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Advances in malignant melanoma: genetic insights from mouse and man

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1. ABSTRACT

Notorious for its proclivity for metastases and resistance to known therapies, malignant melanoma represents a major health concern. Genetic, epidemiological and genomic investigations are highlighting a repertoire of stereotypical mutations that are associated with human melanoma genesis. The functional significance of many of these genetic alterations is being ascertained through the use of *in vivo* mouse models. Insights from human and mouse studies, coupled with the development of novel tools for high-resolution characterization of the melanoma genome, hold promise for the identification of better diagnostic markers and potential therapeutic targets. With the rapid improvements in drug design, these recent advances are generating optimism for the development of better therapeutic options for melanoma patients.

2. INTRODUCTION

Cutaneous melanoma arises from the malignant transformation of pigment-producing melanocytes that reside at the epidermal-dermal junction of the human skin. Both genetic and environmental factors are known to contribute to disease progression. The first report on familial predisposition to melanoma in individuals with pale complexion and light hair color dates back to the 1820s (1). Two centuries later, the genetic and environmental factors that drive melanoma pathogenesis are beginning to be elucidated.

It is now clear that melanoma incidence is influenced by geographical location, such as altitude and latitude, suggesting a link between ultraviolet (UV) light exposure and melanoma risk (2). The current rise in melanoma incidence is attributable, in part, to the

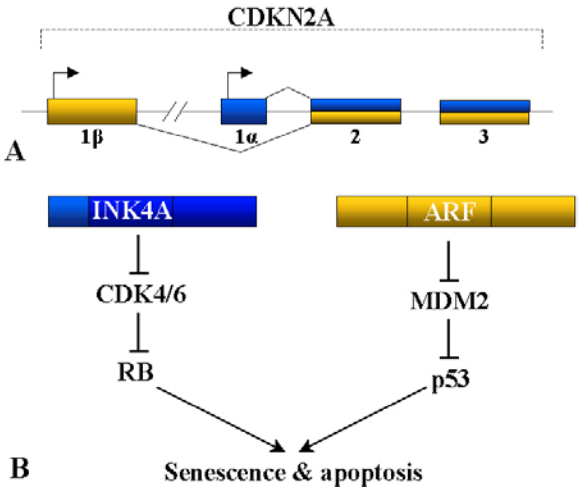

Figure 1. The CDKN2A locus at chromosome 9p21. **A.** The locus has a unique genomic organization and codes for the melanoma suppressors INK4A and ARF (9,10). Two distinct promoters drive the 1α (INK4A) and 1β (ARF) exons and result in alternatively spliced transcripts that share exons 2 and 3. Although shared, different open reading frames within exon 2 give rise to two distinct protein products. **B.** INK4A inhibits CDK4/6-cyclin D-mediated hyperphosphorylation of RB; thereby, insuring that RB is in complex with the transcription factor E2F (11). RB-E2F complexes sequester histone deacetylases that repress transcription resulting in G1 cycle arrest (12). Loss of INK4A allows CDK4/6 to phosphorylate RB, which, in turn, frees E2F to initiate transcription for entry in S phase. The melanoma suppressor ARF blocks MDM2-mediated ubiquitylation and subsequent degradation of p53 (13-16). This helps stabilize p53 and preserves its melanoma suppressive activities. Loss of ARF activity can result in uncontrolled MDM2 degradation of p53, which, in turn, can lead to loss of cell cycle control and compromised tumor suppression.

popularity of sun tanning and the increase in migration or travel of fair-skinned individuals to sun-intense locations (3). On the other hand, recent decline in melanoma incidence in Australia has been attributed to an elevated level of awareness of the dangers of excessive sun exposure (4). Genetic determinants of melanoma risk also include pigmentation patterns of the hair and skin and multiple benign or dysplastic nevi (moles). However, our current understanding of the genetic basis for these phenotypes and the mechanisms through which their underlying genetic lesions can contribute to the genesis of melanocytic tumors remains incomplete.

At present, early detection and prevention are the only effective tools for combating melanoma. With the advent of new technologies and the development of model systems, our insights into the mechanisms of melanoma formation are expanding, along with the potential for developing new and more effective therapies. Genomic investigations are highlighting lesions in melanoma-relevant genes, the functional significance of which is being validated in refined mouse models. These advances will help identify early disease markers and rational targets for drug and biomarker discovery efforts, which should improve drastically the detection and treatment of this disease.

3. GENETICS PREDISPOSITION TO MELANOMA

Key genetic lesions that underlie familial melanoma, which represents approximately 8-12% of all cases (5), have been identified through traditional linkage mapping studies. These analyses have pointed to the two susceptibility genes, *CDKN2A* and *CDK4*, as potent regulators of melanoma genesis. More recently, polymorphic variants of the *pigmentation-associated melanocortin-1 receptor (MC1R)* have been found to be associated with predisposition to melanoma, particularly those variants encoding red hair, freckling, fair skin and sun sensitivity.

3.1. The CDKN2A locus

Identified for its frequent homozygous deletion in cancer cell lines of various types, the 9p21 locus was designated a melanoma susceptibility gene when it was found to be commonly altered in patients with familial melanoma (6-8). The *CDKN2A* locus is unique in that it encodes the two distinct cancer suppressor loci $p16^{INK4A}$ and *ARF* (also referred to as *p14* in humans and *p19* in rodents) (9,10). Both products of the *CDKN2A* locus are potent inhibitors of melanomagenesis, and their loss predisposes to the development of the disease (Figure 1). *INK4A*, which is the founding member of the inhibitor of cyclin-dependent kinase protein family, prevents CDK4/6-mediated phosphorylation and subsequent inactivation of the *RB* tumor suppressor and results in G1 cell cycle arrest (11,12). The product of the alternative reading-frame of the 9p21 locus is *ARF*, which protects against melanoma by inhibiting MDM2-mediated ubiquitination and subsequent degradation of p53 (10,13-16).

3.2. INK4A

Inherited mutation in the *CDKN2A* locus in melanoma-prone families ranges from 25-40% (17,18). Evidence that INK4A is a melanoma suppressor comes from the presence of mutations that target exon 1α, which is the *INK4A*-specific exon within the locus in the germline of melanoma-prone individuals. Recent associations have been found between melanoma incidence and polymorphisms in the 5' and 3' untranslated regions of *CDKN2A*, suggesting that the familial predisposition alleles should be expanded to include mutations that affect the translation efficacy and those that could modulate message stability (19,20).

3.3. CDK4

Germline and acquired sporadic *CDK4* mutations that affect INK4A binding have been described in melanoma patients, reinforcing the importance of *CDKN2A* locus in suppressing melanoma. The most prevalent of these mutant alleles targets CDK4 Arg24, a residue that is conserved between CDK4 and CDK6 (Arg24Cys or Arg24His) and is known to be crucial for binding to INK4A (21-25). As expected, these *CDK4* mutations are

epistatic to *INK4A* loss, supporting the importance of INK4A in the regulation of CDK4/6 activity and, subsequently, inhibiting melanoma development. In accordance, the manifestations of germline *INK4A* and *CDK4* mutations are clinically indistinguishable, with a similar mean age of melanoma onset and number of melanocytic lesions (26). Finally, mice expressing the Arg24Cys mutant form of Cdk4 are susceptible to carcinogen-induced melanoma (27). Interestingly, no Cdkn2a loss or *Trp53* mutations were detected in melanomas arising in this model, suggesting that the Arf-p53 pathway is intact in these tumors and that defective Ink4a-Cdk4-Rb function is specifically driving melanoma genesis in these mice (27).

3.4. ARF

The finding that *CDKN2A* mutations frequently target both *INK4A* and *ARF*, that ARF plays an important regulatory role of p53, and that p53 mutation is rare in melanoma suggest the possibility that ARF may possess melanoma-suppressing properties (28). In support of this notion, somatic mutations that are specific to ARF-coding sequences in the shared exon 2 of *CDKN2A*, and insertions and deletions in *ARF* exon 1β have been reported in two metastatic human melanomas (29-31). Inherited susceptibility to melanoma has also been documented in two patients with germline *ARF* mutations −the first was a 14-kb deletion in exon 1β that spared both *INK4A* and *INK4B* genes, consistent with an *ARF*-specific event, and the second was a 16-bp insertion in exon 1β that generated a frame-shifted ARF mutant defective in cell cycle arrest (32,33). However, a lack of demonstration of intact *INK4A* activity in these lesions precludes designation of *ARF* as a *bona fide* melanoma suppressor. Although partial or total deletion of exon 2 clearly result in abrogation of both p16 and ARF function, there is some evidence that point mutagenesis of exon 2 may still be preferentially targeting the p16 transcript. A recent survey of reported exon 2 point mutations (both germline and somatic) demonstrates a higher proportion of synonymous changes and a lower percentage of nonsense mutations in the ARF reading frame when compared to the p16 reading frame (34). *In silico* modeling of sequence variants that yield missense mutations in both frames further suggests that the impact of the codon change is more severe for p16 than ARF (34). Thus, although ARF clearly exhibits genetic and biochemical evidence of tumor suppressive function, unlike p16, its precise contribution to human melanoma pathogenesis remains to be clarified.

The advent of mouse models to study the tumor suppressive activities of the *Cdkn2a* locus, whose organization is evolutionarily conserved between mouse and man, provided a genetic platform upon which to assess the contributions of Ink4a and Arf to melanoma suppression (10,35-39).

4. MOUSE MODELS FOR GENETIC DISSECTION OF CDKN2A

To dissect the melanoma suppressive capabilities of the *CDKN2A* locus, exons 2 and 3 of murine *Cdkn2a* were deleted using a conventional gene-targeting approach (40). This allele lacked both *Ink4a* and *Arf* and exhibited a high incidence of fibrosarcoma and lymphoma (40). When combined with a constitutively active *HRAS* mutation in the melanocytic lineage (*Tyr-RAS*), *Ink4a/Arf*-null animals were highly prone to cutaneous melanoma, with a short latency and high penetrance (41). Interestingly, melanomas arising in *Tyr-RAS/Cdkn2a*$^{+/-}$ showed a loss of heterozygosity (LOH) pattern of the wild-type allele that always included exon 2, pointing to the need for elimination of both Ink4a and Arf in mouse melanoma development (41).

The role of the p53 pathway in protecting against human melanoma development has remained controversial. In fact, very few mutations or deletions of *TP53* have been reported in human melanocytic tumors (42). Early mouse modeling studies using the SV40 T antigens gave rise to a highly aggressive melanoma phenotype, pointing to a potentially important role for the p53 pathway in melanoma formation (43). Consistent with these observations, later studies on *Tyr-RAS/Trp53*$^{+/-}$ mice also yielded a cutaneous melanoma phenotype (44). Importantly, melanomas arising in the *Tyr-RAS/Trp53*$^{+/-}$ model were associated with LOH for the wild-type *Trp53* allele and retention of *Arf*, which provided direct evidence of the anti-melanoma capabilities of the p53 pathway (42,44). In contrast to melanomas from *Tyr-RAS/Arf*$^{-/-}$ mice, in which Ink4a was lost in approximately 50% of the cases (discussed below), melanomas arising in *Tyr-RAS/Trp53*$^{-/-}$ mice always retained *Ink4a* activity (44). Interestingly, the Rb pathway in *Ink4a*-competent melanomas was frequently inactivated by amplification and overexpression of *Myc*, which can act to relieve the G1 cell cycle block by acting in parallel and downstream of Ink4a (44-46). As in the case of mouse tumors, human melanomas exhibit an increase in *MYC* copy number coupled with overexpression of the oncogene (47,48). Although the basis for the discrepancy in preference for Rb-pathway inactivation between *Arf*- and *Trp53*-null mice is currently unknown, it is clear that there exists cooperation between *Myc* overexpression and *Arf* loss in some murine systems, and that inactivation of *Ink4a* can cooperate with *p53* loss (49,50). Although additional investigations are warranted to dissect the precise roles of these genetic combinations, it is evident that both the RB and p53 pathways play key roles in melanoma suppression.

To address more fully the contributions of the *Cdkn2a* locus to melanoma prevention, *Ink4a*- and *Arf*-specific knockouts have been generated. Surprisingly, mice that were deficient for *Ink4a* but carried a wild-type *Arf* allele exhibited weak spontaneous and DMBA (7,12-dimethylbenzanthracene) carcinogen-induced melanoma susceptibility phenotypes (50-52). Melanoma genesis in *Ink4a*-deficient animals was greatly enhanced in the setting of *Arf* haploinsufficieny, suggesting that *Arf* does, indeed, contribute in *Cdkn2a* melanoma suppression (53). When combined with the *Tyr-RAS* transgene, either the *Ink4a*- or *Arf*-knockout facilitated melanoma formation (53). It is noteworthy that the Rb-pathway was frequently targeted in *Tyr-RAS/Arf*$^{-/-}$ mice, while the p53-pathway was compromised in melanomas from *Tyr-RAS/Ink4a*$^{-/-}$

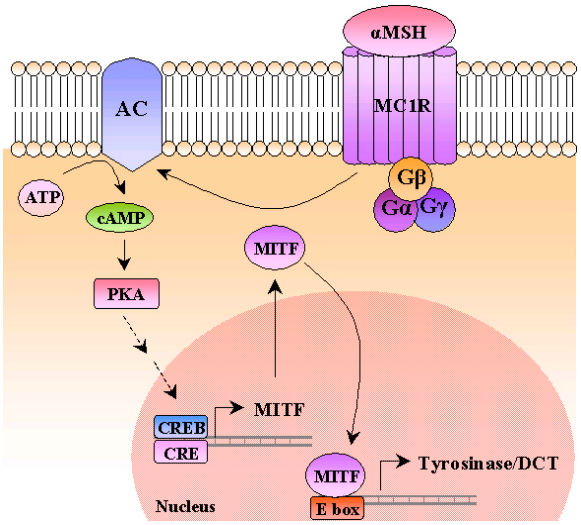

Figure 2. The melanocortin signaling pathway. Melanocytic growth is stimulated by the ligand αMSH, which binds to and activates the G protein-coupled (GPCR) melanocortin-1 receptor MC1R. The G_s family of G proteins (including Gα, Gβ and Gγ) transmits signals from MC1R to adenylyl cyclase (AC), which, in turn, catalyzes the conversion of cytoplasmic ATP to cAMP. Increased levels of cAMP act as a second messenger to activate protein-kinase A (PKA), which, upon activation, translocates to the nucleus where it phosphorylates the CREB (cAMP-responsive-element binding protein) family of transcription factors). Phosphorylated CREBs then induce the expression of genes containing CRE (cAMP-responsive elements) consensus sequences in their promoters, such as the helix-loop-helix transcription factor MITF (64). The transcription factor MITF, which is important for melanocyte differentiation and survival binds to promoters harboring E box consensus sequences (CANNTG), such as those present in the promoters of the pigmentary genes tyrosinase TRP-1 (tyrosinase-related protein-1), and DCT (dopachrome tautomerase), which is also referred to as TRP2 (64).

animals (53). These striking genetic signatures point to both *Ink4a* and *Arf* as two potent suppressors of melanoma *in vivo*, and provide a potential explanation for the need for concomitant regulation of both genes and the evolutionary conservation of the *Cdkn2a* locus.

Taken together, data from *in vivo* mouse models clearly implicate ARF and, thus, the p53 pathway, as a *bona fide* melanoma-suppressing network. Further studies are required to explain the apparent low frequency of *TP53* mutations in human melanomas, in view of the recurrent and early loss of *CDKN2A* and the close association between ARF and p53 in melanoma pathogenesis.

5. MC1R AND THE RHC PHENOTYPE

The 'red hair color' (RHC) phenotype is known to be an independent risk factor for all skin cancers, including melanoma, and is characterized by pigmentary traits that include red hair, fair complexion, inability to tan, and freckling. The *MC1R* gene encodes a seven-transmembrane G-protein-coupled receptor that is expressed in epidermal melanocytes. Upon stimulation by its ligand, *melanocyte-stimulating factor (MSH)*, the MC1R receptor acts as a key determinant of the pigmentary characteristics of the melanocytic lineage (Figure 2). Allelic polymorphisms within the *MC1R* gene underlie, in many instances, pigmentary variation and the human skin phototype (54,55). The most common three MC1R variants −Arg151Cys, Arg160Trp and Asp294His, R151C, R160W and D294H, respectively− are associated with the RHC phenotype (56-61). Carriers of one of the RHC-linked *MC1R* polymorphisms exhibit a reduced epidermal response to UV damage, and, therefore, may be at an increased risk of developing melanoma (58,59,61,61).

The association between *MC1R* polymorphisms and pigmentary traits relates to imbalances in MSH-MC1R regulation of melanin synthesis (63,64). Certain *MC1R* variants shift the pheomelanin-eumelanin balance, favoring the accumulation of the former compound in the skin. Increased pheomelanin can contribute to melanoma pathogenesis by virtue of its diminished UV protective capacity and its mutagenic and toxic properties (65). In accordance with this hypothesis, *in vitro* cell culture studies have demonstrated that *MC1R*-variant human melanocytes are hypersensitive to UV-light exposure and its associated cytotoxic effects (66). *MC1R* polymorphisms are also linked to increased melanoma risk in carriers with dark/olive complexions, as these variants are known for their ability to modify the penetrance of *CDKN2A* mutations (59,61,62,67,68). In a study conducted by Hayward and colleagues on 15 Australian families with a history of melanoma, a single *MC1R* polymorphism was associated with a substantial increase in the penetrance of *CDKN2A* mutations from 50% to 84% and reduced the mean age of melanoma onset from 58 to 37 years (61). Consistent with these observations, an independent study on Dutch families reported an increase in *CDKN2A* melanoma penetrance from 18% to 35% and 55% in patients with one or two *MC1R* variants, respectively (68). From these two studies, it is evident that common RHC MC1R variants can act as modulators of melanoma susceptibility in *CDKN2A* mutation carriers, as can additional modifiers of disease risk (69). Ongoing and future studies should help elucidate the genetic and biochemical bases for the interaction between *MC1R* and other modifiers of melanoma risk in disease pathogenesis.

6. SOMATIC MUTATIONS IN MELANOMA

Mutations of the *CDKN2A* and *CDK4* genes underlie predisposition to melanoma in only a fraction of familial and sporadic cases, pointing to the presence of additional genes that are implicated in disease pathogenesis. Molecular studies in human melanomas and derivative cell lines have uncovered a host of melanoma genes and related pathways, and the melanoma-relevance of a subset of these genetic networks has been validated on a functional level. With the advent of technologies that allow for high-resolution scanning of the melanoma

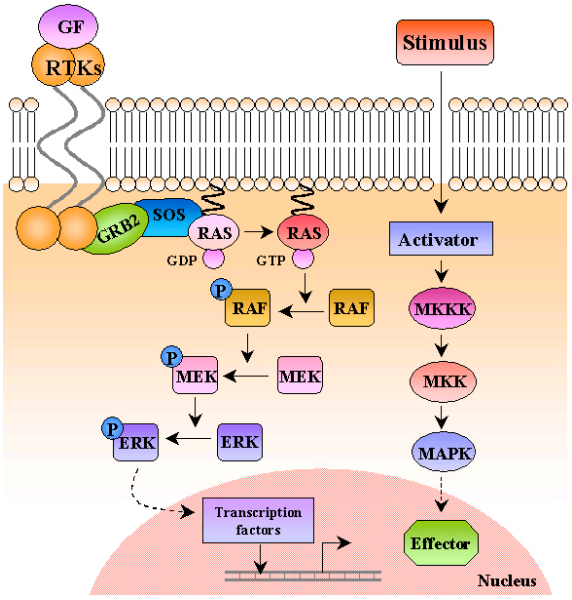

Figure 3. The MAPK-signaling pathway. Mitogen-activated protein kinases (MAPKs) relay a wide range of extracellular signals in response to a host of environmental stimuli (70). At minimum, four distinct MAPK signaling modules exist in mammalian cells. Shown here is a simplified MAPK signaling cascade with the RAS-RAF-MEK-ERK pathway. Binding of growth factors (HGF/SF, for example) to their respective receptor tyrosine kinase receptor (RTKs), such as MET, triggers receptor dimerization and subsequent autophosphorylation of tyrosine residues present on the internal portion of the receptors. Tyrosine phosphorylation activates RTKs and allows them to bind sequence homology 2 (SH2) domains of proteins, such as GRB2. This complex then brings the cytosolic protein son of sevenless (SOS) into close proximity of RAS (HRAS, NRAS or KRAS) on the plasma membrane, and catalyzes the conversion of the inactive GDP-bound RAS into active GTP-bound RAS. Active RAS initiates the signaling cascade by phosphorylating RAF MKKK (cRAF1, ARAF or BRAF), which, in turn, phosphorylates MEK MKKs (MEK1 and MEK2). Activated MEKs phosphorylate the extracellular signal-regulated kinases ERK MAPKs (ERK1 and ERK2), which then translocate into the nucleus and phosphorylate transcription factors or other specific substrates. In addition to the classical RTK dimerization paradigm, GPCR signaling to ERK can be achieved in response to extracellular stimuli through mechanisms that are not fully understood at present (79).

genome, it is, however, increasingly clear that numerous DNA copy number alterations found in human melanomas do not harbor known melanoma targets, and, thus, may contain novel melanoma genes that have yet to be identified.

6.1. The MAPK cascade

The mitogen-activated protein kinase (MAPK) pathway regulates a broad range of cellular responses to cues from the environment (70). MAPK extracellular-signal-regulated kinases 1 and 2 (ERK1 and ERK2) respond to diverse extracellular signals. The best-characterized signaling cascade begins with ligand-induced activation of a receptor tyrosine kinase (RTK), which in turn activates the RAS family of proto-oncogenes (NRAS, HRAS and KRAS). RAS activates the RAF serine/threonine kinases (c-RAF1, BRAF and ARAF). RAF kinases (MAPK kinase kinases (MKKK)) then phosphorylate the MAPK kinase MEK (MKK), which subsequently phosphorylates and activates ERK1 and ERK2 (Figure 3). Although many permutations of MKKK-MKK-MAPK components would appear possible, scaffolding proteins such as KSR have been suggested to confer specificity to a particular cell context (71). Additionally, this pathway is tightly regulated by several phosphatases (e.g. MKP3) whose importance in cancer biology in just becoming appreciated.

Proliferation, differentiation and survival of the melanocytic compartment is controlled in an intricate manner that requires paracrine stimulation from both GPCRs, such as endothelin-1/3 and α-melanocyte stimulating hormone (α-MSH), and RTKs, like basic fibroblast growth factor receptor (bFGFR) and hepatocyte growth factor receptor c-MET (72-76). It is also noteworthy that *Dsk5*, which represents a gain-of-function allele of the *epidermal growth factor receptor* (*Egfr*), has been found to confer an increase in epidermal melanocyte number and a concomitant hyper-pigmentation phenotype in the mouse (77). Also interesting is the fact that ERK activation has been connected to GPCR signaling through cross-talk mechanisms that are still poorly defined, placing the MAP kinase pathway at the intersection between GPCR and RTK signaling ((78,79). Given the potential cross talk between these two pathways, it is conceivable that activating mutations in *RAS*, *RAF* or other MAPK components could stimulate both GPCR and RTK signaling cascades, resulting in loss of extracellular growth-factor regulation. This might help explain the preponderance of MAPK activating mutations in many cancer types, including malignant melanoma.

6.1.1. RAS

Consistent with the frequent involvement of MAPK signaling in tumorigenesis, recent studies have reported gain-of-function *NRAS* point mutations in up to 56% of congenital nevi, 33% of primary melanomas and 26% of metastatic melanocytic tumors ((80,81). However, *NRAS* mutations have rarely been detected in dysplastic nevi, which may point to two distinct pathways of melanomagenesis; one originating from benign melanocytic lesions and the other stemming from dysplastic nevi (82-84). Interestingly, *NRAS* mutations have been reported in nodular melanomas and have been correlated with sun exposure (82,85). On the genomic level, Bastian and colleagues have reported a chromosome 11p amplification in Spitz nevi that encompasses the *HRAS* locus, and, interestingly, found that this amplified allele harbored an activating *HRAS* mutation (86). In line with the phenotype associated with activating *NRAS* mutations in humans, gain-of-function *HRAS* mutations in the melanocytic lineage of transgenic mouse leads to aberrant

proliferation and transformation, a phenotype that is exacerbated by the addition of *Cdkn2a* or *Trp53* inactivating mutations (41,44,87). Recent evidence, however, points to clear differences between *HRAS* and *NRAS* oncogenesis. Whereas transgenic *HRAS* expression results in non-metastatic melanoma, expression of dominant-active *NRas* in Ink4a-deficient mice gives rise to metastasizing melanocytic tumors (41,88). In summary, the genetic data from both human and mouse studies highlight an important role for RAS-signaling in melanoma genesis.

6.1.2. BRAF

Activating mutations in a gene encoding another member of the MAPK signaling cascade, *BRAF*, have been identified in approximately 60% of human primary melanomas and cell lines (89). The vast majority of these *BRAF* mutations target the kinase domain, with 80% of them resulting in a single substitution (V600E) that confers constitutive *BRAF* activation (90). Significantly, BRAFV600E and activating NRAS mutations have been found to be mutually exclusive, supporting the notion that these alleles are functionally redundant in their melanoma promoting properties (89,91). In recent years, *BRAF* mutations have been detected in human nevi of both the benign and dysplastic varieties, which suggests that ERK activation may be an early event in melanoma progression (92,93). Notably, a study by Kumar *et al.* documented an inverse association in sporadic primary melanomas between activating *BRAF* and *NRAS* mutations and allelic loss on chromosome 9 (90). However, the presence of *BRAF* mutations in both benign and malignant lesions supports the need for additional cooperating genetic events to drive a full-fledged melanoma phenotype. More recently, Chudnovsky and colleagues demonstrated that Ras but not BRaf increased melanocytic neoplasia, which was attributable, in part, to the fact that RAS can activate PI3K while BRAF cannot (94). Interestingly, *BRAF* mutations were found to be significantly more common in melanomas occurring on skin subjected to intermittent sun exposure than that exposed to chronic UV (95). Furthermore, the mutated *BRAF* allele was often elevated in copy number, suggesting that *BRAF* is selected for in a subset of melanomas and pointing to the potential existence of distinct pathways of melanomagenesis (95). Ongoing studies should shed light on the genetic lesions that can cooperate with activating *BRAF* mutations in full transformation of melanocytes and on the role of BRAF in melanoma initiation and progression. The role of BRAF in melanocytic proliferation has been reviewed previously (96,97).

6.2. HGF/SF-MET signaling

The HGF/SF ligand binds to and activates tyrosine-kinase receptor c-MET, which is present on the surface of epithelial and melanocytic cells (98). Although HGF/SF-MET signaling normally acts in a paracrine manner, autocrine activation of this signaling pathway has been noted in various tumor types as well as in melanoma (99,100). Not only does HGF/SF stimulate the proliferation and motility of human melanocytes in culture, but it also downregulates E-cadherin and desmoglein-1, resulting in a disruption of normal contact between melanocytes and keratinocytes and a cell scattering phenotype (100,101). Indeed, HGF/SF-MET activation has been linked to melanoma progression, as increased c-MET expression is characteristic of metastatic lesions and gain of the 7q33-qter region that harbors c-MET is detected in late stages of melanoma development (102-104). The melanomagenic effects of HGF/SF-MET signaling appear to be conserved between humans and mice, since studies of murine melanoma cells in explant models have demonstrated Met tyrosine-kinase activity yields a metastatic phenotype (105).

The most convincing evidence to support a role for abnormal HGF/SF-MET signaling in cancer progression comes from the observation that mice ubiquitously expressing a constitutively active HGF/SF transgene develop a variety of tumors, including cutaneous melanoma (106). Despite the relatively low incidence and long latency of melanoma in the transgenic HGF/SF model, it was found that 20% of mice presenting with melanoma develop metastatic lesions, presumably by acquiring autocrine c-Met activation (106). On the other hand, mice that carry a tissue-specific *HRAS* transgene on a *Cdkn2a*$^{-/-}$ background exhibit a high penetrance of melanoma with a very short latency but do not show any evidence of metastases, pointing to a role for MET signaling in melanoma progression (41).

6.3. PTEN and melanoma

LOH and chromosomal studies have revealed deletions and rearrangements that target 10q24-26 in many types of tumors, including melanoma. Approximately 30-50% of human melanomas exhibit 10q LOH that encompasses both the MYC antagonist MXI1 and the tumor suppressor PTEN coding regions (107). PTEN is a well-studied tumor suppressor that negatively regulates the phosphatidylinositol 3-kinase (PI3K)-AKT pathway that relays cell proliferation and survival signals (108). Originally identified as a tumor suppressor by virtue of its homozygous deletion in breast cancers and gliomas, *PTEN* LOH or mutation have also been detected in 5-15% of primary and metastatic melanomas and in 30-40% of melanoma cell lines (109-113). Functionally, ectopic expression of PTEN was found to suppress the growth, tumorigenesis and metastasis of *PTEN*-deficient melanoma cell lines, establishing PTEN as a potent inhibitor of melanoma progression (114).

Inherited PTEN mutations in humans have been linked to three related cancer predisposition syndromes: Cowden Disease, Lhermitte-Duclos Disease and Bannayan-Zonana Syndrome (116-118). In mice, *Pten*-nullizygosity confers embryonic lethality in the mouse, while heterozygous animals exhibit an adult-onset susceptibility phenotype to a wide range of tumor types that is *Pten*-allele- and genetic background-dependent (119-122). Although not observed in *Pten*-heterozygous mice, cutaneous melanomas have been reported, albeit at low incidence, in *Pten*$^{+/-}$/*Cdkn2a*$^{+/-}$ animals, which is suggestive of collaborative tumor suppression in melanoma (122).

Given that only 5-15% of human melanomas display *PTEN* mutations or allelic loss while 10q24 loss is evident in 30-50% of melanocytic tumors, and that reintroduction of *PTEN* into these tumor lines does not suppress cell growth, it is likely that other melanoma suppressors exist within the chromosome 10 locus (114). The prime candidate for the role of melanoma suppressor in the 10q24 deletion region is the MYC antagonist *MXI1*. Supporting evidence for a tumor suppressive role for MXI1 in melanoma development comes from the observations that *Myc* is amplified and overexpressed in RAS-driven melanomas from *Trp53*-null mice (44), and that *Mxi1*-deficient mice display a cancer-predisposition phenotype (123). However, the role of Mxi1 in protecting against melanoma in mice remains largely enigmatic and requires a more detailed examination. It is worth noting that a recent study has revealed high levels of phospho-AKT in 17% of normal nevi, 43% of dysplastic melanocytic lesions, 49% of primary melanomas and 77% of metastatic tumors (124). Therefore, it appears that loss of PTEN tumor suppression may be a more common phenomenon in melanoma than previously thought.

7. GENE-ENVIRONMENT INTERACTION IN MELANOMA

A clear link has long been established between sun and, consequently, UV exposure and skin cancer risk, including that of developing melanoma. Largely based on migration studies, it has been postulated that sun-induced damage during childhood confers a high risk of developing melanoma later in life (2,125-127). The link between sun exposure and melanoma risk is bolstered further by the fact that UV light can induce melanocytic lesions and melanoma in human skin grafts (128,129) and in mice (discussed below).

A significant number of investigations have focused on defining the molecular underpinnings of UV cancer promotion, and several mechanisms have been proposed, including a role of UV as a mutagen and mitogen, a promoter of oncogenic paracrine components and an inhibitor of immune surveillance (3, 130,131). In search of molecular targets of UV mutagenesis, studies have focused on identifying UV signature mutations in melanoma-relevant genes, such as C→T or CC→TT substitutions that result from the repair of UVB-induced damage of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts. *INK4A* emerged as a possible target of UV-induced mutations after the detection of C→T substitutions in the *CDKN2A* locus in human melanomas (132-134). However, the fact that similar mutations have also been observed in human gliomas, which are not linked to UV exposure, undermines the UV-*CDKN2A* connection (135). Perhaps other manifestations of UV radiation could contribute to the genesis of melanoma, such as its ability to induce crosslinks between proteins and DNA, oxidative stress and DNA single-strand breaks, chromosomal deletions associated with double-stranded breaks and altered methylation patterning that can result in epigenetic changes in gene regulation (136-138). Although our knowledge of the mechanisms of UV-mediated carcinogenesis is rudimentary at present, studies on genetically engineered mice is providing valuable insights into the melanomagenic potential of ultraviolet light.

Using *Hgf/Sf* transgenic mice, Merlino and colleagues found that a single mild episode of sunburn in neonatal animals increased dramatically the risk of developing melanoma (139). Interestingly, chronic exposure of adult *Hgf/Sf* transgenic mice to UV light resulted in other skin cancers but not melanoma (140). Furthermore, UV exposure dramatically accelerated the onset and increased the penetrance of melanoma in *Hgf/Sf*$^{+}$*/Cdkn2a*$^{+/-}$ mice (141). To examine the possibility that one or both of the *Cdkn2a* locus products might be targets of UV, *Tyr-RAS* mice neonates that were deficient for either *Ink4a* or *Arf* were treated with a single erythrogenic dose of ultraviolet radiation (142). Only the *Tyr-RAS/Arf*$^{-/-}$ animals developed melanoma, and, surprisingly, melanomas arising in the UV-exposed mice acquired Rb-pathway lesions that were different from those observed in unexposed *Tyr-RAS/Arf*$^{-/-}$ littermates (142). Although *Ink4a* mutations were observed in both spontaneous and UV-promoted melanomas at incidences of 50% and 25%, respectively, *Cdk6* amplification was observed only in UV-induced tumors at a frequency of 50% (142). Strikingly, loss of *Ink4a* and gain of *Cdk6* were mutually exclusive events in these UV-induced melanomas (142). In a recent study, *CDK6* amplification was found to be functionally important in driving melanomagenesis in human cells, as siRNA knockdown of the CDK6 transcript lead to a reduction in melanoma cell growth (143). These findings suggest that Rb pathway components are critical targets of melanomagenesis in the UV-induced *Tyr-RAS/Arf*$^{-/-}$ mouse model (142).

Much in line with findings from the *Tyr-RAS/Arf*$^{-/-}$ model, recent studies have revealed a cooperative effect between UV exposure and *INK4A* mutation in human populations (144). In a study that included 402 melanoma patients and 713 unaffected close relatives from 80 families carrying known *INK4A* mutations, the authors assessed the penetrance of heterozygous *INK4A* mutations as well as potential environmental contributions to melanomagenesis in these individuals (144). The overall penetrance of melanoma by age 80 was found to be 67%, and, whereas only 58% of individuals of European origin developed the disease, 76% from the United States and an astounding 91% from Australia presented with melanoma (144). These observations clearly indicate that the penetrance of *INK4A* mutation is greatly influenced by geographical location, with the highest incidence of melanoma being in mutation carriers from regions of more intense UV exposure. Given the fact that UV light targets the Rb pathway in mice through inactivation of *Ink4a* or amplification of *Cdk6*, it is not surprising that *INK4A* mutation carriers living in locations of high UV exposure (Australia) would be more susceptible to melanoma then those from low UV exposure regions (Europe). Since almost all *CDKN2a* mutant humans are heterozygous, UV exposure may facilitate loss of the WT allele. It is also possible that the propensity to amplify *Cdk6* in mice and its counterpart in humans may be different.

Factors other than geographical location and *INK4A* status can also contribute to melanoma risk. For example, gene-environment interaction between *MC1R* and UV that have yet to be fully elucidated, as well as sun protection and tanning practices that are likely to contribute to melanoma risk in *INK4A* mutation carriers and non-carriers alike.

8. CANDIDATE TARGETS FOR MELANOMA THERAPIES

Traditionally, systemic chemotherapeutic or radiotherapeutic regimens have served, in many cases, as the only treatment options for cancer patients. The recent successes of the tyrosine-kinase inihibitor imatinib (Gleevec) in the treatment of gastrointestinal stromal tumors and chronic myelogenous leukaemia (CML) and the tyrosine kinase inhibitor gefitinib (Iressa), which targets the epidermal growth factor receptor in NSCLC harboring EGFR mutation (145-148) have demonstrated the power of targeted therapies. Genomic technologies and emerging proteomic tools are identifying many potential candidate targets for therapeutic development. In this respect, one of the biggest challenges will be to select those targets that are most essential for tumor maintenance. Another lesson learned from imatinib and gefitinib treatment of patients is the issue of acquired tumor drug resistance, pointing to needs for understanding drug mechanism of action as the basis for designing rational combination therapeutic regimens (149,150).

Inducible transgenic mouse models have served as an invaluable resource for studying the effects of loss of oncogene expression in established tumors, highlighting the requirement for persistent expression of certain oncogenes for tumor maintenance (151). Using such a model, a constitutively active *RAS* allele was found to be required not only for melanoma initiation, but also for tumor preservation (152). Persistent oncogenic *RAS* expression was found to play an essential role in melanoma maintenance by regulating tumor angiogenesis through vascular endothelial growth factor-dependent and -independent mechanisms (152). Given its position immediately downstream of RAS, it would not be surprising if BRAF activity were to also be required for melanoma maintenance. It is noteworthy, however, that RAS signals through several signaling cascades, only one of which is the RAF-ERK pathway. In addition, functional evaluation of the role of BRAF in tumor initiation and maintenance in melanoma models and in a clinical context has yet to be accomplished.

As in the case of many cancers, melanoma arises as a consequence of aberrant communications between tumor cells and the microenvironment milieu. Melanoma cells typically downregulate E-cadherin expression, which disrupts normal melanocytes-keratinocyte adhesion and allows tumors to escape into the dermis and to evade negative growth signals from keratinocytes (153). In the context of a three-dimensional skin reconstruction system, ectopic expression of E-cadherin in melanoma cells restored adhesion with keratinocytes and suppressed melanomagenesis (154). Not only can melanoma cells escape negative growth signal from the surrounding microenvironment, but they can also elicit a paracrine response that stimulates stromal cells to produce growth factors. For example, melanoma cells often expression high levels of c-Met, which drives the production of HGF in dermal fibroblasts and provides the tumor cell with the necessary proliferative signals (100). Furthermore, numerous studies have shown that melanomagenesis is also accompanied by recruitment of endothelial cells in support of angiogenesis (155). The interdependency of melanoma cells on positive growth signals from the microenvironment and tissue vasculature provides a unique opportunity for the development of therapeutic compounds that target these tumor-supporting networks. Stromal and endothelial melanoma-supporting cells may provide better therapeutic targets, since their relative genomic stability would impede their acquisition of drug resistance.

Chromosomal instability is a major driving force behind genomic alteration that can endow a tumor with important growth advantages. Consistent with this hypothesis, melanomagenesis is accompanied by the accumulation of recurrent genomic gains and losses that are believed to contribute to disease progression (156). In fact, successful classification of melanoma has been accomplished through the use of array-based comparative genomic hybridization (aCGH), highlighting a genotype-phenotype correlation between DNA copy number and melanoma subtype (157). Interestingly, spontaneous and carcinogen-induced melanomas that arise in mouse models and derivative cell lines share some of the genomic patterns of gain and loss that are characteristic of human melanocytic tumors (158,159). The evolutionary conservation of these lesions is suggestive of their potential functional significance in driving melanomagenesis in both mice and humans. Using a high-resolution aCGH platform, we have initiated a large-scale analysis of benign nevi, primary and metastatic melanomas (160). We have observed a dramatic increase in the number of recurrent genomic gains and losses during the transition from primary to distal metastatic melanoma (unpublished observations). Although our analyses have rediscovered amplicons and deletions that target genes previously implicated in melanomagenesis, a large number of focal and recurrent events do not harbor known cancer genes (unpublished observations). The targets of a subset of these novel DNA copy number alterations will likely prove to be melanomagenic and will serve as potential targets for future drug discovery efforts. The current standing and future direction in the melanoma therapeutics field has been eloquently reviewed in several recent articles (97,161,162).

9. CHALLENGES AND OPPORTUNITIES

There is no doubt that substantial progress has been made in dissecting genetic networks and key gene-environment interactions that drive melanomagenesis. One of the major challenges will be to translate the enormous body of knowledge that has been acquired into effective therapeutic options for melanoma patients. A good example of how this is being accomplished is a RAF

inhibitor that is presently in clinical trials. Although activated BRAF represents a logical target for drug discovery efforts, its role in melanomagenesis and, possibly, in tumor maintenance have yet to be fully elucidated. The development of a regulatable BRAF model, analogous to the inducible HRAS mouse system, would provide the scientific community with an invaluable tool for defining the role of RAF in melanoma development and tumor maintenance. Furthermore, such an inducible BRAF model would serve as an important tool in understanding mechanisms of tumor escape that would be anticipated if RAF inhibitors were to make their way to the clinic. Finally, the use of pre-clinical models will facilitate the identification of cooperating events in melanoma development, which should help guide combination therapy initiatives, and will serve as a platform for testing the efficacy and safety of new melanoma drugs. In addition to activating *BRAF* mutations, many genetic lesions that are likely to contribute to melanoma progression have yet to be discovered. The rapidly evolving genomic technologies will likely facilitate the identification of melanoma-relevant genes, and, therefore, it will be necessary to assess their functional contributions in tumor progression and maintenance as a first step towards selecting those that would be the most suitable drug targets.

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