The molecular pathology of cutaneous melanoma

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Abstract. Cutaneous melanoma is a highly aggressive cancer with still limited, but increasingly efficacious, standard treatment options. Recent preclinical and clinical findings support the notion that cutaneous melanoma is not one malignant disorder but rather a family of distinct molecular diseases. Incorporation of genetic signatures into the conventional histopathological classification of melanoma already has great implications for the management of cutaneous melanoma. Herein, we review our rapidly growing understanding of the molecular biology of cutaneous melanoma, including the pathogenic roles of the mitogen-associated protein kinase (MAPK) pathway, the phosphatidylinositol 3 kinase [PI3K]/phosphatase and tensin homologue deleted on chromosome 10 [PTEN]/Akt/mammalian target of rapamycin [mTOR] pathway, MET (hepatocyte growth factor), Notch signaling, and other key molecules regulating cell cycle progression and apoptosis. The mutation Val600Glu in the BRAF oncogene (designated BRAF(V600E)) has been associated with clinical benefit from agents that inhibit BRAF(V600E) or MEK (a kinase in the MAPK pathway). Cutaneous melanomas arising from mucosal, acral, chronically sun-damaged surfaces sometimes have oncogenic mutations in KIT, against which several inhibitors have shown clinical efficacy. These findings suggest that prospective genotyping of patients with melanoma, combined with the growing availability of targeted agents, which can be used to rationally exploit these findings, should be used increasingly as we work to develop new and more effective treatments for this devastating disease.

Keywords: Melanoma, cell cycle, pRb pathway, p16INK4A, p14ARF, cyclins, CDKs, Apaf1, Bcl2, MAPK pathway, Ras, BRAF, PTEN, PI3K/AKT pathway, SCF, ckit, HGF, cmet, cmyc, Notch

1. Introduction

As the treatment of metastatic melanoma moves rapidly into the era of targeted therapy, there is an ever growing need to untangle the underlying genetic complexity and cellular signalling heterogeneity of this highly malignant tumor. It is widely hoped that an understanding of how genetic and signalling profiles dictate pharmacologic response will allow for the selection of optimal patient sub-populations for clinical trials.

The molecular pathology of melanoma development and progression is now being deciphered (Fig. 1). Although we have an increasingly good understanding of the genetics and biochemistry of these events, the mechanisms underlying their combinatorial interactions are still poorly defined and need to be fathomed to ultimately lead to more successful therapeutic strategies. The goal of this chapter, therefore, is to highlight some of the issues that have arisen. For the purpose of this chapter we will focus on the molecular events occurring within the melanoma cell, especially those pathways which have been the scope of intensive research over the past decade and which are now targets of an increasing armamentarium of novel therapeutic approaches. Here, we discuss recent findings and hypotheses on the role of growth factor pathways in the molecular pathology of melanoma. The matter of melanoma-stroma interactions is too involved to thoroughly explore here. As the literature on molecular and cellular events in melanoma development and progression is now huge and includes comprehensive reviews [1–5], we will take the liberty of a more subjective view and review molecules and pathways that now take center stage as well as discuss emerging areas and interesting questions in the melanoma field.
Fig. 1. Development of melanoma. This model implies that melanoma commonly develops and progresses in a sequence of steps from nevus lesions, which can be histologically identified in approximately 35% of cases. The model also acknowledges that melanoma may also develop directly from normal cells. The exact role of tumor-initiating or melanoma stem cells remains to be elucidated. Genetic (such as mutations and deletions of critical genes) and epigenetic (such as DNA-methylation leading to gene silencing) alterations of critical pathways as well as the acquired capability of evading apoptosis, progressively disturb normal skin homeostasis leading to melanoma development. Genetic changes are expected at the transition from common acquired (benign) nevus to dysplastic nevus and primary melanoma allowing cells to persist. During melanoma invasion and metastasis increased growth, invasion and stromal ‘landscaping’ by proteolysis occurs, leading to an increased manipulation of the microenvironment and interactions with stromal skin cells.

2. The unmet clinical need: “Melanoma is the tumor that gives cancer a bad name”

Melanoma is the deadliest form of skin cancer and one of the most aggressive of all neoplasms. The incidence of cutaneous melanoma has increased rapidly during the past several decades, and continues to do so at an alarming rate: The National Institutes of Health predicts 62,480 new cases of melanoma in the year 2009, with 8,420 of the patients succumbing to their disease [3]. The majority of these deaths are due to distant metastases from the primary site because melanoma is notorious for its propensity to metastasize. Although localized melanoma is frequently curable by surgical excision, metastatic melanoma is inherently resistant to most systemic treatments, and survival of patients with advanced disease has not improved in more than 30 years [6,7]. Melanoma is poorly responsive to cytotoxic chemotherapy, and thus the survival of patients is based on screening, early detection, and wide resection of the primary lesion. The overall survival for patients with metastatic melanoma ranges from only 4.7 to 11 months, with a median survival of 8.5 months [8].

2.1. Melanoma is characterised by the dysfunction of the epidermal melanin unit

Melanoma arises from the transformation of neural crest-derived melanocytes, the pigment cells of the skin, which reside in the basal layer of the epidermis. We now view melanoma as a complex tissue resulting from disrupted skin homeostasis, rather than focusing on the melanoma cell, and the genes within it, alone. Normal skin homeostasis is maintained by dynamic interactions between the melanocytes and their microenvironment, such as keratinocytes, fibroblasts, endothelial and immunocompetent cells, and the extracellular matrix. Similarly, during transformation and progression of melanocytes to melanoma cells, there are, however deregulated, reciprocal and conspirational interactions between the neoplastic cells and the adjacent stromal cells [9,10].
Under normal physiological conditions, melanocytes and keratinocytes form the ‘melanin unit’ in the epidermis. In this unit, melanocytes and keratinocytes are evenly aligned along the basement membrane zone at a ratio of 1.5–8. Each melanocyte extends with its dendrites into the upper layers of the epidermis, transferring pigment-containing melanosomes to approximately 35 keratinocytes in its unit via dendritic processes. The melanin contained in these melanosomes absorbs and scatters damaging ultraviolet radiation, thereby shielding the nucleic acids on the skin from damage (reviewed in [9–12]).

The undifferentiated keratinocytes of the basal layer regulate melanocyte growth, number of dendrites and expression of cell surface molecules, providing evidence that this highly ordered organizational pattern serves as the structural basis for intercellular regulation. Information is not yet available on how melanocytes and keratinocytes can maintain this lifelong balance, which is only disturbed during transformation into a melanocytic nevus (‘mole’) or a melanoma. It is now generally accepted that melanoma risk is modulated by skin pigmentation patterns, such as those linked to MC1R polymorphisms, and early exposure to ultraviolet (UV) light (reviewed in [13]).

3. Selected components of the cell cycle machinery

Recent excellent reviews summarize our current understanding of cell cycle regulation [14–16]. Mitogenic growth factors promote the entry of quiescent cells into the first gap phase (G1) and initiation of DNA synthesis (S phase) of the cell cycle (Fig. 2). G1 cyclin-dependent kinases (CDKs) serve as positive regulators. D-type cyclins (D1, D2, D3) complex with CDK4 and CDK6 to stimulate their kinase activities, which in turn cause the phosphorylation and inactivation of the retinoblastoma (Rb) tumor suppressor protein. By binding to E2F, Rb recruits histone deacetylases to the promoters of E2F-responsive genes and represses their transcription. Multiple Ras effector pathways (details of which forthcoming) appear to play an important role in the regulation of cyclin D1. Cyclin D1, in part, regulates the kinase activities of both CDK4 and CDK6. These complexes are formed in the cytoplasm and are transported into the nucleus and undergo stimulatory modifications including phosphorylation by CDK-activating kinase (CAK) to yield active holoenzymes. Further into G1, cyclin E complexes with CDK2 and causes additional phosphorylation and inactivation of Rb. With sufficient phosphorylation of Rb, E2F is released and transactivates genes required for S phase entry, including cyclins E and A. CDK inhibitors (CKIs) serve as negative regulators of the Rb pathway. CKIs are classified into two distinct families on the basis of their structural and functional characteristics. The members of the INK4 family of CKIs (p16\textsuperscript{ink4a}, p15\textsuperscript{ink4b}, p18\textsuperscript{ink4c}, and p19\textsuperscript{ink4d}) contain multiple ankyrin repeats and act as negative regulators of CDK4/6 by binding to the catalytic subunit and preventing formation of the active cyclin-CDK complex. The Cip/Kip family of CKIs (p21\textsuperscript{Cip1/Waf1}, p27\textsuperscript{Kip1}, and p57\textsuperscript{Kip2}) is more broadly acting and regulates both CDK4/6 and CDK2 activity. Each member of the family contains a characteristic motif within the amino-terminal region that enables them to bind to both cyclin and CDK subunits. The stoichiometry between CDKs and CKIs is important and determines the activity of Rb and, ultimately, the proliferative state of cells.

3.1. Alterations of the pRb pathway in melanoma

The p16-cyclin D/Cdk4-pRb pathway is deregulated in a large majority of human cancers, either through loss of p16 or pRb function or through deregulated expression of cyclin D or cdk4 (reviewed in [14–16]). Several mechanisms of inactivation of the pRb pathway have been noted in various malignant tumors and cell lines thereof. The most common known ways in which these gene products are dysregulated in neoplasms are through gene deletion (homozygous or hemizygous); inactivating mutations; epigenetic changes such as promoter methylation, transcriptional repression, protein sequestration and inactivation (e.g., viral oncoproteins); and posttranslational modifications (e.g., inactivating phosphorylation events). The p16-cyclin D/Cdk4-pRb pathway as a functional unit is frequently altered in melanoma pathogenesis [14]. In fact, in one study, virtually all (96%) of investigated melanoma cell lines harboured alterations at the DNA level within CDKN2A/p16, CDKN2B/p15, CDK4 and CCND1/cyclin D1 [17]. Inactivation mutations in the pRb ‘pocket’ region, which blocks binding to E2F, are rare in melanoma. Instead, the function of pRb in melanoma is commonly inhibited by hyperphosphorylation and, to a lesser extent, by underexpression [18]. In a recent study, Curtin and colleagues conducted a genome-wide analysis of DNA copy number and mutational analysis of BRAF and NRAS in 126 melanomas from individuals with varying UV exposure histories [19]. They found that amplification of CDK4,
Fig. 2. Cell cycle regulatory proteins. The cell cycle is controlled by a series of complex interactions among multiple proteins that interact in a precisely orchestrated sequence. Illustrated here is a highly schematic summary of the primary protein interactions, with special attention to components involved in the G1 to S transition of the cell cycle. Rb is one of the targets for the enzymatic activity of cyclin-cdk complexes. p21 is transcriptionally regulated by p53 and inactivates various cdks. p27 inactivates the same complexes as p21. p16 and p15 have a more restricted activity and form binary complexes with cdk4 and cdk6, displacing D-type cyclins. TGF-β can downregulate the cyclin E/cdk2 activity by activating the transcription of two inhibitors of cyclin-dependent protein kinases, p21 and p15. A surge in the level of p15 displaces p27 from the complex of cyclin D/cdk4/6 and the resulting p27 binds to the cyclin E/cdk2 complex. This inactivates its kinase activity and blocks cell cycle progression.

...was commonly seen in acral and mucosal melanomas but not observed in tumors with activating \textit{BRAF} or \textit{NRAS} mutations or \textit{Cyclin D1} copy number gains. Furthermore, losses/deletions of the melanoma suppressor \textit{CDKN2A} locus were also more commonly detected in mucosal and acral melanomas, but only in samples without \textit{CDK4} amplification.

### 3.2. p16INK4A/p14ARF in primary specimen

Since its discovery as a CKI in 1993, the tumor suppressor p16 has received widespread attention in melanoma research (see [14] and references therein). A plethora of studies show that in sporadic melanomas, p16 is inactivated through homozygous deletion (6–25%), mutation (0–26%) and methylation (0-10%) in roughly one forth of primary tumors. Based on an immunohistochemical analysis of 103 melanocytic lesions representing all stages in the progression of melanoma, Reed et al. [20] suggest that loss of p16 protein expression is not necessary for melanoma initiation as all \textit{in situ} lesions and the majority of invasive melanomas retained expression of this protein. Loss is potentially more related to invasiveness or the ability to metastasize, because 52% of primary invasive tumors and 72% of metastatic lesions showed partial or no expression of p16. In thin to intermediate (<4 mm) sporadic melanomas, however, the frequencies of LOH at the INK4A locus, INK4A intragenic mutations and promoter methylation are extremely low (10%) [21]. Loss of p16 expression is associated with progression of disease [20], suggesting that other regulatory mechanisms allow for decreased p16 in melanomas.

In this regard, the transcriptional regulatory protein Id1 (inhibitor of differentiation) has recently been identified as a repressor of p16/INK4A transcription [21].
In melanomas, Id1 expression is limited to the in situ component of invasive melanomas and perivascular regions of metastatic tumors. Id1 expression correlates with decreased p16INK4A expression in RGP melanomas, suggesting that Id1 transcriptional repression may represent one of the earliest mechanisms of p16 dysregulation in melanoma initiation. Consequently, a model for p16 inactivation via multiple steps in melanomagenesis has been proposed [21]: Reversible transcriptional inactivation in RGP melanomas (via Id1 or other repressors), subsequent acquired epigenetic changes (e.g., promoter methylation), and finally irreversible genetic alterations (e.g., mutations, deletions) associated with melanoma invasion and metastasis. Taken together, these findings indeed implicate deregulation of the p16/pRb pathway as a critical early event in the development of melanoma.

The INK4A/ARF locus on chromosome 9p21 also encodes another tumor suppressor gene called p14ARF (p19ARF in mice). Exon 1β, located approximately 15kb centromeric to exon 1α of p16, is spliced onto exons 2 and 3 of p16 to generate this alternative transcript. Alternate promoters regulate the independent production of these 2 mRNAs and translation of the p14ARF mRNA occurs in a reading frame alternate to p16. p19ARF acts through MDM2 to inhibit p53 degradation and exon 1β is sufficient for p19ARF-mediated stabilization of p53 function (see [11,16] for review). Thus far, there are no molecular data supporting a tumor suppressive role for p14ARF in sporadic melanoma. Clearly, however, homozygous deletions at the INK4A/ARF locus have the capacity to inactivate both p14ARF and p16 function through the shared second exon.

### 3.3. Expression and prognostic relevance of Cyclins/CDKs/CKIs in melanoma

As in other malignant tumors, certain cell-cycle regulators are differentially expressed during melanoma progression and are independent prognostic markers, albeit with somewhat incongruent results. Florenes and colleagues [11,16,22] observed only rare cyclin D3-positive cells and no cyclin D1-positive cells in benign nevi. In contrast, cyclin D3/cyclin D1 was found to be expressed by 96%/62% of primary and 97%/29% of metastatic melanomas, respectively. Kaplan-Meier analysis revealed that high levels of cyclin D3 is an indicator of early relapse and decreased overall survival for patients with superficial spreading, but not nodular melanoma, whereas cyclin D1 does not have any impact on disease-free and overall survival.

p27Kip1 is strongly expressed by normal melanocytes and benign nevi, whereas in nodular melanomas, the level of p27Kip1 was found to correlate significantly with the thickness of the tumor, with less protein expressed in thicker lesions [23]. Patients having tumors with fewer than 5% p27Kip1-staining cells have a significantly higher risk of early relapse of their disease compared with those expressing moderate or high levels. In contrast, the level of p27Kip1 does not correlate with tumor thickness or disease-free survival in patients with superficial spreading melanomas. Furthermore, p27Kip1 does not appear to have an influence on overall survival for either subgroup. When examined the combined effect of p21Waf1/Cip1 and p27Kip1 on clinical outcome, it was found that analysis of these two CKIs together may have greater prognostic potential than either alone. These data underscore the value of analyzing multiple cell cycle regulatory proteins to obtain the most reliable indication of prognosis [23].

A significant increase of cyclin E and CDK2 expression occurs during tumor progression in melanomas [24]. Cyclins B1, D2, and D3 show significantly increased expression in metastases, but normal or even decreased expression in primary melanomas. In contrast, cyclins A and D1, and CDK1 and CDK4 are expressed very weakly in situ with no significant differences between nevi, primary melanomas or metastases, and histopathological staging. Approximately 30% of primary melanomas and 40% of metastases completely lack p21Waf1/Cip1 expression. In superficial spreading melanomas, a significant correlation between protein expression and tumor thickness was found, with thin lesions showing low protein levels. By comparing primary and metastatic specimens obtained from the same patient, a reduction in p21Waf1/Cip1 staining was observed in the latter [25].

### 4. Selective components of the apoptotic program

Acquired resistance toward apoptosis (programmed cell death) is a hallmark of cancer [26]. The concept that apoptosis might influence the malignant phenotype was first raised in 1972 by Kerr, Wyllie and Currie. However, the importance of apoptosis in the pathogenesis of cancer remained under-appreciated for almost two decades. A comprehensive overview of apoptosis is beyond the scope of this chapter (for review, see refs [27,28]). As a general summary, most apoptotic
pathways involve a sensor that detects a death-inducing signal, a signal transduction network and execution machinery that actively carries out the process of cell death. DNA damage can induce apoptosis through a central sensor, p53, although p53-independent pathways clearly exist (Fig. 3). Many of the signals that elicit apoptosis converge on the mitochondria, which respond to pro-apoptotic signals by releasing cytochrome c. Cytochrome c can interact in a multi-protein complex with Apaf1 and pro-caspase-9, leading to caspase-9 activation and the initiation of a protease cascade. One of the most important modulators is Bcl-2, owing to its ability to affect cytochrome c release from mitochondria and/or modulate the Apaf1/caspase-9 interaction. However, neither Bcl-2 nor Bcl-xL has been shown to be completely cytoprotective against Apaf1/caspase-9 mediated cell death.

Bcl-2 is the founding member of an expanding gene family that includes other anti-apoptotic molecules such as Bcl-xL, as well as pro-apoptotic members such as Bax. A series of enzymes known as caspases are considered the engine of apoptotic cell death. Caspases are cysteine proteases that are expressed as inactive pro-enzymes. The “signaling” caspase-9 associates with Apaf-1 (apoptotic protease activating factor-1), and oligomerization of this complex in the presence of cytochrome c can activate the downstream “effector” caspase cascade. Thus, essential downstream components of p53-induced cell death include caspase-9 and its adapter Apaf-1 [29]. It is now becoming increasingly clear that mutations in many cancer-related genes can disrupt the apoptotic machinery and compelling evidence indicates that apoptotic activity is important in tumor suppression.

4.1. Defects of the apoptotic program in melanoma

p53 was the first tumor suppressor gene linked to apoptosis. p53 mutations occur in the majority of human tumors and are often associated with advanced tumor stage and poor patient prognosis. Moreover, several upstream and downstream components of the p53 pathway (e.g. Mdm-2, p14ARF and Bax) are altered in human tumors [26,30,31] (Fig. 3). What triggers apoptosis during tumor development? A variety of signals appear important [26,31–34]. Extracellular triggers include radiation, growth/survival factor depletion and loss of cell-matrix interactions and cell-cell adherence-based survival signals. In the skin, excessive exposure to UV radiation induces apoptosis, which presumably serves to eliminate heavily damaged cells. UV irradiation induces apoptosis, and loss of p53 function leads to the survival of these damaged cells thereby initiating tumor development. Hence, loss of apoptotic function can impact tumor initiation, progression and metastasis.

p53 mutations were found to be associated with poor prognosis, de novo or acquired resistance, and relapse in a broad field of solid and hematologic malignancies (for review, see refs [31]). Melanoma is a radiation- and chemotherapy-refractory neoplasm and there is no standard therapy for patients with disseminated disease: the commonly used anticancer drugs do not alter the prognosis, i.e. the fatal outcome [8], and are highly heterogeneous in their in vitro effects. However, contrary to other malignancies, p53 mutations are very rare in melanoma, being less than 5%. Tumor-derived p53 does localize to the nucleus in melanoma, however, its transcriptional activity is weak and overexpression of wild-type p53 does not induce immediate cell death. Another intriguing factor is that cells which carry mutated p53 undergo apoptosis when overexpressed with wild-type p53 [35]. This suggests that p53 is kept in an inactive state, either by other factors (such as Apaf-1) or through post-transcriptional mechanism(s), thereby functionally inactivating its pathways. Thus, in melanoma studies fail to correlate p53 mutations with reduced toxicity to anticancer agents.

4.2. Apaf-1

Findings by Scott Lowe and colleagues at the beginning of the decade [36] provided exciting and promising new insights into mechanisms how melanomas become apoptosis-, and thus chemotherapy-resistant, despite a fully functional p53. They show that melanoma cells of different progression stages can avoid apoptosis by inactivating a downstream component of the apoptotic pathway, namely Apaf-1, thus disabling the p53 apoptotic program. What makes the results of Soengas et al. even more interesting is that they showed a) a deletion of one of the alleles in 42% of the specimens tested and b) a reversible “switching off” of the remaining allele by methylation, rather than a permanent deactivation by deletion or mutation. The methylation of Apaf-1 could experimentally be reversed by a methylation inhibitor (5-aza-2dC) and thus its pro-apoptotic function restored. Pre-treatment assessment of Apaf-1 status may therefore improve the therapeutic management of patients with melanoma.
4.3. Bcl-2 and melanoma

Bcl-2 is a strongly antiapoptotic protein that is expressed in normal melanocytes, benign nevi, primary melanoma, and melanoma metastases. At this time, the overall importance of Bcl-2 expression by stage and lesion type remains controversial (reviewed in [37]). Bcl-2 protein is located within the inner layers of the mitochondrial membrane, where it prevents apoptosis by blocking the release of cytochrome C from mitochondria. In this role, Bcl-2 promotes resistance to anticancer therapy and could contribute to enhanced metastatic potential. Moreover, Bcl-2 protein has been found in normal developing melanocytes, and the loss of Bcl-2 function in Bcl-2 knockout mice leads to accelerated disappearance of melanocytes. The mice are viable but rapidly lose hair color and turn from black to white [38]. Drug resistance in melanoma has been partially attributed to overexpression of Bcl-2, which blocks the release of cytochrome C [28]. Experimental transfection of cells with Bcl-2 confers a multidrug resistant phenotype in both hematologic and solid tumor cells [39]. Conversely, pharmacologic reduction or targeted inactivation of Bcl-2 amplifies anticancer responses to chemotherapy in multiple in vivo models, including melanoma [40]. Hence, several factors support a role for Bcl-2 antisense therapy to treat melanoma, based on evidence suggesting a critical role for Bcl-2 in melanoma cell survival.

5. Mitogen-activated protein kinase (MAPK) signal transduction pathways

Mitogen-activated protein kinase (MAPK) pathways are cellular signalling pathways that enable cells to transduce extracellular signals to an intracellular response (Fig. 4) [41,42]. The molecular details of mam-
malian MAP kinase signal transduction pathways activated by stress and inflammation have only begun to be dissected. The impact of these pathways on, amongst others, the pathology of cancer, the side effects of cancer therapy, chronic inflammation, embryonic development, innate and acquired immunity, is profound. Thus it is not surprising that understanding these pathways has attracted wide interest, and in the past 10 years, dramatic progress has been made. Accordingly, it is now becoming possible to envisage the transition of these findings to the development of novel treatment strategies.

5.1. Ras-Raf-MEK-MAP kinase pathway

Since the discovery of the role of Ras oncogenes in carcinogenesis in the early 80s, we have witnessed an explosion of research in the signal transduction area [41,42]. Likewise, a host of growth signaling pathways is now implicated in melanocyte and melanoma biology. They include cell growth and differentiation, melanogenesis, cell fate determination, survival and apoptosis. The Ras gene product is a monomeric, membrane-localized guanosine triphosphate (GTP)/guanosine diphosphate (GDP)-binding (G) protein of 21 kD that functions as a nodal point, a molecular “switch” converting signals from receptor and non-receptor tyrosine kinases on the cell membrane to downstream cytoplasmic or nuclear events (Fig. 4). There are multiple effects that occur after Ras activation, initiating a multitude of cytoplasmic signalling cascades, leading to protein synthesis and regulation of cell survival, proliferation, and differentiation. The precise functions of Ras proteins continue to be elucidated. Ras utilizes a variety of downstream effectors and mediates its actions in mechanistically distinct ways, depending on both species and cell-type identity. In turn, these further complicate our ability to define a simple relationship between Ras and, for example, cell cycle progression. The MAP kinase (MAPK) pathway has emerged as the crucial route between membrane-bound Ras and the nucleus (Fig. 4). This pathway encompasses a cascade of phosphorylation events involving three key kinases, namely Raf, MEK (MAP kinase kinase) and ERK (MAP kinase). The critical nuclear target of the Ras-Raf-MEK-MAP kinase pathway are the transcription factors. Another well-characterized effector of Ras is the phosphoinositide 3-phosphate lipid kinase (P3K)-AKT/PKB pathway (Fig. 4).

Each human cell contains at least three distinct ras proto-oncogenes, which encode four highly related but distinct proteins, H-ras, N-ras, and K-ras (K4A- and K4B-). Activating single-point mutations of the ras gene can result in constitutive activation of Ras protein, thereby continuously stimulating cell proliferation and inhibiting apoptosis. These mutated forms of Ras have impaired GTPase activity. Although they still bind GTPase activating proteins (GAP), there is no “off” sign, since GTPase is no longer activated. Activating mutations are limited to a small number of sites (codons/amino acids 12, 13, 59, and 61), all of which abolish GAP-induced GTP hydrolysis of the Ras proteins. Oncogenic K-ras mutations occur frequently in non-small-cell lung, colorectal, and pancreatic carcinomas; H-ras mutations are common in bladder, kidney, and thyroid carcinomas; and N-ras mutations are found in hepatocellular carcinoma, hematologic malignancies and melanoma [41,42].

5.2. Ras genes and melanoma

Within the ras gene family, only N-ras has a significant association with melanoma progression, whereas H-ras or K-ras are rarely mutated. Mutations in N-ras have since been identified in 15–20% of all melanomas, and are most commonly the result of the substitution from leucine to glutamine at position 61 [43,44]. The presence of N-ras mutations appears to correlate with the vertical growth phase (VGP) and with melanoma arising in chronically UV-exposed body sites. Moreover, N-ras mutations are associated with nodular melanomas and to a lesser extent with lentigo maligna melanoma. Benign and dysplastic nevi did not exhibit ras mutations, however, N-ras mutations in congenital nevi have been observed. N-ras mutations did not correlate with metastasis or survival parameters (reviewed in [3,4,11]).

5.3. BRAF in benign nevi and melanoma

When active in its GTP-bound state, RAS activates a number of downstream effectors, one of which is the RAF family of serine/threonine kinases. There are three isoforms of RAF, namely, A-Raf, BRAF, and CRAF (also called Raf-1) (reviewed in [3,41,42,45]). Once activated, RAF stimulates the MAPK cascade, resulting in the sequential activation of MEK1 and MEK2, which in turn activates ERK1 and ERK2 (Fig. 4). Once activated, the ERKs either activate cytoplasmic targets or migrate to the nucleus, where they phosphorylate transcription factors. In melanocytes, the MAPK (ERK) pathway is activated by growth fac-
Fig. 4. The Ras/RAF/MEK/ERK and the PI3K/AKT signalling pathways in melanoma. Ligand-mediated dimerization of the receptor tyrosine kinases (RTKs) and subsequent autophosphorylation or transphosphorylation leads to their association with a variety of cytoplasmic phosphotyrosine binding proteins. This results in the initiation of a phosphorylation cascade and activation of several downstream pathways involved in cell growth and survival, including the Ras/Raf/MEK/ERK and PI3K/Akt pathways. Stimulation of these pathways transmits a signal to the nucleus resulting in modification of gene transcription patterns that ultimately affects processes such as cell division, apoptosis, adhesion, migration, and/or differentiation.

Proteins released from the local microenvironment, such as stem cell factor (SCF), α-melanocyte-stimulating hormone (α-MSH), and hepatocyte growth factor (HGF). Under physiological conditions, these growth factors only induce a weak stimulation of the MAPK (ERK) pathway that is insufficient to induce melanocyte proliferation. In most melanoma cells, the situation is very different and it has been shown that > 90% of clinical melanoma specimens have continuous hyperactivity in the MAPK (ERK) pathway.

Particularly BRAF has recently attracted enormous biological and therapeutic interest, as the b-raf gene is found mutated in at least 60% of cutaneous melanomas [44], and at a lower frequency in many other human malignancies. This discovery has firmly established the involvement of Raf kinases in cancer. The most common mutation (over 80%) is a V600E change in the activation loop that induces the constitutive activation of catalytic activity [46]. Curiously, in melanoma this mutation is rare in unexposed or chronically sun-damaged skin, but frequent in skin with intermittent sun exposure and often accompanied by amplification of the mutant allele [47]. The importance of localization is underlined by the observation that B-Raf mutations do not occur in melanomas of the uvea [48]. Furthermore, the frequency of BRAF mutations in melanoma is positively linked with genetic variants of the melanocortin-1 receptor in melanocytes [49]. Additionally, melanomas with wild-type BRAF or N-RAS frequently have increases in the number of copies of the genes for cyclin-dependent kinase 4 (CDK4) and cyclin D1 (CCND1), downstream components of the RAS-BRAF pathway [19]. What is more, constitutive MAPK (ERK) activity increases cyclin D1 and down-regulates p27 expression in melanoma cells [50].

Interestingly, however, a high frequency of BRAF V600E mutations (73–82%) has also been reported in common benign naevi, suggesting that BRAF activation might be the critical inducer of these benign precursor lesions [51–53]. Hence, acquisition of the
BRAF V600E mutation seems to be an early event in melanoma development with a high percentage of melanocytic nevi also found to harbor the mutation. Interestingly, the presence of the BRAF V600E mutation alone is not sufficient to oncogeneically transform naevi cells into melanoma [54]. Instead, the forced expression of BRAF V600E in primary human melanocytes leads to an irreversible growth arrest characteristic of senescence [55]. Pathological studies have confirmed these in vitro findings, and showed that most nevi are growth arrested and express many markers of senescence [55]. This process, termed “oncogene-induced senescence,” is thought to be an important mechanism in protecting cells from malignant change [3].

It has been suggested that the presence of a BRAF V600E mutation, rather than an NRAS or low-activity BRAF mutation, predicts for sensitivity to both MEK and BRAF inhibitors. [4,56,57]. Recent investigations have also sought to determine the prognostic relevance of BRAF mutations in melanoma. However, studies to date have failed to identify a link between BRAF mutational status and disease outcome [58]. In another study, patients with BRAF mutation showed longer disease-free survival (median of 12 months) than patients without mutation (median of 5 months), although this association was not statistically significant [59]. From such tiny samplings it would be reckless to draw definite conclusions, but it is interesting to note that thus far no molecule has emerged as a predictive or prognostic maker in melanoma for routine clinical use.

There are also possibilities that BRAF/MAPK signalling may allow melanoma cells to escape immune surveillance through suppression of their highly immunogenic pigmentation antigens [60]. Moreover, it has been shown that treating melanoma cells with an RNAi to V600E BRAF mutation reduces the release of immunosuppressive cytokines from melanoma cells [61].

The melanoma MAPK (ERK) signalling pathway is highly robust, with BRAF-V600E mutated melanoma cells rapidly developing resistance to BRAF inhibition in vitro [62]. Rather than this resulting from an acquired genetic change in the melanoma cells, resistance instead results from a simple switching within the MAPK pathway so that the signal is routed from BRAF to CRAF (Fig. 4). Studies on melanoma cell lines harbouring NRAS mutations have already shown that melanomas can use CRAF as a mechanism to activate downstream MAPK signalling [63]. The presence of a network connection that can easily switch MAPK signalling from BRAF to CRAF (also called Raf-1) is suggestive of a mechanism by which resistance to BRAF inhibitors may be acquired and could also potentially explain side effects observed with BRAF inhibitors [63–65].

6. PTEN and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway

PTEN (phosphatase and tensin homolog deleted on chromosome 10), also known as MMAC1 (mutated in multiple advanced cancers 1) or TEP1 (TGFβ-regulated and epithelial cell enriched phosphatase 1) was recently identified as a tumor suppressor gene located at 10q23.3 and has been shown to be deleted or mutated in a variety of advanced malignant tumors (see [3–5,66,67] for more comprehensive reviews). PTEN is a dual-specificity phosphatase capable of dephosphorylating both tyrosine phosphate and serine/threonine phosphate residues on proteins. It also functions as a major lipid phosphatase, counteracting phosphatidylinositol 3,4,5-triphosphate (PIP3) and phosphatidylinositol 3,4-diphosphate (PIP2), which are required for the activation of PKB/Akt, an important mediator of cell survival protecting cells from apoptosis (Fig. 4). PTEN has been shown to be involved in cell migration, spreading, and focal adhesion formation through direct dephosphorylation and inactivation of focal adhesion kinase (FAK). In addition, PTEN inhibits Shc phosphorylation, thus preventing association of Shc with Grb2/Sos and subsequent activation of the Ras/raf/MEK1/MAPK pathway. PTEN suppresses the stabilization of hypoxia-mediated HIF-1α (hypoxia-inducible favor 1α), which when stabilized through the PI3K/AKT pathway, upregulates VEGF expression. This suggests a possible role for PTEN in angiogenesis. While some of the functions of PTEN have been delineated, the nature of the factors involved in PTEN’s decision towards inducing cell-cycle arrest or apoptosis are still elusive. Germline mutations of PTEN have been found in the autosomal dominant Cowden syndrome, which is characterized by multiple hamartomas, especially of the skin, and an increased risk of breast (25–50% of affected females) and thyroid (10% of affected individuals) cancers as well as, to a lesser extent, malignant melanomas.
Loss of chromosome 10q has been detected in 30 to 50% of both early- and advanced-stage sporadic melanomas, and has been associated with poor clinical outcome. There is a host of studies examining the genomic status of PTEN in melanoma cell lines and non-cultured melanomas of various stages, with incongruous results (reviewed in [3–5,66,67]). Overall it appears that homozygous deletions and intragenic mutations of PTEN occur in the metastatic setting, especially in cell lines. Typically, PTEN expression is lost in up to 30% of melanoma cell lines and 10% of human tumor material. There is also evidence of inactivating PTEN mutations in > 10% of short-term melanoma cell cultures [68]. One study examined the mutational status of both PTEN and NRAS in 53 melanoma cell lines. The authors found in 30% alterations in PTEN and 21% to harbor activating NRAS mutations, with only 1 cell line having mutations in both, suggesting that PTEN and NRAS may function in the same pathway. PTEN can also function as a haploinsufficient tumor suppressor, and it is known that allelic loss of PTEN occurs in at least 58% of melanoma metastases.

With the accumulating knowledge of PTEN inactivation, it appears that there are several mechanisms which lead to PTEN inactivation: Initial work on tumor cell lines almost uniformly suggested that PTEN intragenic mutations, homozygous deletions, and the two structural hits (one structural hit – LOH – followed by the second epigenetic hit) would be the rule across a large variety of tumors. In this regard, studies on melanomas have shown that biallelic structural inactivation occurs by either homozygous deletion at 10q23 or somatic intragenic PTEN mutation plus loss of the remaining wild-type allele. Zhou et al. [69] have proposed that non-cultured melanomas might be somewhat unique in that, although PTEN inactivation is seen to occur by one structural hit (LOH) followed by the second epigenetic hit, PTEN protein expression in melanomas can be biallelically silenced with relatively high frequency by epigenetic phenomena as well: Examination of 4 primary and 30 metastatic melanoma showed little to no expression of PTEN in 65% of the cases. These results would support PTEN as a major tumor suppressor on 10q involved in melanoma tumorigenesis.

6.1. Activation of the PI3K/AKT pathway

Activation of the MAPK (ERK) pathway clearly does not account for all aspects of melanoma biology and there is evidence that other signaling pathways may be equally important (reviewed in [3–5,66,67]). To date, the most widely studied of these is the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway [70,71]. Activation of the PI3K/AKT pathway in melanoma occurs through either paracrine/autocrine growth factors or the loss of expression and/or mutation of negative pathway regulators. In particular, the insulin-like growth factor-1 (IGF-1) is known to aid the growth of early-stage melanoma cells, at least in part, through the activation of PI3K/AKT [72].

The AKT family consists of three members, AKT1–3 [73], which exhibit different expression patterns depending on the cell type. Of these, 43–50% of melanomas have selective constitutive activity in AKT3 [70]. There is evidence that overexpression of AKT3 may occur as a result of copy number increases in the long arm of chromosome 1 [74]. Another mechanism for PI3K/AKT pathway activation in melanoma is through the acquisition of activating E17K mutations in AKT3, but this is thought to be relatively rare [75]. AKT has a critical role in cancer development through its ability to regulate apoptosis through the direct phosphorylation of BAD as well as it effects many other pathways, including the inhibition of forkhead signalling and the inhibition of glycogen synthase kinase-3 [76]. As already mentioned, one of the most critical regulators of AKT is the phosphatase and tensin homolog (PTEN), which degrades the products of PI3K, thereby preventing the activation of AKT. In summary, the mechanism by which the PI3K-AKT pathway is activated in melanoma is not yet fully elucidated, but may involve the loss of expression or functional inactivation of PTEN.

6.2. Entente fatale: For melanoma progression both PI3K/AKT and MAPK (ERK) signalling are required

Data are now emerging from both preclinical and clinical studies that the MAPK (ERK) and the PI3K-AKT pathways have overlapping functions with regard to melanoma biology (Fig. 4). Analysis of melanoma lines and human melanoma samples have shown that those mutational profiles are favoured which simultaneously promote both MAPK and PI3K/AKT signalling [71]. As activating NRAS mutations can stimulate both the MAPK (ERK) and PI3K/AKT pathways, they are rarely found in concert with BRAF mutations and these mutations are mutually exclusive [19]. Likewise, BRAF mutations are frequently found in combination with either PTEN loss/inactivation or activating
AKT3 mutations, again showing a requirement for dual MAPK (ERK) and PI3K/AKT pathway activity [68,71,75].

AKT3 seems to cooperate with the BRAF V600E mutation in melanoma initiation by suppressing MAPK signalling activity through phosphorylation at Ser364/428 of BRAF [77]. It is therefore suggested that AKT3’s ability to downregulate MAPK (ERK) pathway activity may allow early-stage melanoma cells to escape from oncogeneinduced senescence. To date, the strongest experimental proof of concept for the cooperation between the BRAF V600E mutation and PI3K/AKT signalling in melanoma development is derived from the mouse modelling work of Dankort and co-workers [78]. These authors have elegantly shown that the conditional expression of melanocyte-specific mutated BRAF alone led to the development of benign melanocytic hyperplasia but not melanoma. Full transformation to melanoma occurred only when the BRAF-V600E mutation was activated in the presence of PI3K/AKT activity after suppression of PTEN expression.

Hence, the intracellular melanoma signalling network is highly interconnected with a great deal of functional redundancy built into it (Fig. 4). Studies have already shown that single-agent MEK inhibition is relatively ineffective at inducing melanoma regression in both pre-clinical models [56] and in early-phase clinical trials (reviewed in [79]). The disappointing clinical activity seen after single-agent MEK inhibition parallels with that observed with single-agent sorafenib [80], and activity seen after single-agent MEK inhibition parallels with that observed with single-agent sorafenib [80], and it is therefore suggested that AKT3’s ability to downregulate MAPK (ERK) pathway activity may allow early-stage melanoma cells to escape from oncogeneinduced senescence. To date, the strongest experimental proof of concept for the cooperation between the BRAF V600E mutation and PI3K/AKT signalling in melanoma development is derived from the mouse modelling work of Dankort and co-workers [78]. These authors have elegantly shown that the conditional expression of melanocyte-specific mutated BRAF alone led to the development of benign melanocytic hyperplasia but not melanoma. Full transformation to melanoma occurred only when the BRAF-V600E mutation was activated in the presence of PI3K/AKT activity after suppression of PTEN expression.

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7. Stem cell factor (SCF)/c-kit signalling pathway as the driving oncogenic event in distinct subtypes of melanoma

c-KIT is a transmembrane receptor tyrosine kinase (RTK) to the PDGF/CSF-1 receptor subfamily, whose aberrant activation is implicated in the progression of gastrointestinal stromal tumors (GIST) and some acute myeloid leukemias (reviewed in [82]). The c-KIT receptor ligand is the glycoprotein Stem Cell Factor (SCF), which is also known under a variety of other names including steel factor (SF). c-KIT is known to recruit and activate a number of intracellular signalling pathways implicated in tumor progression, such as phosphoinositide-3 kinase (PI3K)/AKT, Src, mitogen-activated protein kinase (MAPK), janus kinase (JAK)/signal transducers and activators of transcription (STAT) and phospholipase-C (PLC)-γ [82].

The role of c-KIT signalling in melanoma has been controversial; although c-KIT activity is critical to melanocyte development and migration, its expression tends to be lost in most melanomas [83]. Progression of melanoma towards the invasive and metastatic phenotype is associated with loss of expression of c-kit (see [11,82,84] for review). c-Kit plays a pivotal role in normal growth and differentiation of embryonic melanoblasts. SCF is highly mitogenic for human melanoblasts and melanocytes in culture and for melanocytes in vivo. In mice, c-kit has been mapped to the dominant white spotting (w) locus; its ligand is the product of the steel (sl) locus on chromosome 10. Mutations in the w or sl locus, or injection of neutralizing anti-c-KIT antibodies into pregnant mice, results in the piebald phenotype, characterised by white spotting of the fur and attributed to a local reduction of the number of cutaneous melanocytes. Mutations in the c-kit receptor also have been identified in human patients with piebaldism, suggesting that normal function of c-kit is required for human melanocyte development and migration [11,82].

Several early pathological studies have demonstrated that the progression of melanoma is associated with a loss of expression of c-kit [11,82]. The vast majority of metastatic lesions and cell lines do not express detectable levels of the c-kit receptor. Transfection of c-kit into c-kit negative, highly metastatic human melanoma cells significantly inhibited tumor growth and metastasis in nude mice [85]. Exposure of c-kit-positive melanoma cells in vitro and in vivo to SCF triggered apoptosis of these cells but not of normal melanocytes. Loss of c-kit receptor may thus allow melanoma cells to escape SCF/c-kit-mediated apoptosis [85]. On the basis of the aforementioned results it was assumed that c-KIT was primarily a regulator of melanocyte behavior and therefore dispensable for melanoma growth.

However, the recent publication showing the presence of activating c-KIT aberrations (either amplification and/or mutation) in mucosal (39%) and acral (36%) melanomas, as well as melanomas arising on skin with chronic sun damage (28%), has renewed interest in c-KIT signaling in melanoma [86]. In addition, subsequent studies have shown that c-KIT is
Table 1
Selected genetic alterations in cutaneous melanoma

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alteration frequency/type(s)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>BRAF</td>
<td>50–70% mutated (e.g., V600)</td>
<td>[44]</td>
</tr>
<tr>
<td>NRAS</td>
<td>15–30% mutated</td>
<td>[44,71]</td>
</tr>
<tr>
<td>AKT3</td>
<td>overexpressed; 43–50% selective constitutive activity</td>
<td>[70]</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>6–44% amplified (depending on subtype: chronic sun exposure/face, acral and mucosal melanoma)</td>
<td>[19,118]</td>
</tr>
<tr>
<td>CDK4</td>
<td>amplification commonly seen in acral and mucosal melanomas</td>
<td>[19]</td>
</tr>
<tr>
<td>MITF</td>
<td>10–16% amplified</td>
<td>[119]</td>
</tr>
<tr>
<td>Notch</td>
<td>overexpression</td>
<td>[110,112]</td>
</tr>
<tr>
<td>c-met</td>
<td>overexpression</td>
<td>[97,98]</td>
</tr>
<tr>
<td>c-kit</td>
<td>amplification and/or mutation: 22–39% (mucosal), 36% (acral), 28% (sun-damaged skin), 15% (anal)</td>
<td>[86–88]</td>
</tr>
<tr>
<td>c-myc</td>
<td>overexpression</td>
<td>[100,120,121]</td>
</tr>
<tr>
<td>CDKN2A (p16INK4A)</td>
<td>30–70% deleted, mutated or silenced</td>
<td>[122]</td>
</tr>
<tr>
<td>PTEN</td>
<td>5–20% deleted or mutated</td>
<td>[71,101,123]</td>
</tr>
<tr>
<td>APAF-1</td>
<td>40% silenced</td>
<td>[36]</td>
</tr>
<tr>
<td>p53</td>
<td>10% lost or mutated</td>
<td>[124]</td>
</tr>
</tbody>
</table>

expressed in 88% of oral mucosal melanomas, and that at least 22% of these harbored activating mutations [87]. In these instances, most of the c-KIT expression was in the in situ component, with strong expression reported in the invasive portion in only 22% of cases. Another recent paper also reported the presence of the activating L576P mutation in c-KIT in 15% of anal melanomas, a mutation that the authors showed to be imatinib sensitive in vitro [88]. What’s more, work from our own laboratory has further identified a subset of melanomas that lack BRAF mutations, with constitutive c-KIT signaling activity resulting from c-KIT/CDK4 co-overexpression [89]. Although the initial clinical trials of the c-KIT inhibitor imatinib mesylate in melanoma were negative, some dramatic responses have been seen in patients with very high c-KIT expression and/or documented activating mutations, fostering the belief that focused studies in patients selected on the basis of c-KIT mutational status will yield more encouraging results [82].

8. Hepatocyte growth factor (HGF)/scatter factor (SF)/c-Met tyrosine kinase receptor

c-Met is a receptor tyrosine kinase (RTK) that has been known to stimulate the invasive growth of cancer cells, increase their metastatic potential, and is also known to be expressed and mutated in a variety of solid tumors [90]. The structure of c-Met consists of a disulfide-linked α–β heterodimer with a molecular weight of 190 kDa (see [11] for review). The physiological ligand for c-Met is the hepatocyte growth factor (HGF), which is also known as scatter factor. HGF is a multifunctional cytokine acting as a mitogen, motogen, and morphogen for many epithelial cells. HGF is physiologically secreted by cells of mesenchymal origin and acts on neighboring epithelial cells through a paracrine loop [91]. Hepatocyte growth factor (HGF) is a multifunctional cytokine acting as mitogen, motogen and morphogen for many epithelial cells through its tyrosine kinase receptor c-Met, which is present in epithelial cells and melanocytes. HGF is physiologically secreted by cells of mesenchymal origin and acts on neighboring epithelial cells through a paracrine loop. However, coexpression of HGF and c-Met occurs in a variety of transformed cells and tumors both in vitro and in vivo and has been shown to be involved in tumor development and invasion (reviewed in [11]).

HGF/c-Met signaling has been implicated in the development of melanoma [92]. HGF stimulates proliferation and motility of human melanocytes [93]. In transgenic mice that ubiquitously expressed HGF, ectopic localization of melanocytes and hyperpigmentation in skin were observed and melanoma arose spontaneously. In these mice, ultraviolet radiation-induced carcinogenesis was accelerated (reviewed in [94]). It has been suggested that c-Met autocrine activation induced development of malignant melanoma and acquisition of the metastatic phenotype [95].

Many missense mutations of c-Met have been reported in a variety of cancers, with the majority of them identified in the cytoplasmic activation loop tyrosine kinase domain. c-Met activating point mutations in the kinase domain are implicated as the cause of hereditary papillary renal carcinoma. In addition, kinase domain mutations have been observed in sporadic papillary re-
nal carcinoma, ovarian cancer, childhood hepatocellular carcinoma, and gastric cancer [90].

In normal skin, c-Met is present on epithelial cells and melanocytes, whereas HGF is produced mainly by mesenchymal cells and, consequently, interacts with its receptor in a paracrine manner [96]. HGF is a mitogen of human melanocytes, and overexpression of c-Met correlates with the invasive growth phase of melanoma cells [97]. c-Met is overexpression is also associated with patient survival [98]. Studies by our group have shown that most of melanoma cells, but not normal melanocytes, produce HGF, which can induce sustained activation of its receptor [91]. Hence, an autocrine HGF/c-Met signaling loop may be involved in the development of melanomas. Consistent with this, prolonged HGF stimulation induces the down-regulation of the intercellular adhesive molecule E-cadherin that is implicated in the control of melanocyte proliferation [91]. Finally, in transgenic mice that ubiquitously expressed HGF, ectopic localization of melanocytes and hyperpigmentation in skin were observed, and melanoma arose spontaneously. In these mice, UV radiation-induced carcinogenesis was accelerated [99]. It is suggested that c-Met autocrine activation induced the development of malignant melanoma and the acquisition of the metastatic phenotype [95]. More recently, Puri and co-workers showed that a small-molecule tyrosine kinase inhibitor of c-Met inhibits growth of implanted IGR39D melanomas highly overexpressing c-myc spontaneously formed macroscopic metastases (lymph nodes and lung) in severe combined immunodeficient (SCID) mice, whereas less prominent c-myc overexpression caused the development of only lung micrometastases [103]. These findings suggest that constitutive high c-myc expression in human melanoma results in a more aggressive growth behavior both in vitro and in vivo, and favors metastasis.

More recently, it has been suggested that one of the major functions of c-myc overexpression in melanoma progression is to continuously suppress BRAFV600E or NRASQ61R-dependent oncogene-induced senescence [104].

10. Notch signaling and melanoma

The Notch signaling pathway is an evolutionarily conserved signaling cascade that affects cell fate decisions and many differentiation processes during both embryonic and postnatal development (see [105] for review). Notch signaling is critical for developing and maintaining tissue homeostasis. Its pathway comprises a family of transmembrane receptors and their ligands, negative and positive modifiers, and transcription factors. To date, 4 mammalian receptors (Notch1 through Notch4) and at least 5 ligands (Delta 1, 3, and 4 and Jagged 1, 2) have been identified. Binding of the ligand renders the Notch receptor susceptible to metalloprotease- and γ-secretase–mediated proteolytic cleavage, which in turn results in the release of the Notch intracellular domain (NIC) from the plasma membrane and its subsequent translocation into the nucleus.

Notch signaling has also been implicated in a variety of neoplastic malignancies reviewed in [106–108]). Notch can function as a tumor promoter or a suppressor depending on the cell type and context. In experimental mouse models of skin tumorigenesis, Notch1 shows antitumor activity. Ablation of Notch1 in keratinocytes leads to spontaneous development of basal cell carcinoma–like tumors, likely as a result of reactivation of the β-catenin signaling pathway [109].

Recent data suggest that Notch activation may also play a role in melanoma progression. Our group has previously shown that activation of Notch signaling promotes the progression of early-stage melanoma cell lines in a β-catenin–dependent manner both in vitro and in vivo [110]. Blocking Notch signaling suppressed whereas constitutive activation of the Notch1 pathway enhanced primary melanoma cell growth both
in vitro and in vivo yet had little effect on metastatic melanoma cells. Activation of Notch1 signaling enabled primary melanoma cells to gain metastatic capability. Furthermore, the oncogenic effect of Notch1 on primary melanoma cells was mediated by β-catenin, which was upregulated following Notch1 activation. Inhibiting β-catenin expression reversed Notch1-enhanced tumor growth and metastasis, therefore suggesting a β-catenin–dependent, stage-specific role for Notch1 signaling in promoting the progression of primary melanoma.

What’s more, microarray profiling comparing the gene expression of normal human melanocytes to human melanomas revealed up-regulation of Notch target genes in melanoma cells, suggesting activation of the Notch signaling pathway in melanoma [111]. More recently, an analysis of cell lines and patient lesions showed that Notch activity is significantly higher in melanomas than their nontransformed counterparts. Notch and Notch target genes are up-regulated in both melanoma lesions and melanoma cell lines. Notch receptors 1, 2, and 4 are overexpressed in melanoma cell lines and lesions, particularly when compared against primary melanocytes or normal human skin [112].

Moreover, Notch1 signaling is activated in melanoma cells, but not melanocytes, and constitutive Notch1 activation confers transforming properties to primary melanocytes in vitro. The use of a constitutively active, truncated Notch transgene construct (N1IC) was exploited by Pinnix and co-workers to determine if Notch activation is a “driving” event in melanocytic transformation or instead a “passenger” event associated with melanoma progression. N1IC-infected melanocytes displayed increased proliferative capacity and biological features more reminiscent of melanoma, such as dysregulated cell adhesion and migration. Specifically, ectopic N1IC expression induced gross morphologic changes, increased growth, adhesion, migration, and survival, and resulted in the loss of E-cadherin expression and up-regulation of MCAM, two well-characterized events in melanoma development. In addition, MCAM was identified as a direct Notch target due to the presence of two high-affinity CSL binding sites present in the MCAM promoter. The N1IC oncoprotein conferred anchorage-independent growth, increased survival, and loss of contact inhibition; suppression of Notch signalling decreased the growth of melanoma cell lines, whereas primary melanocytes were unaffected. Taken together, these data suggest that deregulation of Notch signaling plays a specific role in promoting a transformed phenotype in human melanocytes and define the importance of Notch signaling in human melanoma.

Gene expression analyses supported these observations and aid in the identification of MCAM, an adhesion molecule associated with acquisition of the malignant phenotype, as a direct target of Notch transactivation. N1IC-positive melanocytes grew at clonal density, proliferated in limiting media conditions, and also exhibited anchorage-independent growth. Hence, Notch alone is a transforming oncogene in human melanocytes, a phenomenon not previously described for any melanoma oncogene [112].

Our aforementioned microarray data describing Notch pathway activity is underscored by a recent report by Hoek and colleagues that revealed up-regulation of Notch2 and Hey1 in a separate but distinct panel of malignant melanoma cell lines, suggesting a role for Notch activation in the transformation of melanocytes [111]. Previous immunohistochemical studies on early-phase melanoma lesions have shown overexpression of full-length Notch1 protein in melanoma tissue compared against benign human nevi [110] and normal human skin [113].

What’s more, our current studies strongly suggest that constitutive Notch signaling is associated with melanocyte transformation and melanoma tumorigenesis. Of particular significance is the ability of a γ-secretase inhibitor to selectively inhibit the growth of melanoma cell lines. These findings are consistent with a recent report identifying a γ-secretase inhibitor that induced effective apoptosis in human melanoma cells while sparing melanocytes [114].

These findings were recently confirmed and expanded by Bedogni and co-workers who found that Notch1 signaling is elevated in human melanoma samples and cell lines and is required for Akt and hypoxia to transform melanocytes in vitro [115]. Notch1 facilitated melanoma development in a xenograft model by maintaining cell proliferation and by protecting cells from stress-induced cell death. Hyperactivated PI3K/Akt signaling led to upregulation of Notch1 through NF-κB activity, while the low oxygen content normally found in skin increased mRNA and protein levels of Notch1 via stabilization of HIF-1α. Taken together, these findings demonstrate that Notch1 is a key effector of both Akt and hypoxia in melanoma development and identify the Notch signaling pathway as a potential therapeutic target in melanoma treatment.
11. Conclusions and outlook: A changing treatment paradigm based on molecular classification

Cutaneous melanoma can be perceived as a disease of communication between and within skin cells. The molecular aberrations are pleiotropic, but growth factor receptor and mitogen-activated protein kinase (MAPK) pathways feature prominently. Following the discovery that the overwhelming proportion of cutaneous melanomas have constitutive activity in the extracellular signal regulated kinase (ERK) pathway, there has been considerable interest in pharmacologically targeting this pathway using small molecule inhibitors [3, 67]. Although there is evidence to suggest that the presence of the BRAF V600E mutation is predictive of response to BRAF and MEK inhibitors [116], recent clinical trials on MEK inhibitors have not led to the expected favorable results [3, 4]. BRAF/MAPK signaling may be more heterogeneous than first thought and locally regulated by the microenvironment [56, 104]. It also is possible that other factors, such as enhanced phosphoinositide-3-kinase/AKT signaling activity, may further influence response to BRAF/MEK inhibition [117].

As yet, very little is known about the factors underlying resistance to BRAF inhibition in the BRAF V600E–mutated melanoma population. A greater understanding of the genetic basis of response to BRAF and other inhibitors is essential in selecting the most appropriate patient sub-population for future clinical studies and developing strategies to overcome inherent resistance.

Although many melanomas harbor either activating mutations in BRAF or NRAS, there remains a substantial, yet little known, group of tumors without either mutation. We have recently suggested that co-overexpression of KIT/CDK4 is a potential mechanism of oncogenic transformation in some BRAF/NRAS wild-type melanomas. This group of melanomas may be a subpopulation for which KIT inhibitors may constitute optimal therapy [89].

We now also realize that there are differences in the genetic profiles of melanomas that originate from skin that is either chronically sun-damaged (as defined by the appearance of solar elastosis) or skin that lacks sun-induced damage [4]. Thus, melanomas that arise on skin with chronic sun-induced damage have a low incidence of BRAF mutations and instead showed increased cyclin D1 copy number. Frequent amplifications of cyclin D1 also occur in distinct histologic subtypes of melanoma [118].

As we have seen, multiple studies support the existence of distinct genetic pathways associated with melanoma development, mirroring different molecular and clinical behaviour. The complexity of the signaling observed in melanoma, and outlined in this book chapter, calls for a network-biology approach and a combination of targeted therapies. The challenge for melanoma investigators is to identify the key signalling “hubs” or relay stations in this melanoma network. Although we have made great strides in unravelling the mysteries of melanoma genetics and molecular biology, the important task now facing clinicians and researchers is to translate these discoveries into novel therapeutic strategies that will improve patient outcomes.

During the past three decades the translation of molecular and immunological studies in melanoma biology into useful clinical correlates or novel efficacious therapies has been overwhelmingly disappointing. Even though molecular markers and novel therapeutic compounds look promising in small-scale studies, the vast majority have failed to prove clinically useful in large-scale (phase III) studies. Today, in 2010, we clearly stand at the crossroads of melanoma therapy. In view of the plethora of targeted agents currently under clinical investigation and our emerging knowledge of molecular sub-classifications, we may already have the pharmacologic arsenal that could control melanoma if targeted to molecularly defined patient subgroups or if administered in biologically appropriate combinations. Clearly, therefore, pharmacogenomic analysis of melanoma populations may be a suitable strategy for the further subclassification of melanoma, ultimately leading to more “personalized” therapy approaches in the future. Lastly, to improve the outcome of melanoma and to determine the biological mechanisms of efficacy or failure, future clinical studies with targeted agents will also require more stringent monitoring of biological endpoints.

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