysis of each population. Using transgenic mice in which the melanocyte lineage was tagged by LacZ (Mackenzie et al. 1997), it was observed that senile graying is associated with progressive depletion of the stem cell pool (Nishimura et al. 2005). Melanocyte stem cell depletion was preceded by the stochastic appearance of pigmented melanocytes within the bulge region—a population that was not maintained, suggesting that it may represent a dead-end route away from the stem cell pool. Similar observations were made in aging human hair follicles (Nishimura et al. 2005). In addition, several murine models of accelerated graying have been analyzed, and demonstrate either an accelerated version of the age-related process (*Mitf^{tr/rit/vit}*) or abrupt and selective stem cell loss (*Bcl2^{-/-}*). Interestingly, graying in the *Bcl2*-deficient background is dramatically rescued by simultaneous deficiency of the proapoptotic family member BIM (Bouillet et al. 2001).

The cellular and molecular events regulating apoptosis of the melanocyte population during the regressive (catagen) phase of the hair follicle cycle are being revealed by systematic analyses (Sharov et al. 2005) that may identify critical steps in homeostatic regulation of this lineage. Collectively these studies are beginning to define not only the genes that play essential roles in melanocyte survival, but also the molecular contexts for the further characterization of signaling and transcriptional pathways that interact with those genes. Moreover, it is plausible that lineage-specific survival pathways and factors may emerge as mechanistically important players in the genesis of melanoma, as exemplified by MITF discussed above.

Due to the combination of obvious phenotype, germline heritability, and modest (if any) effects on organismal survival, analyses of pigmentation mutants have led to the discovery of numerous melanocyte viability genes. One of the best characterized human conditions of syndromic pigmentation is the autosomal dominant condition Waardenburg Syndrome, named for the Dutch ophthalmologist who characterized patients exhibiting “core” pigmentation and deafness defects (Type 2) or others exhibiting the core defects plus other recurring phenotypic abnormalities (Types 1, 3, and 4). Unique features of each subtype, and their associated genes, are outlined in Table 1. The core phenotype of melanocyte deficiency is characterized by a combination of ventral spotting (typically the belly in mice, the forelock in man) and variable degrees of deafness. Hearing deficiency stems from a requirement for melanocytes within the stria vascularis of the cochlea (inner ear)—a requirement whose role appears to involve maintenance of endolymphatic potassium required for the auditory nerve action potential (Steel 1995). Genetic causes of Waardenburg Syndrome are now well characterized (for review, see Price and Fisher 2001). The gene whose mutation is associated with the “core” pigmentation/deafness phenotype is the MITF transcription factor (although MITF mutation occurs in only a minority of Waardenburg Syndrome Type 2 patients, termed Type 2A). The Waardenburg-associated mutations represent a striking epistatic

Table 1. Human syndromic pigmentation/deafness genes

Syndrome	Gene(s)	Affected lineage(s)	Symptoms
Waardenburg Type 1	Pax3	Melanocyte, musculoskeletal	White hairlock, deafness, craniofacial defects
Waardenburg Type 2a	MITF	Melanocyte	White hairlock, deafness
Other 2	Slug	Melanocyte	White hairlock, deafness
Waardenburg Type 3	Pax3	Melanocyte, musculoskeletal	White hairlock, deafness, limb deformities
Waardenburg Type 4	Sox10 ET1, EdnrB	Melanocyte, enteric ganglia	White hairlock, deafness, Hirschsprung's syndrome (megacolon)

series in which essentially every culprit gene is mechanistically linked to regulation of MITF expression or activity. These genes, *Pax3*, *Slug*, *Sox10*, *endothelin 1*, and *endothelin receptor B* are transcriptional regulators of MITF expression (*Pax3* and *Sox10*), transcriptional targets of MITF (*Slug*), or activators of MAPK that directly phosphorylate MITF (ET1 and EdnrB) (Hemesath et al. 1998; Watanabe et al. 1998; Bondurand et al. 2000; Lee et al. 2000; Potterf et al. 2000; Verastegui et al. 2000; Sanchez-Martin et al. 2003).

MITF, the master regulator

As an essential factor for melanocyte development, analysis of the mechanistic role(s) of MITF is likely to shed light on the wiring of critical signaling pathways within the lineage. Mutation of MITF was originally described in the *microphthalmia* mouse (*Mitf^{mi/mi}*), which arose >50 yr ago (Silvers 1979). These mice exhibit complete absence of neural-crest-derived melanocytes, hyperproliferative/dysfunctional retinal pigment epithelium (RPE), deficient mast cells, and osteopetrosis (due to osteoclast defects). Whereas the RPE and mast cell defects are beyond the scope of this review, the melanocyte and osteoclast defects are pertinent to both melanoma and other human malignancies based on clues from related transcription factors. The melanocyte defect arises very early in development, with recognizable progenitor cells (Dct+) disappearing very shortly after their emergence from the neural crest (Hodgkinson et al. 1993). Heterozygous mutant mice or humans with a single mutant *Mitf* allele typically display ventral white spotting due to diminished melanocytes within this watershed zone, which requires the greatest migration from the (dorsal) neural crest. It is uncertain at present why ventral spotting in humans is selective for hair follicles (rather than epidermal melanocytes), although the phenotype suggests a greater migratory or survival "strain" on the lineage. Regardless, complete deficiency of *Mitf* results in complete absence of melanocytes, suggesting that *Mitf* is essential for lineage survival, or proliferation, or to prevent transdifferentiation toward other neural crest lineages (such as glia or neurons). The identification of signaling pathways residing upstream and downstream of *Mitf* has helped to illuminate the context in which it functions.

Pigmentation versus survival Although *Mitf* plays a central role in the pigmentation pathway, it is noteworthy

that the phenotypic consequence of *Mitf* deficiency primarily involves lineage survival, rather than pigmentation/differentiation. While there is at least one *Mitf* mutant allele that displays altered pigmentation (*Mitf^{brownish}*) (Steingrimsdottir et al. 1996), the others (including the founder allele *Mitf^{mi}*) are devoid of melanocytes, rather than failing to make pigment within viable melanocytes. The latter situation occurs in albinos, which are unpigmented due to mutations within genes essential for pigmentation, without affecting melanocyte viability. It is also a potential possibility that *Mitf*'s role in lineage survival could be a fleeting role during a transient developmental stage (with only a pigmentation role in adulthood). However, this is unlikely, given the phenotype of the *Mitf^{vit}* allele. *Mitf^{vit}* encodes a mutation mapping to the helix-loop-helix (HLH) domain that produces a mild hypomorphic biochemical behavior in DNA-binding activity (Hemesath et al. 1994; Steingrimsdottir et al. 1994). *Mitf^{vit/vit}* mice are born with nearly normal coat color (occasional white spot), but undergo accelerated graying (with melanocyte loss) within ~3–12 mo, demonstrating a post-developmental requirement for *Mitf* in continued survival of the melanocyte lineage.

Mitf is certainly not the only gene implicated in melanocyte survival. Some, such as *Sox10* and *Pax3*, are transcription factors epistatically linked to *Mitf* (for review, see Price and Fisher 2001). Another group includes growth factor or receptor genes, such as *endothelin 3*, *endothelin receptorB*, *stem cell factor*, and *c-Kit*. The list of all cytokine/receptor genes essential for melanocyte survival is undoubtedly underestimated by the likelihood that other melanocyte survival genes may also be essential for overall survival of the organism, thus precluding the recognition of their role in pigment cell defects in humans (or other species). Nonetheless, these known mutations clearly provide vital survival signals, possibly mediated by the PI3Kinase and/or MAPK pathways. In fact, one melanocyte-specific target downstream of *c-Kit*/MAPK signaling is MITF, which has been shown to be directly phosphorylated by Erk kinase in a manner that measurably enhanced affinity for the p300 transcriptional coactivator (Hemesath et al. 1998; Price et al. 1998a). In addition, the identical phosphorylation (plus an additional one mediated by Rsk kinase) triggered ubiquitylation and degradation of *Mitf* (Wu et al. 2000; Xu et al. 2000), suggesting a post-translational regulatory event that is under tight homeostatic control. It is incompletely understood whether the net conse-

quence of this MITF phosphorylation is enhanced transcriptional activity, diminished activity (associated with decreased protein stability), or a combination thereof—perhaps segregating among specific transcriptional target genes. Furthermore, as already mentioned, nearly all human melanomas harbor MAPK activation, the majority of which occur through BRAF or NRAS activating mutations. One biochemical consequence among the myriad of MAPK targets is robust and constitutive Erk-mediated phosphorylation of Mitf. Indeed, mutant BRAF has been shown to down-regulate MITF levels (Wellbrock and Marais 2005), and correspondingly, MITF overexpression (in a specific context) could inhibit growth-stimulatory effects of mutant BRAF. MITF has also been shown to undergo a variety of additional post-translational modifications, including phosphorylation by p38 kinase (Mansky et al. 2002), GSK3 β (Takeda et al. 2000a), and sumoylation (Miller et al. 2005; Murakami and Arnheiter 2005), each of which has been linked biochemically to modulate MITF transcriptional activity.

The MITF locus is complex, with multiple distinct promoter/exon units residing within an ~200-kb locus (Hershey and Fisher 2005). Alternative splicing links the various initial exons to the common downstream coding exons. The most 3' of these promoters appears to direct melanocyte-specific expression of an MITF isoform whose expression is specific to the melanocyte lineage (whereas the others are not) (Fuse et al. 1996). This promoter is the best studied, and contains regulatory elements that respond to cAMP and Wnt signaling as well as Sox10, Pax3, and other transcription factors of importance in neural crest lineage development.

MITF as an oncogene Additional evidence in support of MITF's functionally important role in melanocyte growth/survival came from studies identifying genomic amplification of its genetic locus in 10%–20% of human clinical melanoma specimens (see above). Is MITF a plausible human oncogene? After all, its biochemical activities in mediating melanocytic pigmentation/differentiation are well established, and these are hardly activities associated with oncogenesis. Aside from the direct evidence for MITF's amplified state in a fraction of melanomas, other studies have strongly linked MITF to several nonmelanocytic neoplasms that arise through genomic alterations in close relatives of MITF.

Early biochemical characterization of MITF revealed that its basic/HLH/leucine-zipper (bHLHzip) DNA-binding/dimerization domain placed it as a relative of the MYC oncoprotein family (Hemesath et al. 1994). However, MITF was not found to form functional heterodimers with members of the MYC subfamily of bHLHzip factors. Instead, a different set of three closely related transcription factors, TFE3, TFEB, and TFEC, were found to even more closely resemble MITF, with the capacity to homo- or heterodimerize and bind DNA together with MITF. This MITF-containing subfamily of bHLHzip transcription factors was termed the MiT family (Hemesath et al. 1994). In addition, elegant genetic studies by Steingrimsson et al. (2002) revealed an ability

of TFE3 to functionally replace MITF activity in osteoclast development, suggesting biochemical redundancy in the activities of these closely related family members.

TFE3 was the first family member to be identified as a fusion oncogene (Sidhar et al. 1996; Weterman et al. 1996; Clark et al. 1997). A significant fraction of pediatric papillary renal cell carcinomas (now termed “translocation-associated” renal cell carcinomas) was seen to harbor translocations that fused the TFE3 to a variety of alternative 5' partner genes. In each case, the bHLHzip of TFE3 was preserved. Other fusions of TFE3 were seen to occur in alveolar soft parts sarcoma (Ladanyi et al. 2001), where a recurring 5' fusion to the ASPS gene was identified. Although there was ambiguity over which portion (or portions) of the fusion oncoproteins conferred oncogenic activity, the situation was considerably clarified with the identification of TFEB translocated papillary renal carcinomas (Davis et al. 2003; Kuiper et al. 2003). Interestingly, these tumors were identified, in part, by their unexpected expression of several melanocytic markers in combination with an isolated, recurring translocation localizing to 6p21 (Argani et al. 2001). The fusion gene was informative because the 5' partner gene (from chromosome 11) fused upstream of TFEB's translation initiation codon and did not apparently contribute open reading frame sequences, but rather promoter-swap mechanism to the fusion gene. This mode of translocation oncogenesis was reminiscent of Myc-immunoglobulin translocations in lymphoid malignancies, and suggested that transcriptional dysregulation of TFE3 or TFEB expression is likely to represent an oncogenic event. It is thus highly plausible that gene amplification, another mode of Myc family dysregulation in cancer, would represent an oncogenic event through which MITF contributes to melanomagenesis. Another recently identified MiT tumor is Clear Cell Sarcoma (also called Melanoma of Soft Parts). This sarcoma is occasionally pigmented and expresses a variety of melanocytic markers (Granter et al. 2001) as well as a recurring translocation that fuses the EWS and ATF1 genes (Fujimura et al. 1996). Recent data have suggested that EWS-ATF1 functions by oncogenically activating MITF expression (via EWS-ATF1's role in mimicking melanocyte-stimulating hormone [MSH] signaling), since MITF is essential for viability of Clear Cell Sarcoma (Davis et al. 2006).

Transcriptional targets of Mitf Numerous technical approaches have been used to identify genes whose expression is modulated by Mitf in melanocytes. Using candidate approaches, multiple pigmentation-associated factors have been thus recognized, although it is noteworthy that factors other than MITF are undoubtedly important in their regulation as well, because the endogenous genes are not always predictably up- or down-regulated by modulation of Mitf expression alone (Gaggioli et al. 2003). Other transcription factors of importance in the regulation of potential Mitf targets include CREB/ATF1, SOX10 (Jiao et al. 2004; Ludwig et al. 2004), Pax3 (as a negative regulator and potential media-

tor of melanocyte stem cell maintenance) (Lang et al. 2005), OTX2 in retinal pigment epithelium (Martinez-Morales et al. 2003), CBP and MAZR (in mast cells) (Ogihara et al. 1999; Morii et al. 2002), Pu.1 in osteoclast target genes (Sato et al. 1999; Luchin et al. 2001; Cassady et al. 2003; So et al. 2003; Partington et al. 2004), Wnt/LEF (Yasumoto et al. 2002), and probably others as well.

Although the list of identified transcriptional targets of *Mitf* is growing rapidly, one plausible classification of these genes is through the broad groups of differentiation/pigmentation-associated factors versus proliferation/survival-associated genes. These designations undoubtedly represent some degree of oversimplification, yet may be helpful in linking *Mitf* biochemistry to major pathways with which it is associated. Two interesting reported transcriptional targets are the cyCDK inhibitors (CDKi) *INK4A* (Loercher et al. 2005) and *p21* (Carreira et al. 2005). Both potentially provide links to melanocytic differentiation although neither alone is apparently essential for melanocyte differentiation/pigmentation, since knockouts of both appear normally pigmented. Still, *p16^{In4a}* is an unequivocally important melanoma genetic locus in man (see above), and these studies suggest a means through which loss of CDKi activity could permit (or reveal) an oncogenic activity of *Mitf* that is otherwise constrained by the CDKi (Merlino 2005).

Pigmentation and differentiation targets. Pigmentation-associated transcriptional targets of MITF include the pigment enzyme genes tyrosinase, TRP1, TRP2/Dct, which were recognized to contain an evolutionarily conserved consensus promoter/enhancer element that matches the MITF recognition sequence (Bentley et al. 1994; Hemesath et al. 1994; Yasumoto et al. 1994). Additional differentiation-associated genes include *Pmel17/silver/gp100* (which encodes the melanoma diagnostic epitope HMB45) (Halaban et al. 1996; Baxter and Pavan 2003; Du et al. 2003), *MelanA/Mart1* (Du et al. 2003), *Melastatin (TRPM1)* (Miller et al. 2004; Zhiqi et al. 2004), *AIM1* (ocular albinism 4 gene) (Du and Fisher 2002), *Ocular albinism 1 gene (OA1)* (Vetrini et al. 2004), *VMD2* (Esumi et al. 2004), *HIF1 α* (Busca et al. 2005), *Plasminogen activator inhibitor-1* (Murakami et al. 2006), numerous mast cell targets of MITF including *Prostaglandin D2*, multiple mast cell genes including proteases, various adhesion molecules and others (see Ito et al. 2004; Morii et al. 2004; Takeda et al. 2006 and references therein), and *melanocortin 1 receptor (MC1R)* in mast cells and possibly in melanocytes (Adachi et al. 2000; Smith et al. 2001; Aoki and Moro 2002). Numerous additional genes are being identified through expression microarrays, and are liable to reflect the multiple steps involved between signaling, expressing, packaging, and exporting pigment.

Proliferation and survival targets. Significantly fewer genes have been identified to date as potential mediators of the MITF-dependent survival or proliferation. *Bcl2* is one such gene, which was also notable for the melanocytic viability phenotype associated with its knockout in the mouse (McGill et al. 2002; Nishimura et al. 2005). *CDK2* is another example of a MITF target

selectively in the melanocyte lineage (Du et al. 2004). This finding stemmed from analysis of the *pmel17/gp100* locus, which virtually overlaps the *CDK2* gene and appeared to use a common enhancer element to regulate expression of both genes (whereas the MITF-binding site was dispensable for *CDK2* expression in nonmelanocytes). For uncertain reasons, melanoma cells were also seen to selectively require *CDK2* expression, because knockdown of *CDK2* affected their cell cycle and viability, but not the viability of a variety of nonmelanocytic cancer cell types (Du et al. 2004) as had previously been observed by others (Tetsu and McCormick 2003). Importantly, neither *Bcl2* nor *CDK2* appears to explain MITF's requirement for melanocyte viability during development, because the phenotype of MITF homozygous deficiency is profoundly worse than either alone, and expression of neither gene in MITF-knockdown melanoma cells is capable of rescuing viability. Evidence has also been obtained that suggests that *Slug* may be a direct transcriptional target of *Mitf* (Sanchez-Martin et al. 2002). This finding is of note because of the same authors' discovery of several Waardenburg Syndrome Type 2 patients with (germline or somatic) deletion of *Slug*, thereby implying an epistatic relationship between *Mitf* and *Slug*. The potential importance of *Slug* in lineage survival is highlighted by its recent discovery as a transcriptional target of *p53* and a transcriptional repressor of the apoptotic factor *PUMA* (Wu et al. 2005). Furthermore, relevance of *Slug* to melanocyte biology is reinforced by the demonstration that *Slug* contributes to invasive behavior of human melanocytes transformed with defined genetic events (Gupta et al. 2005). The cytokine receptor *c-Met* has also recently emerged as a direct transcriptional target of *Mitf* (McGill et al. 2006). Additional targets of MITF are likely to mediate its survival/proliferation effects, and it remains to be seen whether some of these genes might serve as surrogate drug targets for the MITF oncogenic pathway.

Wnt signaling

Wnt signaling has long been linked to major developmental decisions in neural crest derivatives. Among the various lineages derived from neural crest progenitors, Wnt pathway overactivation has been shown to bias differentiation toward the melanocytic lineage (Dorsky et al. 1998), consistent with the possibility that it regulates the lineage commitment decision. Indeed, zebrafish studies by Raible, Moon, and colleagues demonstrated that canonical Wnt pathway activation resulted in up-regulated *Mitf* expression via discrete TCF/LEF sequence elements within proximal melanocyte-specific promoter of the *Mitf* gene (Dorsky et al. 2000). Importantly, the investigators found that enforced *Mitf* expression was able to measurably rescue melanoblast survival in Wnt-deficient embryos, providing functional evidence that *Mitf* is a developmentally essential Wnt target. Additional studies identified similar biochemical relationships between Wnt effectors (β -catenin, TCF/LEF) and *Mitf* expression in murine and human melanocytes and

melanoma cells (Takeda et al. 2000b; Widlund et al. 2002). Interactions have also been observed between LEF-1 and MITF, suggesting that MITF may function as an alternative coactivator of certain Wnt-signaling targets (independent of β -catenin) (Yasumoto et al. 2002).

Although a relatively high incidence of stabilizing β -catenin mutations has been observed in melanoma cell lines (Rubinfeld et al. 1997), a much lower incidence of such mutations has been detected in primary clinical melanoma specimens. However, a significant fraction of clinical melanoma lesions do exhibit immunohistochemical evidence of nuclear β -catenin localization (Rimm et al. 1999), signifying activation of the canonical Wnt pathway, thus an activity (albeit biochemically incompletely defined as yet) through which this upstream regulator of Mitf may be contributing to melanoma survival/proliferation. While it is likely that β -catenin acts on other genes, Mitf is an important downstream target, as cell line studies showed that lethality caused by Wnt pathway suppression (by dominant-negative TCF) could be rescued by enforced expression of Mitf (Widlund et al. 2002). Collectively, these observations are consistent with the view that Mitf plays an essential role in at least certain melanoma cell lines.

Distinct from the effects of the canonical Wnt pathway's regulation of Mitf, gene expression studies of human melanoma have identified the Wnt5a signaling pathway as a participant in melanoma progression (Weeraratna et al. 2002). Wnt5a expression correlated with invasive behavior in vitro, as well as with increased tumor grade in clinical specimens. Overexpression of Wnt5a appears to enhance the invasion capability of melanoma cells, while blockade of Frizzled 5 (the Wnt5a receptor) can suppress invasiveness. The Wnt5a pathway in melanoma cells was found to be associated with PKC activation, rather than β -catenin targeting (Weeraratna et al. 2002).

MSH signaling

The signaling pathway triggered by MSH is thought to represent one of the most important regulators of pigmentation (Fig. 3), although other pathways clearly exist (Barsh 1996; Fitch et al. 2003; Van Raamsdonk et al. 2004; Lamason et al. 2005), and will likely provide valuable insights to the pigmentation process. MSH binds to its receptor, Mc1R, in a manner that is directly competed by the agouti protein in nonhuman mammalian species (the role of agouti in man is less clear). Successful competition by agouti results in fur/hair bands with characteristic yellow stripe patterns, whereas the nonagouti phenotype (due to deficient agouti protein) produces uniform black pigmentation associated with unimpeded access to Mc1R by MSH and is responsible for the "black" color of the commonly studied C57bl/6 mouse strain.

MC1R is a seven-transmembrane G-protein-coupled receptor (GPCR) expressed on epidermal melanocytes. It is highly polymorphic in human populations, and its allelic variation is the principle determinant of pigment phenotypes and skin phototypes in humans (Valverde et

Figure 3. Melanocortin signaling α -MSH stimulates the cAMP pathway via MC1R, a seven-transmembrane GPCR on melanocytes. Heterotrimeric G-protein complexes downstream of MC1R stimulate adenylyl cyclase (AC), which catalyzes the production of cyclic AMP (cAMP). Elevated cAMP triggers (via intermediates) the phosphorylation/activation of the CREB (cAMP-responsive element binding) family of transcription factors, triggering recruitment of the CBP/p300 coactivators and transcriptional activation of multiple target genes. In melanocytes, one critical CREB target is MITF, a bHLHZip protein that transactivates genes important for pigmentation/differentiation as well as other cellular processes (see text for details). Among these are genes that produce synthesis and maturation of melanosomes, specialized melanin-containing vesicles that are exported to neighboring keratinocytes in the epidermis.

al. 1995; Sturm 2002). Three common variants of MC1R (R151C, R160W, and D294H) have been associated with the so-called Red Hair Color (RHC) phenotype (Box et al. 1997, 2001; Smith et al. 1998; Flanagan et al. 2000; Palmer et al. 2000; Bastiaens et al. 2001a,b), characteristic of individuals with red hair, fair complexion, an inability to tan, and a tendency to freckle. Carrying a single RHC MC1R variant is associated with a significantly diminished ability of the epidermis to respond to damage by UV (Flanagan et al. 2000; Box et al. 2001; Healy et al. 2001), presumably contributing to increased melanoma risk (Palmer et al. 2000; Healy et al. 2001). However, the observation that RHC MC1R variants are associated with increased melanoma risk even in individuals with dark/olive complexions (Palmer et al. 2000; Healy et al. 2001; Kennedy et al. 2001) suggests that MC1R have additional pigment-independent effect(s) on melanoma risk.

In general, those MC1R variants that robustly transduce MSH signals to activate adenylate cyclase, result in dark pigmentation phenotypes. In contrast, those Mc1R variants that produce weak or absent cAMP responses to MSH stimulation are associated with red/fair skin pigmentation and phototype (Kadekaro et al. 2003). Downstream from cAMP activation, multiple events occur, including phosphorylation/activation of CREB/ATF1 transcription factors that, in turn, activate expression of the Mitf promoter in melanocytes (Bertolotto et al. 1998;

Price et al. 1998b). The precise kinase(s) responsible for CREB/ATF1 phosphorylation are likely to include Rsk kinase (Bohm et al. 1995) and/or Protein Kinase A. Other consequences of cAMP stimulation have also been suggested, including activation of RAF/MAPK (Busca and Ballotti 2000) and inhibition of the PI3K pathways (Khaled et al. 2003), in addition to activation of the canonical PKA response. While CREB and ATF1 likely contribute directly to transcriptional regulation of certain components of the pigmentation machinery, it is probable that their activation of *Mitf* plays an important role as well, with *Mitf* likely imposing tissue-specific expression of these genes. The pheomelanin (red pigment) to eumelanin (brown/black pigment) switch occurring at the level of MSH/Mc1R signaling likely represents alternatively low- versus high-level *Mitf* expression, with correspondingly low versus high tyrosinase (and tyrosinase-related-protein) enzymatic metabolism of tyrosine to either thiol-reduced red-blond pigments or non-thiol-reduced brown-black pigments.

One potential challenge to the simple MSH signaling schema is the fact that cAMP and CREB/ATF1 activation is highly ubiquitous, yet *Mitf* activation (and pigmentation) is highly tissue restricted. One clue to how this restriction occurs came from the observation that the melanocyte-specific *Mitf* promoter is nonresponsive to cAMP in nonmelanocytes (Bertolotto et al. 1998; Price et al. 1998b), and also lost responsiveness to cAMP within the melanocyte lineage in the absence of an upstream promoter region (Huber et al. 2003). The cooperating site was mapped to a Sox10 consensus element, and enforced Sox10 expression was seen to permit cAMP responsiveness of endogenous *Mitf* gene expression within nonmelanocytes (Huber et al. 2003). Collectively these studies linked regulation of pigmentation, cAMP signaling, and *Mitf* expression to the Sox10 (Waardenburg Syndrome Type 4) gene.

Emerging paradigms for melanoma therapeutics

The treatment of advanced melanoma has witnessed numerous clinical innovations and recurrent frustrations over the past several decades. While primary cutaneous melanoma often can be effectively treated with surgical resection alone, the 5-yr survival of patients with regional lymph node spread hovers just above 50%, and disseminated melanoma represents one of the most treatment-resistant of all human malignancies, with a median survival of 6–9 mo (Balch et al. 2001). At the same time, this disease has consistently provided tantalizing glimpses into possible therapeutic avenues that, if actualized, might prove broadly transforming to cancer medicine. Rare but profound clinical responses to immune modulation (for review, see Wolchok and Livingston 2001) and frequent genetic lesions affecting the hotly targeted MAP kinase pathway (Davies et al. 2002) represent two prominent examples in this regard. Although both immunotherapy and targeted therapy have encountered many more failures than successes thus far, recent advances in both arenas suggest that melanoma

may offer a proving ground for emerging therapies capable of altering the natural history of molecularly defined tumor subtypes.

Dacarbazine (DTIC) and hydroxylurea remain the only FDA-approved chemotherapeutic agents in common use for metastatic melanoma. Between 10% and 20% of patients will experience a clinical response to DTIC; however, the response duration is short (4–6 mo), and no randomized, controlled trial has ever compared patient survival following DTIC treatment to that achieved with either best supportive care or placebo (Eigentler et al. 2003). The additional combination of chemotherapy agents with antimelanoma activity (e.g., platinum compounds, taxanes, or other alkylating agents) may improve the patient response rate, but these strategies also increase the toxicity of therapy and have not enhanced overall survival. Temozolomide, which shares the same active metabolite as DTIC, can cross the blood–brain barrier; thus, this agent has garnered attention as a possible systemic agent for the treatment of metastatic melanoma involving the brain and central nervous system. However, this agent has not demonstrated enhanced efficacy relative to DTIC alone in randomized trials (Middleton et al. 2000). For this reason, temozolomide failed to receive FDA approval for use in metastatic melanoma. Together, these grim realities underscore an urgent need for improved systemic treatment of this highly resistant malignancy.

Targeted therapy in melanoma

The MAP kinase pathway The prevalence of activating BRAF and NRAS mutations and consistent downstream activation of ERK suggests that activation of the MAP kinase signaling cascade may constitute an obligatory rite of passage in the transformation of melanocytes. Such dependency may present a therapeutically vulnerable point of attack (Fig. 4). Several lines of experimental evidence affirm this notion, particularly in melanomas harboring the BRAF^{V600E} mutation. First, the MAP kinase pathway exhibits constitutive activation in BRAF^{V600E} melanoma cells (as measured by ERK activation), which is required for melanoma proliferation in this context (Hingorani et al. 2003; Karasarides et al. 2004; Wellbrock et al. 2004). Second, depletion of BRAF, but not A-RAF or C-RAF, by small interfering RNA (siRNA)-mediated knockdown inhibits both ERK activation and cell growth while also triggering apoptosis in BRAF^{V600E} melanoma cells (Hingorani et al. 2003; Wellbrock et al. 2004). Consistent with these effects, BRAF depletion also abrogates both colony formation in soft agar and tumor xenograft formation by BRAF^{V600E} melanoma cells. Moreover, inducible RNAi experiments have shown that knockdown of BRAF attenuates growth of BRAF^{V600E} melanoma xenografts in an ERK-dependent manner (Hoflich et al. 2006). Silencing of activated NRAS by siRNA knockdown also decreases phospho-ERK levels and triggers apoptosis in NRAS-mutant melanoma cells (Eskandarpour et al. 2005). Together, these

Figure 4. Melanoma pathways and targeted therapeutics. Several investigational agents designed to inhibit cell-autonomous melanoma pathways or augment ant melanoma immune mechanisms have entered clinical development. Proteins that have been targeted in melanoma clinical trials are indicated (red bars and bold arrow). The MAP kinase pathway (*bottom left*) has been targeted by RAF and MEK inhibitors, the PI3 kinase/AKT pathway (*bottom right*) by TOR inhibitors, and the cell cycle by CDK inhibitors (*bottom middle*). Drugs inhibiting the Hsp90 chaperone protein may lead to degradation of several activated oncogenes (see text for details). The Bcl-2 antiapoptotic oncoprotein has been targeted by an antisense agent (see text). Aside from IFN- α and IL-2, recent attempts to enhance melanoma immunotherapy include toll-like receptor agonists (*top left*) and CTLA-4 antibody blockade (*top middle*). These are predicted to generate a more effective T-cell-mediated immunotoxicity to melanoma cells. Strategies to interdict RTK such as c-kit may prove fruitful in the future against defined melanoma subtypes.

data suggest that melanomas carrying activating BRAF or NRAS mutations may exhibit an “oncogene addiction” phenotype involving the MAP kinase pathway.

Despite these intriguing observations, the MAP kinase pathway has not yet been fully credentialed as a melanoma target for several reasons. First, the MAP kinase dependency operant in cultured BRAF^{V600E} melanoma cell lines and xenografts may become attenuated *in vivo* because of the well-characterized redundancies in ERK activation by numerous autocrine and paracrine factors (Lazar-Molnar et al. 2000). Second, although BRAF mutations occur early in melanoma genesis, they are clearly insufficient for melanocyte transformation; thus continued BRAF^{V600E} activity in the context of all other cooperating events may not be essential for melanoma maintenance *in situ*. Moreover, although ectopic BRAF^{V600E} expression transforms certain murine melanocytes, it is incapable of transforming immortalized human melanocytes by itself (Chudnovsky et al. 2005); rather, it requires the cooperative actions of additional genes, such as *MITF* (Garraway et al. 2005). BRAF^{V600E} therefore seems a much weaker melanoma oncogene than are the RAS family members. Furthermore, siRNA-mediated interdiction of the MAP kinase pathway in NRAS-mutant melanoma cells yields only limited effects on ERK activation and cell proliferation (Wellbrock et al. 2004). These data raise the possibility that BRAF^{V600E} mutations and, by extension, aberrant MAP kinase activation may influence tumor establishment but prove insufficient for maintenance in human melanomas.

Given the prominence of MAP kinase signaling in many tumor types, several small molecules that target this pathway have entered clinical development. Sorafenib (BAY 43-9006) was one of the first such compounds to be evaluated in clinical trials; this bi-aryl urea exhibits *in vitro* inhibition of both wild-type and mutant BRAF kinase activities in the 20–40 nM range (Ahmad

and Eisen 2004). Sorafenib also inhibits C-RAF and VEGFR-3 at low to subnanomolar concentrations, while exhibiting higher nanomolar activity *in vitro* against PDGFR, VEGFR-2, c-KIT, and FLT3. Despite its robust anti-RAF activity *in vitro*, the potency of sorafenib against MAP kinase activity in whole cells has proved more variable. IC₅₀ values for this compound against BRAF^{V600E} melanoma cells are 5–6 μ M (50- to 100-fold higher than that required for *in vitro* kinase inhibition) (Karasarides et al. 2004); concentrations as high as 10–20 μ M are required for full phospho-ERK inhibition (Panka et al. 2006). In xenografts, daily administration of 50 mg/kg sorafenib partially inhibited phospho-MEK activity, and attenuated (but did not abrogate) tumor growth. Despite its promising preclinical properties, the preliminary efficacy data for sorafenib in melanoma patients appears more modest. In a recent phase I/II clinical trial involving 22 melanoma patients, only one partial responder was observed, while 12 patients had stable disease with a median duration of only 4 mo (the BRAF and NRAS mutation status of these patients is not yet available) (Flaherty et al. 2005). Sorafenib received FDA approval for the treatment of metastatic renal cancer, a malignancy whose pathophysiology involves angiogenesis, but where BRAF mutations have not heretofore been observed. Thus, the clinical efficacy of sorafenib may derive more from its anti-VEGFR activity than from BRAF blockade.

Nonetheless, cautious optimism persists for the development of targeted therapeutics against the MAP kinase pathway in melanoma. Several second-generation small molecule inhibitors of RAF and MEK exhibit *in vitro* potencies exceeding that of sorafenib. For example, the recently described RAF inhibitor RAF-265, previously known as CHIR-265 (Novartis, Inc.), shows pharmacologic IC₅₀ values of <100 nM against BRAF-mutant melanoma cell lines (e.g., 50- to 100-fold more potent

than sorafenib in the same genetic context) (Jallat 2006). RAF-265 dosed daily at 100 mg/kg achieved tumor regression in BRAF^{V600E} melanoma xenografts, which correlated with phospho-MEK and cyclin D1 inhibition, as well as p27 up-regulation. In addition, a series of small molecules has been purported to selectively target the V600E variant of BRAF, resulting in submicromolar IC₅₀ values against BRAF^{V600E} melanoma cells (<http://www.plexxikon.com>). Presumably, these and other emerging RAF inhibitors with enhanced potency and selectivity may afford more robust MAP kinase pathway interdiction in clinical trials.

Since MEK1 and MEK2 serine-threonine kinases constitute the critical downstream effectors of BRAF in the MAP kinase cascade (Fig. 4), MEK inhibition also offers an attractive means of targeting tumors with this dependency. Toward this end, several small molecule MEK inhibitors are also in development. Although clinical trials of the first-generation MEK inhibitor CI-1040 (Pfizer, Inc.) were confounded by its suboptimal pharmacokinetic and pharmacodynamic parameters (Rinehart et al. 2004), the second-generation oral inhibitor PD0325901 blocks MEK1/2 at much higher potency (Sebolt-Leopold and Herrera 2004). Recently, an unbiased pharmacologic analysis of NCI-60 cell lines stratified based on the presence or absence of BRAF^{V600E} mutation revealed that compounds targeting MEK kinase were among the most potent of >40,000 compounds examined against BRAF^{V600E} cells (Solit et al. 2006). Interestingly, this selective MEK sensitivity was not observed when the same cell lines were classified based on RAS mutation status. Testing of CI-1040 and PD0325901 against a largely independent panel of BRAF^{V600E} and NRAS^{Q61R} melanoma cell lines and xenografts yielded similar results. These data suggest that clinical trials of melanoma patients stratified according to BRAF^{V600E} mutation may enrich for patients most likely to benefit from second-generation RAF and MEK inhibitors. In a recent phase I/II trial of PD0325901 that enrolled 41 patients (27 melanoma patients), melanoma was the only tumor type in which partial disease responses were observed, and such responses were associated with strong phospho-ERK inhibition (Lorusso et al. 2005). Although genotype (BRAF and NRAS status) correlation for the responders is not yet available from this trial, these results raise the possibility that RAF and MEK inhibitors dosed to complete target inhibition may prove effective against a defined subset of human melanomas.

The PI3 kinase/AKT pathway A large body of evidence suggests that the TOR pathway acts as a major effector of the AKT oncogenic signal downstream of PI3 kinase activation (described above) (Fig. 4). Pathway components associated with increased cell growth, such as phosphorylation of p70S6 kinase, are sensitive to the TOR inhibitor rapamycin (for a recent review, see Wullschlegel et al. 2006). The premise that TOR may mediate downstream tumorigenic functions of activated AKT has generated intense enthusiasm around the idea that TOR inhibitors might prove efficacious in melanoma and

other malignancies characterized by frequent PTEN deficiencies. However, the predicted efficacy of TOR inhibitors in melanoma was questioned a priori by a tissue microarray study that examined TOR pathway profiles across a spectrum of human tumors (Xu et al. 2004). Here, none of the 10 melanomas examined expressed the triad of low PTEN, high phospho-S6 kinase, and high phospho-AKT predicted based on in vitro cell line studies to confer rapamycin sensitivity. Consistent with this observation, a phase II trial of the TOR inhibitor temsirolimus (CCI-779) by Wyeth that enrolled 33 melanoma patients resulted in only one partial response (Margolin et al. 2004). These data suggest that single-agent TOR inhibition has limited efficacy in melanoma, and that future trials must either combine other agents with TOR inhibitors or develop robust molecular diagnostic methods to predict TOR dependency in appropriate cancer patients.

Cyclin-dependent kinases The observation that CDKN2A is frequently deleted in melanoma (Chin 2003), combined with the role of cyclin-dependent kinases such as CDK2 in melanoma proliferation (Du et al. 2004), implicates the p16/CDK/pRb pathway as an additional melanoma dependency with possible therapeutic implications. Indeed, unopposed activation of CDK2 as well as CDK4/6 mediates hyperphosphorylation of all Rb pocket proteins in melanoma (Halaban 1999). CDK2 inhibition, in particular, may offer a theoretical therapeutic index in melanoma, because melanoma cell lines appeared to exhibit particular dependence on this kinase, whereas normal tissues (and nonmelanoma cancers) appeared to tolerate CDK2 inhibition (Tetsu and McCormick 2003; Du et al. 2004). These findings have prompted the clinical development of several agents capable of inhibiting CDKs. Flavopiridol, which inhibits CDK1, CDK2, CDK4, and CDK7, represents the best characterized CDK inhibitor. This compound promotes cell cycle arrest and apoptosis in many cancer cell lines; however, flavopiridol has thus far been ineffective as an anticancer agent in several clinical trials (Shapiro 2004). Several additional CDK inhibitors currently at various stages of development (e.g., UCN-01, CYC202, and BMS-387032) may provide greater selectivity among these kinases; their efficacies against melanoma and other solid tumors remain to be determined.

Global regulators of protein homeostasis The ubiquitous chaperone protein Hsp90 mediates proper folding and activation of many cell signaling proteins and transcriptional activators (Fig. 4). Known Hsp90 client proteins include several well-known oncogenes, such as the EGF, HER-2, and MET RTKs, AKT family members; and the CDK4/6 cell cycle regulators (for review, see Workman 2004). Of particular relevance to melanoma, Hsp90 appears to bind and stabilize the activated protein conformations of BRAF, as well as other oncogenes that result from gain-of-function mutations (da Rocha Dias et al. 2005; Grbovic et al. 2006). These observations may in part explain the often substantial increases in Hsp90 activity

observed in tumor cells as compared with normal cells.

In principle, pharmacologic blockade of Hsp90 might simultaneously interdict multiple cellular mechanisms operant in advanced melanoma as well as other solid tumors. If so, Hsp90 small molecule inhibitors may approximate combinatorial actions against multiple hallmark tumor mechanisms. Toward this end, the ansamycin antibiotic geldanamycin and its less toxic derivative 17-allylaminogeldanamycin (17-AAG) have been shown to effect potent inhibition of Hsp90 *in vitro*, resulting in degradation of oncoproteins such as RAF, CDK4, and HER-2, and efficient tumor growth inhibition in several tumor model systems (for review, see Kamal et al. 2004). Accordingly, several phase I clinical trials using varying 17-AAG dosing schedules have been conducted (Banerji et al. 2005; Goetz et al. 2005; Grem et al. 2005; Ramanathan et al. 2005). At high doses (ca. 450 mg/m² per week), evidence of clinical efficacy has occasionally been achieved; two melanoma patients have been reported whose disease stabilized for as long as 2 yr in response to 17-AAG treatment (for review, see Workman 2004). However, development of this agent has been stunted by limited drug solubility and potency in the setting of frequent and diverse toxicities. Therefore, recent efforts in this arena have shifted toward clinical trials using lower 17-AAG doses in combination with conventional chemotherapeutic agents, or to the identification of novel Hsp90 inhibitors with improved potency and therapeutic indices (Llauger et al. 2005). For example, IPI-504 is a novel, water-soluble Hsp90 inhibitor in clinical development that is converted to 17-AAG *in vivo*; this may enable targeted delivery of higher doses into tumor tissue (<http://www.ipi.com>).

Targeting apoptosis in melanoma: inhibiting Bcl2 In recent years it has become clear that resistance to apoptosis promotes both tumor survival and refractoriness to cytotoxic chemotherapy (Johnstone et al. 2002). Normal melanocytes appear to elaborate several intrinsic and extrinsic anti-apoptotic mechanisms that may be exploited in melanoma; these include the expression of melanin and other factors that protect against photo-toxic DNA damage, as well as prosurvival signals from the autocrine and paracrine factors mentioned above (for review, see Soengas and Lowe 2003). Aggressive melanoma tumors and cell lines often manifest low apoptotic indices (Glinsky et al. 1997). Moreover, signaling pathways such as the MAP kinase and PI3 kinase/AKT cascades that undergo aberrant activation in melanoma produce well-characterized tumor survival effects (for recent reviews, see Wada and Penninger 2004; Kharas and Fruman 2005). In melanoma, MAP kinase signaling prevents apoptosis through several mechanisms, including the inhibition of mitochondrial Smac/DIABLO release in response to TRAIL (Zhang et al. 2003), up-regulation of Bcl2 expression via MITF (McGill et al. 2002), and inactivation of the Bad proapoptotic protein (Eisenmann et al. 2003). These observations have fueled a heightened interest in anti-apoptotic melanoma therapeutic strategies.

Melanoma oncogenic growth factors such as NGF and SCF induce the expression of the Bcl-2 oncoprotein (Zhai et al. 1996), as does MITF (McGill et al. 2002), which is also deregulated in some melanomas, as described above. Similarly, the "classic" melanoma growth factor bFGF subverts inhibitory signals from TGF- β that reduce Bcl-2 levels (von Willebrand et al. 2005). Expression of activated NRAS in melanoma cell lines can also up-regulate Bcl-2 (Borner et al. 1999). Both normal melanocytes and melanoma cells express Bcl-2 (Plettenberg et al. 1995), and several studies have correlated elevated Bcl-2 with adverse clinical and pathological factors such as tumor ulceration and patient survival, particularly in thicker primary melanomas (Leiter et al. 2000; Ilmonen et al. 2005). Moreover, germline knockout of Bcl-2 results in death of melanocyte stem cells within the hair follicle at a discrete developmental state (Nishimura et al. 2005), demonstrating a critical role for Bcl-2 in the melanocyte lineage. Additional BCL anti-apoptotic proteins such as Mcl-1 and Bcl-XL are also expressed at various stages of melanoma and may influence both tumor progression and chemoresistance (Tron et al. 1995; Selzer et al. 1998; Skvara et al. 2005). Thus, targeting BCL family members either alone or in combination with conventional cytotoxic agents has been predicted to offer therapeutic benefit in this malignancy.

Extensive clinical experience now exists with Genasense (G3139; Genta, Inc.), an antisense therapeutic directed against Bcl-2 (Badros et al. 2005; Marcucci et al. 2005; O'Brien et al. 2005; Tolcher et al. 2005). Encouraging phase II trial results, including evidence of target modulation, have been reported in hormone-refractory prostate cancer and relapsed multiple myeloma (Badros et al. 2005; Tolcher et al. 2005). Genasense has also been tested in a large phase III study that randomized 771 metastatic melanoma patients to receive DTIC plus either Genasense or placebo. Here, the addition of Genasense to DTIC failed to increase patient survival relative to DTIC alone (median survival 9.0 mo vs. 7.8 mo; $p = 0.18$). Thus, like Temozolomide (described above), Genasense failed to receive FDA approval for use in metastatic melanoma (a summary report from the Genasense FDA review team is available at http://www.fda.gov/ohrms/dockets/ac/04/slides/4037S1_02_FDA-Kane-Yang%20.ppt).

A retrospective view of the preclinical and clinical data surrounding Bcl-2 inhibition presents several important considerations that should influence future targeted therapeutic attempts in melanoma and other cancers. One issue pertains to rigorous target validation: In the case of Bcl-2, the scientific literature projects marked discordance concerning the extent to which Bcl-2 levels correlate with melanoma progression (Bush and Li 2003). In fact, some reports have suggested that melanoma progression is associated with loss of Bcl-2 expression (van den Oord et al. 1994; Vlaykova et al. 2002). A large study of anti-apoptotic gene expression levels from melanoma sentinel lymph nodes suggested that only survivin expression correlated significantly with clinical outcome, with Bcl-2 showing no association (Gradilone et al.

2003). In one sense, these data might imply that Bcl-2 constitutes a suboptimal melanoma target. However, given the known oncogenic function of Bcl-2 in many human cancers, an alternative explanation is that its anti-apoptotic mechanism may operate primarily within a restricted melanoma subset; for example, those tumors characterized by strong Bcl-2 immunostaining, or other predictive molecular alterations. Thus, lessons from the Bcl-2 experience support future patient selection based on key cellular dependency metrics, where possible.

The above notion underscores an emerging premise relevant to targeted therapeutics across all human tumors: In general, biomarker panels capable of predicting drug sensitivity need to be developed in parallel to the relevant investigational agents. In some cases, a critical somatic genetic alteration by itself may serve as the informative biomarker; the available data for RAF and MEK inhibitor studies in BRAF^{V600E} melanomas endorse this idea. More commonly, biomarkers will likely require empiric determination and iterative validation based on unbiased omics-derived correlates from large clinical trials. Such predictive biomarker signatures will pave the way for rational clinical trial designs that stratify patients a priori based on the likelihood that their tumors will respond to the agent in question. Finally, the Bcl-2 experience offers a reminder that clinical trials of targeted agents increasingly require concomitant pharmacodynamic studies that link tumor response to effective target interdiction. Such analyses have proved informative in MEK inhibitor trial (described above), where the suboptimal performance of CI-1040 was attributable to incomplete target inhibition. Toward this end, melanoma provides a favorable setting for in situ pharmacodynamic testing; often, patients with advanced disease contain numerous dermal metastases that can be biopsied with minimal risk.

Melanoma immunotherapy

The longstanding recognition that primary melanoma lesions may exhibit regions of spontaneous regression, combined with histologic evidence that T-cell infiltration of melanoma tumors correlates with better prognosis (Clemente et al. 1996), places this malignancy at the forefront of immunotherapeutic approaches in human solid tumors. The idea of harnessing the immune system against solid tumors offers conceptual appeal because of the unparalleled target specificity achievable in principle through immune recognition of tumor antigens. Unfortunately, several decades of translational science in cancer immunotherapy have not yet succeeded in altering the natural history of advanced melanoma. Instead, these efforts have unwittingly demonstrated the diverse and sophisticated avenues by which melanoma cells can resist immune destruction. Protective tumor mechanisms include immunoediting, lack of expression of “danger signals” recognized by the immune system, induction of tolerance and/or a refractory inflammatory environment, and elaboration of immunosuppressive factors. Despite these challenges, immunomodulatory

agents have consistently triggered rare but profound melanoma tumor responses, suggesting that some patients with metastatic disease may be cured by these regimens. This observation stands in marked contradistinction to the experience with conventional chemotherapy described above. Moreover, recent advances in biological understanding of immune regulatory mechanisms justify a guarded optimism that future immunotherapeutic strategies might enjoy an even larger measure of success in defined melanoma subtypes.

Interferon- α (IFN- α) and interleukin-2 (IL-2) anchor the existing immunotherapeutic repertoire for melanoma treatment. IFN- α is given as adjuvant therapy for patients whose primary lesion poses a high risk for relapse (either because of adverse histologic features or spread to regional lymph nodes); in this setting, it improves both relapse-free and overall survival (Kirkwood et al. 1996, 2001). The response rate to IFN- α in metastatic melanoma is ~16%; up to one-third of these may be prolonged (Agarwala 2003). IL-2 therapy also yields response rates ranging from 15% to 20% in advanced melanoma; complete remissions (which are often durable) may occur in ~4%–6% of patients (Atkins et al. 1999). Despite these encouraging results, both IFN- α and IL-2 regimens are hampered by substantial and frequently dose-limiting toxicities, which have curbed their widespread use in metastatic disease.

The toxicity associated with systemic IL-2 and IFN- α treatment has led to dose modifications and a series of combinatorial regimens collectively termed biochemotherapy or chemioimmunotherapy (Richards et al. 1992; Atkins et al. 1994). Several of these regimens have yielded encouraging tumor response rates (Legha et al. 1998; Eton et al. 2002); however, no large randomized trial has yet demonstrated a survival benefit. These ambiguous results have thus far precluded widespread application of biochemotherapy in advanced melanoma.

Melanoma vaccines The toxicities and limited efficacy of existing immunotherapeutic approaches provides a rationale for less toxic alternatives to harnessing the immune system against melanoma. Robust methods to identify melanoma-specific antigens recognized by CD4⁺ and CD8⁺ T-lymphocytes have fueled a diverse array of melanoma vaccine efforts in recent years, including some that exploit enforced expression of the immunostimulatory cytokine GM-CSF. While a comprehensive discussion of their design and underlying immunologic principles lies beyond the scope of this review (and has been discussed in several recent reviews, Dranoff 2002; Sondak and Sosman 2003; Speiser et al. 2003; Talebi and Weber 2003), three broad melanoma vaccine categories may be recognized: (1) those that use lymphocyte-derived tumor antigens; (2) vaccines derived from whole tumor cells; and (3) the use of dendritic cells pulsed with melanoma antigens. These strategies have been tested both alone and in combination with novel adjuvants aimed at boosting the resulting anti-tumor immune response. Induction of a specific immune response is frequently demonstrable in melanoma patients following

vaccination; however, meaningful clinical responses are much less common. Efforts are ongoing to test systemic immunotherapeutic agents after vaccination to see if the vaccine-primed immune system can garner a more effective tumor regression in this context.

Toll-like receptor agonists Dendritic cells, monocytes, and other innate immune cell types express Toll-like receptors (TLRs), a family of transmembrane proteins that direct initiation of innate immune responses and enhance subsequent adaptive responses to a variety of insults (Pasare and Medzhitov 2003). TLR activation augments the host immune response in several ways (Fig. 4), including cytokine/chemokine activation, induction of phagocytosis by host cells, and increasing expression of costimulatory molecules (e.g., CD80 and CD86) on dendritic cells, thereby effecting more efficient T-cell activation. Many of these same immune activities should be beneficial in anti-tumor immunity, particularly in the case of melanoma, since TLR actions appear critical in the dermal immune environment. Thus, several groups have begun to investigate pharmacologic approaches toward TLR activation in melanoma immunotherapy.

The imidazoquinolones, which include imiquimod and resiquimod (R-848), are well-known immune-modifying compounds that possess TLR agonistic activity, particularly against the TLR7 isoform. TLR7 agonists can eradicate tumor cells both in cell culture and in vivo (Schon et al. 2003). Imiquimod, which is used clinically to treat anogenital human papillomavirus (HPV) infections, also demonstrates anti-tumor activity against numerous benign and malignant human skin neoplasms. Administration of imiquimod as a topical cream has achieved partial or complete regressions in dysplastic nevi, lentigo maligna, basal cell cancers, and melanoma skin metastases (Ahmed and Berth-Jones 2000; Naylor et al. 2003; Schon et al. 2004; Noel and Kunzle 2005; Zeitouni et al. 2005; Dusza et al. 2006). Moreover, preliminary evidence suggests that this agent may enhance the potency of anti-melanoma vaccines (Craft et al. 2005). Direct application of imiquimod to these cutaneous lesions is associated with natural killer (NK) cell infiltration within days, followed by tumor cell lysis and macrophage infiltration; this response is consistent with immune-mediated killing. Decreased BCL-2 expression within neoplastic cells appears to precede tumor cell death. Whether imiquimod or other TLR agonists can safely be administered as systemic agents is uncertain; however, these findings suggest that this form of immune modulation might prove useful as palliative management within the subset of melanoma patients whose metastatic disease is confined to the skin.

CTLA-4 antibody blockade An essential component of tumor immunotherapy involves T-cell activation in response to tumor-specific antigens. However, effective T-cell stimulation also requires a costimulatory signal; the absence of such costimulation results in anergy or death

of the T cell. CD28, the major costimulatory receptor for T cells, binds to CD80 or CD86 ligands expressed on antigen-presenting cells, as shown in Figure 4. CTLA-4 is a member of the CD28 family and is expressed only on activated T cells; this protein binds CD80 and CD86 with high affinity. However, in contrast to CD28, CTLA-4 appears to attenuate T-cell function. Consistent with this observation, altered CTLA-4 activity contributes to pathogenesis in several mouse models of autoimmune disease. Genetic studies in humans link specific variant CTLA-4 alleles to autoimmune thyroid disease and insulin-dependent diabetes, among others (for review, see Kristiansen et al. 2000). Moreover, CTLA-4 appears to contribute significantly to maintenance of T-cell tolerance to self antigens present on both normal and tumor cells (Takahashi et al. 2000).

Anti-CTLA-4 monoclonal antibodies have recently entered clinical development. Blockade of CTLA-4 function by these antibodies can trigger or exacerbate autoimmunity, but also enhances immune-mediated tumor rejection in mouse models (Leach et al. 1996; van Elsas et al. 1999; Egen et al. 2002). A phase I study of this strategy was reported by Hodi et al. (2003) and demonstrated significant biologic activity in subsets of previously immunized patients with advanced disease. MDX-010 (Mederex, Inc.), a fully humanized anti-CTLA-4 antibody, yielded an encouraging tumor response rate in a phase II trial that enrolled 56 metastatic melanoma patients (Atia et al. 2005), including several patients whose responses persisted for >2 yr. Systemic autoimmunity constitutes the major toxicity associated with CTLA-4 blockade; this can be severe or even lethal if not identified promptly. In the future, administration of anti-CTLA-4 after a trial of a melanoma vaccine may augment a more specific anti-melanoma immune response while allowing patients to receive a less toxic dose (Sanderson et al. 2005; Hodi et al. 2006). Overall, the CTLA-4 experience highlights both the promise of melanoma immunotherapy and the challenges of uncoupling a robust anti-tumor response from the predictable and often dangerous immune-mediated side effects (Dranoff 2005).

Future directions in melanoma therapy

Expanding knowledge of melanoma somatic genetics and advances in anti-tumor immunity provide a rich ground for targeted small-molecule and immunotherapeutic deployment. Anti-angiogenesis therapy may also offer promise in melanoma; indeed, advanced melanomas often become highly vascular and bleed easily, particularly when metastatic to brain. Recent work suggests that many metastatic melanomas exhibit down-regulation of the AP-2 transcription factor; this leads to increased MCAM/MUC18 and thrombin receptor expression, which, in turn, activates angiogenic factors such as IL-8 (for review, see Bar-Eli 1999). Fully humanized monoclonal antibodies directed against MCAM/MUC18 and IL-8 inhibit melanoma growth and angiogenesis in mouse xenografts (Huang et al. 2002; Mills et al. 2002). These intriguing findings merit additional study, particu-

larly in light of the recent successes of anti-angiogenic monoclonal antibody therapy in other solid tumors.

At the same time, use of the above regimens as single agents may at best benefit subsets of melanoma patients and prove unlikely to achieve long-term remissions; therefore, combinations of targeted and/or immunotherapeutic options may expand their cumulative clinical benefit. On the other hand, the toxic side effects of even “targeted” therapies remain considerable. Thus, a major priority for future clinical trials will be the concordant development of genetic or molecular markers that may be used by clinical pathologists and medical oncologists to allow prospective selection of patients most likely to benefit from emerging therapeutic regimens. Biomarker-based patient stratification will therefore improve the chances of therapeutic success while minimizing unnecessary patient exposure to drug toxicities.

BRAF mutation currently represents the leading candidate for a genetic biomarker; presumably, this variant will predict responders to RAF or MEK inhibition, as described above. The demonstration of c-Kit mutation in 1%–2% of melanoma patients justifies efforts to evaluate the therapeutic efficacy of imatinib in this patient subpopulation. Identification of predictive biomarkers for response to immunotherapies (e.g., IL-2, INF- α , and anti-CTLA-4) is equally important and remains an area of active investigation. Recently, a nested study of 200 patients from an ongoing randomized trial found that the presence of either vitiligo or autoantibodies in the serum of metastatic melanoma patients 1 mo after initiation of IFN- α treatment was strongly correlated with increased patient survival (Gogas et al. 2006). These findings raise the possibility that biomarkers indicative of the presence of sensitive melanoma antigens or an effective antitumor immune response might similarly prove to be predictive of future responders to other systemic immunotherapeutic regimens.

While traditionally considered nondrugable, the increasing recognition that transcription factors (e.g., MITF) may play oncogenic roles in melanoma has driven efforts to devise alternative approaches to target this protein class. One such approach involves the use of peptide to block transcription factor activities. In melanoma, ATF2 has been targeted in this fashion with impressive preclinical results (Bhousmik et al. 2002, 2004). These observations are particularly intriguing given evidence that ATF2 nuclear localization correlates with prognosis in human melanoma specimens (Berger et al. 2003). An additional strategy involves elucidating the critical downstream target(s) of oncogenic transcription factors, some of which may prove to be more amenable to small-molecule compound inhibition. Finally, elucidation of these pathways stands to offer novel targeted strategies at prevention (D’Orazio et al. 2006).

The unprecedented propagation of melanoma genetics, genomics, and biological understanding justifies an optimistic view of its future therapeutic outlook. Undoubtedly, the road ahead will remain stunted with challenges and occasional setbacks; however, salient tumor depen-

dencies that underlie definable melanoma subtypes have begun to emerge. In partnership with versatile in vitro and in vivo model systems, increasingly rigorous target validation and rational drug development efforts, and judicious use of correlative omics studies for robust biomarkers, melanoma promises to provide an instructive framework for progress against even the most brittle of human malignancies.

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