REVIEW

Malignant melanoma: modern black plague and genetic black box

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References to 'black cancer' and 'fatal black tumors with metastasis' date to the writings of the legendary Greek physician Hippocrates in the fifth century B.C. However, it was not until the time of Napoleon's Prussian Campaign in 1806 that René Laennec, inventor of the stethoscope, provided the first description of melanoma as a disease entity in his presentation to the Faculté de Médecine in Paris (Bennett and Hall 1994). His 1812 manuscript reporting a case of disseminated melanoma also marks the first published use of the word 'melanoma' (Laennec 1812). Several years later, a truly remarkable conceptual leap was made in the context of melanoma by a general practitioner William Norris (Norris 1820) who arrived at the conclusion that melanoma is a hereditary disease. In his 1820 manuscript, Norris wrote: *'it is remarkable that this gentleman's father...died of a similar disease. This tumor...originated in a mole and it is also worth mentioning that, not only my patient and his children had many moles..., but also his own father and brothers....These facts, together with a case that has come under my notice, rather similar, would incline me to believe that this disease is hereditary.'* This prescient statement is all the more striking by the fact that it was made nearly half of a century before the genetic paradigm was articulated by Gregor Johann Mendel in *The Transactions of the Brunn Natural History Society* in 1866 and 1869 (see Hecht 1989).

Today, cancer biologists stand resolute in the concept that melanoma has strong genetic underpinnings. This position has been strengthened by the identification of genomic hot spots (and their resident genes) in which structural alteration correlates with the genesis and/or progression of melanoma. More recent efforts to understand the biological impact of these melanoma-associated genetic lesions have yielded a series of scientific observations that has captured center stage in the field of cancer genetics. These new molecular insights are indeed welcome because they come at a time when melanoma has emerged as a true health crisis. As a cancer type, incidence of melanoma is rising at a rate second

only to lung cancer in women and, on its present course, the lifetime risk will reach 1 in 75 Caucasians in the US by the year 2000 (Rigel et al. 1996). In contrast to many other cancer types, it is notable that melanoma affects a much younger population and is notorious for its propensity to metastasize early in the course of the disease and for its poor response to current therapeutic modalities (Herlyn 1993).

The purpose of this review is to provide a current view of the key genetic lesions associated with the development of melanoma including those involving the *INK4a/ARF* tumor suppressor locus, the *ras* family oncogenes, and the receptor tyrosine kinases and their ligands. These pathways in particular are emphasized here because their pathogenetic roles in melanoma have been verified on several experimental levels. This review will not attempt to catalog the vast number of genetic and gene expression changes observed in melanoma; several comprehensive reports have capably summarized such information (Albino 1995; Greene 1997; Welch and Goldberg 1997; Haluska and Hodi 1998).

Clinical aspects of melanoma

Melanoma represents a collection of diseases as defined by pathological and clinical criteria. Histologically, the classification of melanoma reflects the location and/or depth of involvement (Fig. 1). For example, the *in situ* melanoma, characterized by the radial growth pattern, is confined primarily to the epidermis. In contrast, the 'vertical growth phase' of a malignant melanoma denotes a transition to a more aggressive and lethal condition, which is characterized by tumor invasion into the underlying dermis and subcutis, and often presages metastasis to distant sites. Although melanoma tends to spread to nonvisceral sites such as skin or lymph nodes, the most common visceral sites involved in metastatic melanomas are lung, liver, brain, bone, and small intestine (Elder 1987).

The search for prognosticators of future metastatic disease and survival has been a major preoccupation of dermatopathologists. Among the many factors assessed, the total tumor thickness emerged as the best independent determinant of prognosis for nonmetastatic melanomas.

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The system described by Breslow, which measures tumor thickness from the uppermost nucleated layer of the epidermis (i.e., the granular cell layer) to the greatest depth of tumor invasion, has gained acceptance as the classification system of choice as a result of its ease, reproducibility, and objectivity. By comparison, Clark's classification system correlates anatomic level of invasion (Fig. 2), coupled with mitotic index, to prognosis. This system, although it offers more information, can be more cumbersome and may lack reproducibility (for review, see Barnhill et al. 1993).

Despite the long history of clinical recognition and the enormous efforts in pathological classification, the precise pathogenetic mechanisms leading to melanoma still are not well understood. Several features of this disease have provided evidence for genetic and epigenetic etiologies. For example, the contribution of sun exposure to the risk of developing melanoma appears firmly accepted, although the precise mode of exposure (e.g., intermittent intense exposure vs. chronic exposure) associated with increased risk has not been defined. It is also clear that skin color is an important modifier of melanoma risk. Dark-skinned individuals have substantially lower risks of melanoma incidence than fair-skinned populations given the same levels of UV exposure (Barnhill et al. 1993). Together, the fact that solar radiation

increases the risk for melanoma in some, and that the magnitude of this effect is modifiable by a known heritable factor, that is, skin type, suggest that identifiable genetic predisposing factors exist for melanomagenesis.

Other evidence supporting a genetic predisposition is the fact that 10% of melanoma patients have a history of an affected family member. In some kindreds, melanomas occur in the setting of the familial atypical multiple mole and melanoma (FAMMM) syndrome, also known as the dysplastic nevus syndrome (DNS). Members of these affected kindreds develop a large number of atypical moles or dysplastic nevi early in life and they succumb to overt melanoma with a higher penetrance and earlier onset than sporadic melanoma (for review, see Haluska and Hodi 1998).

Although the precise definition of dysplastic nevus is still controversial, most dermatologists agree that the density of dysplastic nevi correlates with melanoma risk in a patient (Tucker et al. 1997). Some evidence even suggest that dysplastic nevi are not only markers of risk but also a potential precursor. These include the observations that many superficial spreading melanomas, the most common subtype (Table 1), are associated with pre-existing nevi, and that approximately one-third of these melanomas are associated with nevus remnants with atypical features (for review, see Rhodes 1993). More-

Table 1. *Clinical classification of melanomas*

Subtype	Frequency	Common sites	Median age of diagnosis (years)	Duration of RGP	Others
Superficial spreading melanoma	70%	trunk of men; legs of women	44	1–5 years	potential precursor lesion = dysplastic nevus
Nodular melanoma	10%–30%	trunk of men; legs of women	53	6–18 months	
Acral lentiginous melanoma	most common in darker skin types	palms; soles; nails	65	3 years	
Lentigo maligna melanoma	<5%	sun-damaged skin of head and neck	65	3–15 years	precursor lesion = lentigo maligna

over, among the subset of familial melanomas, >70% of the primary melanomas are associated with pre-existing dysplastic nevi (Greene et al. 1985). In a series of studies on melanoma-prone kindred cohort, it was shown that almost all of the family members with cutaneous melanoma also had dysplastic nevi, and only members with dysplastic nevi developed new melanoma during the period of follow-up (Greene et al. 1985). These clinical observations strongly implicate the dysplastic nevus as a precursor lesion, much as the relationship described for colonic polyps and colorectal carcinoma (Kinzler and Vogelstein 1996). The search for linked loci responsible for melanoma predisposition has yielded some promising candidates. Table 2 summarizes some of these chromosomal lesions and in the review that follows, we discuss those loci experimentally proven to be involved in the genesis and progression of melanoma.

Loss of the 9p21 tumor suppressor—*sine qua non* of melanomagenesis

The familial melanoma locus at 9p21

Several years ago, a series of cytogenetic, linkage, and molecular analyses of familial and sporadic melanomas provided compelling evidence that loss of a tumor suppressor residing at 9p21 represents a rite of passage for

virtually all melanomas. The ensuing detailed molecular analysis of this locus has resulted in one of the most intriguing stories in cancer biology today.

The 9p21 locus in man, and the syntenic region on mouse chromosome 4, contains the closely linked *INK4a* and *INK4b* genes whose products, p16^{INK4a} (*MST1/CDKN2*) and p15^{INK4b}, act as negative regulators of the cell cycle (Kamb 1995). These genes are members of the INK4 (inhibitor of the cyclin-dependent kinase 4) family, which also include p18^{INK4c} and p19^{INK4d} (Harper and Elledge 1996). On the molecular level, INK4 family members execute their growth arrest activities through interaction with and inhibition of the G1 cyclin-dependent kinases, CDK4 and CDK6. CDK4/6 play prominent roles in the phosphorylation of the retinoblastoma tumor suppressor gene product, pRb, the major gatekeeper of the cell-cycle restriction point. Left in its hypophosphorylated state, the pRb protein constrains cell-cycle growth through its active transcriptional repression of genes required for exit from the resting G₁ phase and progression through the DNA synthetic (S) phase of the cell cycle (Fig. 3) (for review, see Sherr 1996). Loss of p16^{INK4a} function tips CDK4/6 towards a more highly activated state, resulting in pRb hyperphosphorylation with associated derepression of pRb-regulated genes and unscheduled entry into S phase.

Interest in the p16^{INK4a} gene reached a new high with

Table 2. *Chromosomal alterations in cutaneous malignant melanoma*

Chr	Alterations	Candidate genes	Involvement	Reference
1 p	Deletion; translocation; LOH		LOH in advanced melanomas; Chr 1 transfer → suppresses metastasis, but not tumorigenicity, of MelJuSo	see, e.g., Miele et al. (1996); Dracopoli et al. (1998)
6q22-27	Deletion most common		40%–75% LOH in late stage melanomas; Chr 6 transfer → conflicting results regarding tumorigenicity vs. metastasis in different cell lines	for review, see Trent et al. (1990); Welch et al. (1994); Miele et al. (1996); Welch and Goldberg (1998)
7	Amplification	<i>EGFR</i>	Increased expression in melanomas; level correlates with aggressiveness	Koprowski et al. (1985); de Wit et al. (1992a,b)
9p21	LOH; deletion; intragenic mutation	<i>INK4a</i>	dysplastic nevus; early melanoma; hereditary predisposition	see text
10q24-26	LOH	<i>MXI1</i> ; <i>PTEN</i> / <i>MMAC1</i>	dysplastic nevus; early melanoma	see text

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Figure 3. The RB-regulated G1/S transition in the mammalian cell cycle. In G₁, hypophosphorylated Rb binds the E2F transcription factor, resulting in transcriptional repression of E2F-responsive genes. In response to growth promoting signals, the CDK4/cyclin D complex participates in the phosphorylation of pRB, causing release of pRB from promoter-bound E2F, activation of E2F-responsive genes, and subsequent entry into S phase. The kinase activity of CDK4/cyclin D is negatively regulated by p16^{INK4a}, a member of the *INK4* G1-specific cyclin-dependent kinase inhibitor (CKI) family.

the seminal discovery of a second unrelated non-CKI protein, encoded by an *INK4a* locus reading frame distinct from that of p16^{INK4a} (Quelle et al. 1995). This new reading frame initiates in a different first exon (exon 1 β) and continues in an alternative reading frame in the shared exon 2 (Fig. 4). The novel protein was named p19^{ARF} for alternative reading frame protein and, to denote the fact that the *INK4a* gene encodes two distinct products, it is now referred to as the *INK4a/ARF* locus.

p19^{ARF} functions as a potent growth suppressor as evidenced by its ability to induce a G1 and G2 phase arrest (Quelle et al. 1995, 1997), block oncogenic transformation, and enhance apoptosis (Pomerantz et al. 1998). Most important is the finding that these p19^{ARF} activities require p53 (Kamijo et al. 1997; Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998), thus linking a single locus to both of pRb (p16^{INK4a}) and p53 (p19^{ARF}). The biochemical basis for the p53 dependency of p19^{ARF} functions appears to relate to the ability of p19^{ARF} to bind Mdm2, block Mdm2-induced degradation of p53, and stabilize p53 protein (Kamijo et al. 1998; Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998). Although not evolutionarily conserved, the mouse p19^{ARF} protein has also been shown to bind p53 directly in the absence of Mdm2 (Kamijo et al. 1998), suggesting an additional Mdm2-independent mechanism for p19^{ARF} function. Moreover, the observation that p19^{ARF} levels are elevated in p53-deficient cells (Quelle et al. 1995; Stott et al. 1998) had raised the possibility that there existed a regulatory feedback loop between ARF and p53 on the level of gene expression. Consistent with this view is the finding that p53 itself can down-regulate expression of p19^{ARF} (Stott et al. 1998), possibly through repression of the 1 β promoter.

In melanoma, as well as other 9p21-associated cancers, the 9p21 region most commonly sustains homozygous deletions in sporadic cases (see references in Funk et al. 1998; Haluska and Hodi 1998; Kumar et al. 1998; Mat-

sumura et al. 1998; Ruiz et al. 1998; Wagner et al. 1998) and both deletions and point mutations in familial cases (Hussusian et al. 1994; Kamb et al. 1994; Gruis et al. 1995; Fitzgerald et al. 1996; Flores et al. 1998; MacKie et al. 1998; Platz et al. 1998; Soufir et al. 1998) often eliminating *INK4b* and *INK4a/ARF* (for review, see Chin et al. 1998; Haluska and Hodi 1998). The high incidence of deletion has fueled an ongoing debate centered on whether each of these genes contributes to the tumor suppressor activity encoded within 9p21. With respect to p16^{INK4a}, its designation as a suppressor of human neoplasia appears to be conclusive. Most convincing are reports describing germline mutations that inactivated the p16^{INK4a} specific exon 1 α in familial melanoma (Hussusian et al. 1994; FitzGerald et al. 1996). Many other melanoma-associated mutations affecting the p16^{INK4a} reading frame have since been described and the majority of these mutations target the ankyrin repeat region of p16^{INK4a} (Hussusian et al. 1994; Kamb et al. 1994; Liu et al. 1995), a domain known to play a role in CDK4/6 interaction (Serrano et al. 1993; Brotherton et al. 1998; Russo et al. 1998). That the p16^{INK4a}-CDK4 relationship is indeed relevant to melanoma is further supported by the identification of germline CDK4 mutations in two melanoma-prone families (Zuo et al. 1996; Soufir et al. 1998). This mutation disrupts the p16^{INK4a} interaction domain, thereby rendering CDK4 refractory to inhibition by the INK4 proteins (Wolfel et al. 1995). Finally, in addition to gene deletion and point mutations, loss of p16^{INK4a} function can result from methylation-induced transcriptional silencing (Gonzalez-Zulueta et al. 1995; Kamb 1995), although this mode of inactivation does not appear to be common in melanoma (Gonzalzo et al. 1997).

Unlike the case for p16^{INK4a}, definitive data linking p15^{INK4b} to 9p21-associated cancers have yet to emerge. For example, there are no reports of mutations exclusively targeting the p15^{INK4b} open reading frame in human cancers. Furthermore, it is notable that p15^{INK4b}-deficient mice exhibit an extremely weak cancer-prone phenotype (E. Latres and M. Barbacid, pers. comm.). In contrast, the case of p19^{ARF} as a tumor suppressor in humans appears to be a stronger, albeit inconclusive, one. The strongest existing evidence includes identification of mutations or deletions impacting exclusively on the p19^{ARF} 1 β exon in some T-ALL cases (Gardie et al. 1998). However, germline p19^{ARF}-specific mutations have yet to be reported in humans, and most tumor samples harboring p19^{ARF} loss show concomitant deletion or mutation of p16^{INK4a} and/or p15^{INK4b} sequences (for review, see Chin et al. 1998). Nevertheless, a p19^{ARF}-specific knockout in the mouse generates a strong cancer-prone condition (Kamijo et al. 1997) and p19^{ARF} overexpression can suppress Myc/Ras transformation in cell culture (Pomerantz et al. 1998). These organismal and cell culture studies indicate that p19^{ARF} possesses potent tumor suppressor activity, thus elevating the likelihood that its ortholog, p14^{ARF}, serves a similar role in human tumors. Given the high frequency of dual inactivation of p19^{ARF} and an INK4 family member in hu-

Figure 4. The 9p21 genomic locus. Within a relatively short genomic distance, the 9p21 locus (and its syntenic region in mouse) encodes for three negative regulators of the cell cycle: p15^{INK4b}, p16^{INK4a}, and p19^{ARF}. The *INK4b* gene encodes for p15^{INK4b}, also a G₁-specific CKI, similar to p16^{INK4a}. The *INK4a/ARF* gene utilizes alternative first exons and common downstream exons to encode two distinct products. The exon 1α bearing transcript encodes p16^{INK4a}, a G₁-specific CKI (see Fig. 3). The exon 1β bearing transcript encodes p19^{ARF}. p19^{ARF} neutralizes Mdm2, thereby stabilizing p53 levels and enhancing p53 activity (see text). Thus, the *INK4a/ARF* locus encodes two proteins functioning upstream of both the Rb and p53 pathways.

man cancers, it is tempting to speculate that the stringent antitumor mechanisms of humans (relative to those in mice) may necessitate dysregulation of both Rb and p53 tumor suppressor pathways for efficient transformation.

The unique genomic organization of *INK4a/ARF* may help explain why *INK4a/ARF* is a frequent target of tumorigenesis, in that a single genetic hit would result in the simultaneous disruption of these two key antioncogenic mechanisms. On a related note, the placement of p19^{ARF} along the p53 pathway (Kamijo et al. 1997, 1998; Pomerantz et al. 1998; Stott et al. 1998; Zhang et al. 1998) and its role as an ‘enhancer’ of p53 activity may also account for the very low incidence of p53 mutations in melanoma (for review, see Chin et al. 1998). An absence of p53 mutations also holds true in a mouse model for melanoma generated in the *INK4a/ARF*-deficient background (see below) (Chin et al. 1997). This observation led us to propose that p19^{ARF} inactivation would lessen the genetic requirement for loss of p53 itself in melanoma (Chin et al. 1997), a reciprocal relationship analogous to the observation that sarcomas harboring MDM2 amplification do not harbor p53 mutations (Oliner et al. 1992). The tight functional interrelationship of p19^{ARF} and p53 is also supported by the observation that deletion of p19^{ARF} or p53 are common, but mutually exclusive, events during the process of cellular immortalization in mouse fibroblasts (Kamijo et al. 1997).

The lack of deletions/mutations in both *INK4/ARF*

and p53 in the same human cancers also appears to extend to other types of carcinomas. A revisited mutation analysis has been performed by Pomerantz et al. (1998) for carcinomas of the bladder, oral region, and lung and for lymphoid neoplasms, such as B-cell chronic lymphocytic leukemia and Hodgkin’s and Non-Hodgkin’s lymphomas (Gruis et al. 1995; Newcomb et al. 1995; Breen et al. 1996; Hangaishi et al. 1996; Heinzel et al. 1996; Kinoshita et al. 1996). In >500 tumors analyzed, the mutation rates were 18% for p16^{INK4a}, 14% for p53, and 4% for both. *INK4a* point mutations that result in a single amino acid change in the p16^{INK4a} ORF were reanalyzed to determine the genetic status of the p19^{ARF} ORF. Only nine (<2%) of 405 evaluable cases harbored both p53 and p19^{ARF} mutations. Because all nine cases also had alteration in p16^{INK4a}, it remains possible that p19^{ARF} mutation was incidental to that of p16^{INK4a} in those tumors. In fact, the most common point mutation in the p19^{ARF} ORF (a P93L substitution; Quelle et al. 1995b) has been shown to be fully active in cell-cycle arrest (Quelle et al. 1997) and in suppression of Myc/Ras transformation and in stabilizing p53 in the presence of high Mdm2 levels (Pomerantz et al. 1998). The distinctly uncommon occurrence of co-mutation of p53 and p19^{ARF} lends support to the widely held view that they operate through a common tumor suppressor pathway.

Interestingly, despite the strong link between *INK4a/ARF* and melanoma in humans, mice doubly null for p16^{INK4a} and ARF (exon 2 + 3 deletion) failed to develop melanoma (Serrano et al. 1996). Rather, the most common malignancies encountered in this model were fibrosarcomas and B-cell lymphomas—two cancer types that also arise in humans inheriting a defective copy of the *INK4a* gene (Cordon-Cardo 1995). Among the possible explanations for the lack of melanoma in the *INK4a/ARF*-null mice is the need for cooperating genetic lesions; one likely candidate considered early in the course of these studies was activation of the Ras pathway. This view stemmed from the ability of Ras to elicit two very different cellular responses—senescence or transformation—in the presence or absence of intact *INK4a/ARF* function, respectively (Serrano et al. 1997). The lack of Ras-induced transformation in the presence of intact mortality pathways is consistent with an earlier transgenic mouse study showing that melanocytic expression of activated H-Ras^{val12} did not result in melanocytic transformation (Powell et al. 1995). In this particular Ras model, metastatic melanoma is observed only following exposure to the carcinogen DMBA (Gause et al. 1997). These results imply that, although Ras activation may play an etiological role in melanoma, other cooperative genetic lesions are clearly required.

Pathogenetic role for activated ras in melanoma?

Historically, activating ras mutations have been among the most common proto-oncogene mutations in human cancers. Despite its prominence in many other cancer types, early studies attempting to establish a causal role for activated Ras in melanoma generated conflicting re-

sults. For example, stable transfection of activated H-ras into cultured mouse (Wilson et al. 1989; Ramon y Cajal et al. 1991) and human (Albino et al. 1992) melanocytes was shown to induce phenotypic characteristics of fully transformed melanoma cells, such as growth inhibition by protein kinase C activator, anchorage independence, tumorigenicity in nude mice, and chromosomal instability. The status of INK4a/ARF was not examined in these cultures. On the other hand, Albino et al. (1989) reported that the frequency of activating *ras* mutations (predominantly N-ras) was significantly higher in cultured melanoma cell lines (24%) than in noncultured melanomas (5%–6%). This observation raised the possibility that *ras* mutations may be a consequence of the inherent genomic instability of transformed cells further accentuated by adaptation to culture. By contrast, Van't Veer et al. (1989) has observed a frequency of N-ras mutations in primary melanomas approaching 20%. Other groups have detected a higher frequency of *ras* mutations (N-ras more than H-ras) in metastatic and recurrent melanomas (Ball et al. 1994; Jafari et al. 1995; Wagner et al. 1995), suggesting a role for Ras activation in disease progression rather than initiation. These data correlate well with expression studies showing increased Ras immunoreactivity in late stage melanoma (Yasuda et al. 1989). In addition, activating N-ras and H-ras mutations have been documented in metastatic melanoma cell lines through use of the NIH/3T3 transformation assay (Albino et al. 1984). Taken together, the consensus view would favor a role for N-ras and H-ras mutations in promoting progression toward a more advanced stage of melanoma.

In an effort to obtain formal genetic proof in support for this Ras hypothesis, a transgenic mouse model was generated in which activated Ras was expressed in the melanocytes of the INK4a/ARF null mouse. Against the wild-type *INK4a/ARF* background, spontaneous cutaneous melanomas emerged at a very low incidence and with a long latency (Chin et al. 1997). Significantly, these rare melanomas showed spontaneous deletion of both alleles of *INK4a/ARF*. Correspondingly, melanomas arising in *INK4a/ARF* heterozygotes sustained a consistent loss of the remaining wild-type *INK4a/ARF* allele. Finally, on an *INK4a/ARF*^{-/-} background, these transgenic animals developed spontaneous cutaneous melanomas with high penetrance after a short latency (Chin et al. 1997). Therefore, Ras activation can cooperate with INK4a/ARF deficiency in vivo to accelerate the genesis of melanoma. Lastly, as mentioned above, similar to human melanoma, the *p53* gene remained in a wild-type configuration in these mouse melanomas (Chin et al. 1997). Thus, the Ras-INK4a/ARF mouse melanoma profile recapitulates some of the prominent genetic features observed in human melanoma, namely activation of the Ras pathway, deletion of the *INK4a/ARF* tumor suppressor, and absence of *p53* mutation. However, despite these similarities, a curious distinction of the mouse Ras/INK/ARF^{-/-} model is its lack of metastasis. This difference is intriguing given the presumed role for Ras activation in promoting invasive and metastatic behavior in a number of cell culture-based experiments (see

above). Together, these data suggest that activation of the Ras pathway, while necessary for metastatic activity, may be not sufficient to induce this hallmark feature of melanoma. Such findings have directed attention to other signaling pathways, particularly those linked to a class of transmembrane receptors known collectively as receptor tyrosine kinases (RTKs).

RTKs in the normal and neoplastic melanocyte

RTKs play pivotal roles in the regulation of virtually all basic cellular processes, including proliferation, differentiation, migration, and survival. RTKs are transmembrane polypeptides containing both an extracellular ligand-binding domain and a cytoplasmic tyrosine kinase domain that signals to a number of pathways including the Ras/MAPK pathway, phosphatidylinositol 3-kinase (PI3-K), among others. An understanding of how RTKs and their signaling pathways influence the behavior of transformed melanocytes has advanced significantly through a series of transgenic experiments comparing the consequences of Ras activation (see above) versus activation of pathways at the level of the receptor (see below). In addition to these cancer studies, much has been gained from the study of normal melanocyte development, particularly in various mouse mutants with pigmentation defects. Thus, to better frame the RTK-cancer connection, we will begin with a brief review of the role of RTKs in normal melanocyte biology.

RTKs in normal melanocyte development

Melanocytes of the integument, choroid, and ear originate from the neural crest, a transient embryonic structure derived from the dorsal neural tube. When appropriately instructed, a subpopulation of neural crest cells undergo epithelial/mesenchymal conversion, migrate laterally below the epidermis, and ultimately give rise to skin and ear melanocytes (Le Douarin 1982; Bennett 1993). It is now appreciated that this developmental process is tightly regulated through the orchestrated activities of multiple and perhaps sequential RTKs (see below). The essential roles of RTKs in normal melanocyte development have been demonstrated through the analyses of a variety of pigmentation defects, particularly piebaldism.

Piebaldism is a condition characterized clinically by patches of white skin and hair. Within these vitiliginous areas, there is a complete absence of melanocytes as a result of aberrant migration and survival. Spontaneous coat color mutations mapping to either the dominant white spotting (*W*) locus or the steel (*Sl*) locus have long been known to cause piebaldism in homozygous mice (for review, see Bennett 1993). The coat color gene at the *W* locus was later determined to be *c-kit*, a proto-oncogene encoding a RTK (Chabot et al. 1988; Geissler et al. 1988), and that at the *Sl* locus to code for its ligand, the steel or mast cell growth factor SLF (reported by several laboratories; for review, see Witte 1990). SLF-Kit signaling appears to be required for functions such as lateral migration and survival of murine melanocyte precursors

in vivo (Nishikawa et al. 1991; Steel et al. 1992; Wehrle-Haller and Weston 1995; Kunisada et al. 1998). The relevance of this pathway to human melanocyte development has been established by identification of *c-kit* mutations in piebald patients (Fleischman et al. 1991; Giebel and Spritz 1991).

Other RTKs are involved in murine melanocyte development as well. For example, the patch (*Ph*) gene, which encodes the platelet-derived growth factor receptor α (PDGFR α), is also associated with white spotting in mice (Stephenson et al. 1991; Soriano 1997). More recently, analysis of melanocyte precursors in transgenic mice expressing hepatocyte growth factor/scatter factor (HGF/SF) has implicated its RTK, *c-met* (Bottaro et al. 1991; Naldini et al. 1991), in melanoblast survival, differentiation, and/or migration (Takayama et al. 1996; Kos et al. 1998).

The growing number of RTKs implicated in melanocyte development may reflect their unique and cooperative roles in normal melanocyte biology. This view is supported by experimental analysis of normal melanocyte growth and differentiation in cultures. Unlike many cell types, human melanocytes absolutely require growth factor supplementation for survival and growth in vitro. Several growth factors have been identified that stimulate mitogenesis through RTKs expressed in melanocytes, including SLF, HGF/SF, and basic fibroblast growth factor (bFGF) (for review, see Halaban 1996). No single growth factor is sufficient for a full proliferative response (Matsumoto et al. 1991; Halaban et al. 1992). Interestingly, these factors have distinct effects on the differentiation program of melanocytes—HGF/SF stimulates, bFGF inhibits, whereas SLF is not required for differentiation (Dotto et al. 1989; Becker et al. 1992; Halaban et al. 1992; Murphy et al. 1992; MacKenzie et al. 1997). The proliferative and differentiation profiles of these growth factors suggest that the RTK pathways activated by them may execute unique functions, at least in part, during melanocyte development (Halaban et al. 1992). The study of how these pathways functionally interrelate in the context of melanoma represents an area of enormous experimental opportunity.

RTKs and malignant melanoma

In view of the dependence of melanocytes on complex RTK signaling for proper orchestration of basic cellular functions essential for development and homeostasis, it is not surprising that RTKs (and components of their signaling pathways) are frequent targets of the transformation process in melanocytes. For examples, the human epidermal growth factor receptor (*EGFR*) proto-oncogene, which maps to chromosome 7p12–13, is overexpressed (Koprowski et al. 1985; de Wit et al. 1992b; Ellis et al. 1992) in late stage melanomas harboring amplified copies of chromosome 7 (Trent 1991; Bastian et al. 1998). The FGF receptor (FGFR) also appears to play a role in melanoma. Contrary to normal melanocytes, which require supplementation of various growth factors in culture, virtually all melanoma cells can produce bFGF and

grow autonomously through the creation of an bFGF–FGFR autocrine loop (Halaban et al. 1988; Becker et al. 1989, 1992). Such a bFGF–FGFR autocrine loop has been shown to be essential for melanoma growth in vivo (Yayon et al. 1997; Wang and Becker 1997), again indicating involvement of RTK signaling in melanoma development.

There have been numerous reports, sometimes conflicting, describing the expression levels of genes encoding various growth factors and their RTKs in primary and metastatic melanomas as well as their derivative cell lines (see, e.g., Albino 1992; Shih and Herlyn 1994; Halaban 1996). When these studies are viewed collectively, a common theme appears to be the acquisition of autocrine signaling loops. These loops include members of the FGF family and their receptors; transforming growth factor α (TGF α) and its receptor, the EGFR; and B61 and its receptor, epithelial cell kinase (ECK). Other RTKs showing strong expression include Erb-B2, IGF1R, and Met. Robust *c-met*, but not *HGF/SF*, expression has been observed in melanoma tissue and cells, and in some cases has been associated with progression to the metastatic state (Natali et al. 1993; Saitoh et al. 1994; Rusciano et al. 1995; Hendrix et al. 1998). In human interconverted uveal melanoma cells, which are highly aggressive and preferentially disseminate to the liver, *c-met* expression has been correlated to high mitogenic response and invasiveness (Hendrix et al. 1998). In contrast, Kit, a RTK which can inhibit melanoma growth, exhibits a loss or reduction in its expression in most melanomas (Funasaka et al. 1992; Lassam and Bickford 1992; Zakut et al. 1993; Gutman et al. 1994).

In addition to autocrine growth promoting effects, a number of RTK-activating growth factors characteristically produced by melanoma cells, such as the FGFs and PDGFs, can also exert profound influences on the immediate microenvironment through paracrine signaling mechanisms (for review, see Rodeck and Herlyn 1991; Shih and Herlyn 1994). These include promotion of angiogenesis, extracellular matrix degradation and adhesive interactions, and suppression of immunologic response and antiproteolytic activity. These biological endpoints of RTK signaling have been proposed to account for the apparent association of RTK activation with metastatic progression in human and murine melanoma.

Perhaps the most convincing experimental evidence implicating RTK signaling pathways in the genesis and progression of melanoma comes from studies of animal models. One well-characterized model is a fish of the genus *Xiphophorus*, which develops melanoma in specific association with aberrant expression of the activated *Xmrk* gene; *Xmrk* encodes a novel transmembrane RTK related to the EGFR (Wittbrodt et al. 1989; Schartl and Adam 1992). In the mouse, ectopic transgene expression of an activated RTK encoded by the *ret* oncogene induced widespread skin hyperpigmentation and multifocal melanocytic tumors (Iwamoto et al. 1992). These transgenic mice did not demonstrate spontaneous metastasis of primary tumors, although established cul-

tured cells from *ret*-induced tumors were metastatic when reintroduced into Balb/c nude mice as transplants (Taniguchi et al. 1992). The efficacy of activated *ret* in inducing melanoma was somewhat unexpected considering that *c-ret* expression is undetectable in normal melanocytes and melanoma cell lines (Nakamura et al. 1994; Easty et al. 1995). One possible interpretation of these data is that RTKs may play redundant roles by engaging and activating similar pathways integral to the development of melanoma.

More recently, a transgenic murine model was described in which mouse HGF/SF overexpression induced the stepwise development of cutaneous melanoma and, in ~20% of tumor-bearing mice, ultimate progression to metastatic disease, a process that typically took more than a year (Takayama et al. 1996, 1997; Otsuka et al. 1998). Melanoma-genesis was driven in this model through constitutive activation of overexpressed endogenous Met by autocrine expression of the *HGF/SF* transgene. The relatively long latency indicates that HGF/SF overexpression is necessary, but not sufficient, for the development of malignant melanoma. This model therefore encourages an analysis of candidate genes associated with acquisition of the metastatic phenotype, such as p16^{INK4a}, p19^{ARF}, and other RTKs, and the application of various techniques capable of identifying genes that are differentially expressed during late stages of melanoma progression.

Progression to the invasive-metastatic state mandates the acquisition of a variety of essential attributes, including the ability to degrade local extracellular matrix, produce appropriate cell adhesion molecules, migrate into adjacent tissue and vessels, colonize a distant site, and stimulate angiogenesis. Ectopically expressed HGF/SF may facilitate metastasis because it can inherently engage pathways playing an essential role in all these processes (for review, see Matsumoto and Nakamura 1996). Most significantly, HGF/SF can potentially dissociate, or 'scatter' sheets of epithelia into individual mesenchymal-type cells in vitro, and regulate, through c-Met, epithelial/mesenchymal conversion and migration of skeletal muscle precursor cells from the somitic dermomyotome (Bladt et al. 1995). The same type of conversion process occurs during the formation of melanoblasts in the neural crest. It has long been appreciated that crest-derived cells, and melanoblasts in particular, are unusually invasive cell types (Erickson et al. 1980). Perhaps constitutive activation of Met in mature melanocytes predisposes to future metastatic capability by encouraging conversion to a more mesenchymal and invasive cell type, providing a potential explanation for the notoriously aggressive and deadly nature of melanomas. This is a testable hypothesis that can now be addressed in transgenic mouse models expressing wild-type or mutant derivatives of *c-met* in melanocytes.

Molecular pathways governing metastasis: lessons from Met and Ras

The genetic dissection of pathways playing an essential

role in the metastatic behavior of melanoma represents an important focus of the field. The distinct clinical outcomes of transgenic mice expressing activated Ras or HGF/SF in melanocytes has been particularly informative. As mentioned above, the melanocyte-specific expression of activated *H-Ras* on an INK4a/ARF-deficient background led to the development of spontaneous cutaneous melanoma with high penetrance and short latency. These tumors were highly invasive but did not progress to a metastatic phenotype. In contrast, transgenic mice overexpressing HGF/SF developed malignant melanoma with a relatively high incidence of metastasis, albeit with a longer latency. How can we account for these phenotypic differences?

In this regard, what is known about RTK downstream signaling pathways in general, and Met pathways in particular, is enlightening. Typically, growth factor binding induces RTK dimerization and activation of the cytoplasmic tyrosine kinase domain, resulting in RTK autophosphorylation at multiple intracellular tyrosine residues, and subsequent phosphorylation/activation of specific substrates (Weiss and Schlessinger 1998). The main function of the autophosphorylated tyrosines is to bind to and activate specific receptor targets containing Src-homology-2 (SH2) domains. These include a variety of catalytic transducers, noncatalytic signal amplifiers, and molecular adaptor proteins (Schlessinger and Ullrich 1992; Pawson 1995). For the generic RTK, each autophosphorylation site is utilized by a specific SH2 protein, and therefore for a particular pathway and cellular response. Unlike other RTKs, Met has two closely spaced autophosphorylation sites, Y1349 and Y1356, embedded within tandemly repeated degenerate sequences. Both sites are required for signal transduction, and each is able to bind multiple SH2-containing proteins, including signal transducers such as PI3-K, phospholipase C- γ (PLC- γ), Stat3, Src, and Grb2, as well as the Grb2-associated docking protein, Gab1 (Ponzetto et al. 1994, 1996; Weidner et al. 1995, 1996).

Studies using specific inhibitors and Met mutants have begun to assign specific factor binding sites on Met and their corresponding downstream pathways to the various HGF/SF-induced biological activities (Fig. 5). For example, Grb2 and subsequent Ras/MAPK and/or JNK activation are mandatory for maximum proliferative response and complete transformation (Ponzetto et al. 1996; Rodrigues et al. 1997); PI3-K activation is clearly required for HGF/SF-induced scattering (Royal and Park 1995; Derman et al. 1995; Khwaja et al. 1998), and has been reported to be a transducer of tumor invasiveness (Kotelevets et al. 1998); PLC- γ has been implicated in cell chemotaxis (Derman et al. 1996); association with Src correlates with a highly metastatic state (Mao et al. 1997); and full induction of the urokinase proteolysis network (Jeffers et al. 1996) involves both Ras and PI3-K (Besser et al. 1997). On the other hand, the complex process of branching morphogenesis is dependent on a STAT pathway, PI3-K, and Grb2-dependent Gab1 binding (Derman et al. 1996; Fournier et al. 1996; Weidner et al. 1996; Nguyen et al. 1997; Boccaccio et al. 1998).

Figure 5. Schematic representation of the correlation between HGF/SF-induced, Met-associated biological activities and their corresponding signal transduction pathways. Colored components are those that have been shown experimentally to be required, but not necessarily sufficient, for a full biological response. This figure, based largely on genetic data, has been simplified for the purposes of discussion; in reality, complex biological activities undoubtedly occur through coordination and integration of multiple pathways. A full proliferative response in Met-expressing cells requires functional Ras, which is activated through Grb2/SOS (purple) or Shc (not shown). PI3-K has clearly been implicated in cell scattering; analysis of Met mutants with impaired Grb2 binding showed that the PI3-K that binds most directly to Met (yellow) is sufficient for implementing the HGF/SF-induced scatter phenotype. The relative contribution of the Gab1-associated PI3-K in scattering is not known, but is not needed. Branching morphogenesis has been shown to require Grb2/Gab1 binding, as well as activation of Stat and PI3-K pathways (green). This schematic presumes the involvement of the Gab1-related PI3-K, but the relative contribution of the two PI3-K binding sites in morphogenesis is largely unknown. See text for details and references.

Even a cursory examination of the complexities of the Met signaling network (Fig. 5) reveals that Ras activation represents but a subset of downstream effector pathways of Met. Although Grb2-mediated Ras activation is absolutely required for *c-met*-associated transformation (Fixman et al. 1996; Ponzetto et al. 1996; Bardelli et al. 1997), the former has been shown to be insufficient for metastasis. Giordano et al. (1997) have recently demonstrated that a *c-met* mutation that superactivates the Grb2-mediated Ras/MAPK pathway but prevents binding of other signal transducers confers enhanced transforming capability, but extinguishes metastatic potential. Interestingly, an invasive phenotype was rescued by coexpressing a complementary mutant Met that could recruit all factors not requiring Grb2, including PI3-K (Giordano et al. 1997). Furthermore, expression of constitutively active PI3-K fully rescued the ability of cells to metastasize, indicating that simultaneous activation of Ras and

PI3-K pathways is required to unleash the metastatic potential of Met (C. Ponzetto, pers. comm.). The fact that Ras/MAPK activation is not sufficient to induce the metastatic phenotype provides a plausible rationale for the behavioral differences observed between the metastatic HGF/SF and the nonmetastatic H-Ras transgenic melanomas.

The genomic frontier of melanoma

The *INK4a/ARF* locus is clearly the most prominent melanoma susceptibility locus in humans, however numerous studies indicate that it is not the only one. The existence of other disease loci has been made evident from melanoma kindred that do not exhibit linkage to 9p21 and from cytogenetic data uncovering recurrent genomic alterations. Specifically, alterations on human chromosomes 1, 6, and 10 appear to be common events (for review, see Welch and Goldberg 1997). Although a convincing disease gene candidate has yet to be identified for these loci, a region on the long arm of chromosome 10 (10q24–26) have captured substantial attention due to its involvement in a wide spectrum of human cancers. Loss of heterozygosity and chromosomal rearrangements spanning 10q24–26 have been observed in melanoma (Parmiter et al. 1988; Robertson et al. 1998), malignant gliomas (Albarosa et al. 1995; Chiariello et al. 1998), lymphomas (Speaks et al. 1992), and prostate adenocarcinomas (see below), providing strong evidence for the presence of one or more tumor suppressors within this locus. Specifically, the Myc antagonist Mxi1 and the phosphatase PTEN/MMAC1 have emerged as prime candidates based upon the fact that germline deletion of either gene leads to development of a cancer-prone condition in the mouse (Di Cristofano et al. 1998; Schreiber-Agus et al. 1998). Furthermore, intragenic mutations have been observed in human prostate cancers (Eagle et al. 1995; Cairns et al. 1997; Prochownik et al. 1998). To clarify the roles of Mxi1 and PTEN/MMAC1 in melanoma pathogenesis, current efforts are focused on comprehensive mutational analyses of Mxi1 and PTEN/MMAC1 in primary human melanoma samples as well as genetic cooperation studies in the mouse. Along these lines, a newly developed and elegant in vitro loss of heterozygosity–chromosome transfer assay has pointed to 10q23 as the chromosome 10 locus bearing a potent growth suppressor of melanoma cells (Robertson et al. 1998). PTEN/MMAC1 appears to be at least one of the genes in this region targeted for loss and its re-introduction into PTEN-deficient melanoma cells suppresses growth (Robertson et al. 1998). Interestingly, ~60%–70% of sporadic melanomas express wild-type PTEN/MMAC1 (Guldberg et al. 1997; Tsao et al. 1998), and the reintroduction of PTEN/MMAC1 into such melanoma cells appears to have no growth suppressive effect (Robertson et al. 1998). Such findings are consistent with the existence of additional melanoma suppressor genes residing on chromosome 10.

Evidence for a melanoma locus at chromosome 1p36 has accumulated from the linkage analysis of individuals

with early onset of disease, predisposition toward multiple primary melanoma, and/or the presence of multiple dysplastic nevi. An autosomal dominant mode of inheritance for the 1p36-linked lesion for melanoma and DNS has been proposed although some questions still surround the relevance of this locus to melanoma susceptibility (for review, see Greene 1997; Welch and Goldberg 1997). For chromosome 6, cytogenetic rearrangements spanning 6q22–27 have been observed in both early- and late-stage primary tumors (Welch et al. 1994; Miele et al. 1996; for review, see Welch and Goldberg 1997). The findings that the *EGFR* locus is amplified and that overexpression of EGFR appears to correlate with tumor aggressiveness, have pointed to EGFR as a potential candidate RTK that is directly involved in the human melanomagenesis (see Table 2). Moreover, the ability of Met activation to promote melanoma in an experimental mouse model (described above), although not yet confirmed in the human disease, reinforces the view that activation of pathways extending from RTKs in general may contribute to the pathogenesis of melanoma.

Ongoing and foreseeable efforts promise to yield a rich harvest of genes crucial to the development of malignant melanoma and, with such genes in hand, emphasis will shift towards how their dysregulation contributes to the melanoma process. Genetic verification of a causal role for these genes/loci will remain an important exercise particularly because many of the genetic lesions identified to date, with few exceptions, have come from the cytogenetic analysis of far-advanced tumors, particularly metastatic disease. This fact, coupled with the inherent genomic instability of malignant melanoma, emphasizes the need for molecular studies to utilize primary, early stage disease samples. In addition, a key challenge to the melanoma gene hunter is the need to appreciate that melanoma actually represents a diverse collection of diseases, often grouped into one molecular heap. Thus, a better genetic roadmap of tumor progression from melanocyte to metastatic melanoma will emerge with careful attentiveness to tumor stage and histologic subtype. On another level, the melanoma field has a strong need for tractable genetic systems to aid in the discovery, verification, and functional analysis of the growing collection of melanoma susceptibility genes. Mouse models are likely to provide such systems as available data suggest that mouse and human melanoma are similar in etiology (Chin et al. 1997). If this proves true, then the meticulous construction of mouse models harboring genetic lesions encountered in human melanoma should provide at least one complementary approach to the genomic efforts in human melanoma. Beyond their obvious impact on the basic understanding of molecular pathogenesis and gene discovery, these melanoma mouse models would find great use in both chemopreventive and chemotherapeutic efforts directed towards eradication of this modern black plague.

Acknowledgments

We thank Drs. Mark Jacobson, Meenhard Herlyn, Nicole

Schreiber-Agus, Tal Raveh, Carola Ponzetto, and Morag Park for critical reading of the manuscript, and members of the DePinho lab for helpful comments. We are also grateful to Dr. Ponzetto for communicating data prior to publication. L.C. is supported by a grant from the National Institutes of Health (NIH) (K08AR02104-01). Work in the DePinho lab is supported by grants from the NIH (R01HD28317, R01EY09300, and R01EY11267) and from the American Heart Association. R.A.D. is an American Cancer Society Research Professor and a recipient of the Irma T. Hirsch Award.

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Genes Dev. 1998, **12**:

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