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Growth Factors and Oncogenes as Targets in Melanoma: Lost in Translation?

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Abstract

If untreated at early stages, melanoma becomes a highly aggressive cancer with rapid metastasis to distant sites. Although cell biologic analyses have uncovered a multitude of signaling pathways involved in melanoma genesis and progression – including the MAPK, PI3K, and FAK pathways – efficacious therapies that target these cellular components have remained elusive. Genome-wide technologies such as microarray chips and array comparative genomic hybridization have generated genetic information that can identify cellular mechanisms critical for the induction and maintainence of the malignant phenotype. Thus, such data can guide the choice of a biologically relevant drug. However, these techniques have also identified melanoma as a genetically and biologically highly heterogeneous disease that likely requires individually tailored therapies based on the patient¹s individual genetic and biologic alterations. In addition, these techniques have generated a large body of data on candidate melanoma genes that await extensive functional validation to separate so called “driver” from “passenger” events. In this review, we cover several advances in melanoma therapeutics and their current limitations as well as emerging genomic, proteomic, and epigenetic strategies for the identification of critical cellular dependencies that may be tractable to therapeutic targeting.

Keywords

melanoma; growth factors; oncogenes; therapy; melanoma genome

THE DISEASE

Melanoma arises from melanocytes, specialized pigmented cells that reside in the skin, at the choroidal layer of the eye, the gastrointestinal and genitourethral mucosal surfaces and the meninges. Melanocytes produce melanins, the pigments responsible for skin and hair color. In

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the skin, melanocytes reside between keratinocytes in the basal layer of the epidermis and in the hair follicles and produce melanin to a variety of direct and indirect stimuli such as ultraviolet (UV) radiation. Thereby, melanocytes fulfill a key role in protection of our whole body from environmental stress factors such as damaging UV radiation, which can induce skin cancer.

There are an estimated 2–3 million cases of newly diagnosed skin cancers across the world each year and melanoma accounts for around 132,000 cases of these (World Health Organization, http://www.who.int/uv/faq/skincancer/en/index1.html). The incidence rates for cutaneous melanoma have risen faster than those of any other malignancy in the Caucasian population over the last 30 years. From 1976 to 2003, the Central Malignant Melanoma Registry of Germany has documented a 3-fold increase in the incidence of cutaneous melanoma, reaching 10.3 and 13.3 newly diagnosed melanomas for males and females, respectively, per 100,000 people per year [1]. In the USA incidence rates are 17.2 (males) and 12.1 (females) per 100,000 population [2], highest incidence rates are observed in Australia with 38.5 (males) and 29.5 (females) per 100,000 people (Globocan 2002 database, http://www-dep.iarc.fr/globocan/downloads.html).

Although melanoma accounts for only 4% of all skin cancers, it is responsible for 80% of deaths from skin cancer. Nowadays, timely diagnosis and treatment of melanoma during the earliest stages of its evolution is of critical importance to patient survival. Whereas surgical resection is curative in most patients with early in situ and radial growth phase melanomas (and around 80% of melanomas can be treated this way), only 14% of patients presenting with locoregional and/or distant metastasis survive for 5 years, despite all therapeutic efforts [3].

ENVIRONMENT AND PREVENTION OF THE DISEASE

In view of the epidemiological, the clinical, and the emerging experimental evidence linking melanoma incidence to UV exposure and skin phototype, primary (sun protection) and secondary (early detection) prevention strategies have been key areas to efforts for reduction of disease incidence and severity in the last centuries.

In Queensland, Australia where secondary prevention started in the 1960s and primary prevention in the 1980s, Coory et al. found sustained increases in age-standardized incidence, but stabilization of mortality rates, with a shift to detection of more early (in situ) melanomas [4, 5]. Similar results have been reported from the Central Malignant Melanoma Registry of Germany with a decrease of age-standardized mortality accompanied by a shift towards the detection of thin melanomas in patients younger than 70 years [1]. These data are being regarded as reflecting the success of earlier detection of melanoma and the authors conclude that continuation of secondary preventive strategies is warranted.

In contrast, strategies to primary prevention (sun protection) in Queensland have shown at best some tentative signs that some younger-age groups may be experiencing trend improvements [5]. Similar evidence comes from the USA, where sun protection campaigns have failed to substantially reduce melanoma incidence [6]. These results may also be reflected by epidemiologic studies that identify UV radiation-exposure, as compared to other risk factors for developing melanoma, as a surprisingly modest one (1.7-fold) in an unselected population [7]. However, the effect of UV radiation is governed by variations (polymorphisms) in particular genes that affect both the defensive response of skin (particularly its impact on cutaneous immune function) and the risk of developing melanoma, *e.g.* by induction of growth factors and reactive oxygen species (ROS) [8, 9]. At the molecular level, exposure to UV radiation increases pigmentation through release of α-melanocyte-stimulating hormone (α-MSH), which interacts with its receptor melanocortin receptor 1 (MCR1) to induce the expression of enzymes producing melanin on the surface of melanocytes. Pigmentation as a response to UV radiation

[10] is modulated by polymorphisms of MCR1, which functionally reduce the activity of this receptor [11] (as in light-skinned and red-headed individuals with skin phototype I [10]) and are significantly associated with an increased risk for melanoma [12].

CLINICAL AND MOLECULAR PATHOLOGY OF CUTANEOUS MELANOMA

Cutaneous melanoma presents clinically with four different major subtypes [13]. By far the most common form (around 75%) is superficial spreading melanoma. It presents clinically as a macule or variably raised plaque with distinct irregular coloration. It is the third most common cancer in young people in the USA and accounts for around 50% of melanoma cases in non-Caucasians [9, 14]. In contrast, the second most common form (around 15%) of melanoma is nodular melanoma, which constitutes a raised nodule, with or without a macule in the background. Acral lentiginous melanoma (ALM) is usually found on the palms, the soles and the nail beds. It accounts for about 5% of melanomas in the Caucasian and for about 50% in the non-Caucasian population. Lentigo maligna (around 5%) is generally a flat macule occurring at chronically sun-exposed body sites of the elderly and is associated with the lifetime dose of UV radiation received.

The Clark model of the progression of melanoma emphasizes the stepwise transformation of melanocytes to melanoma. The model depicts the proliferation of melanocytes in the process of forming melanocytic nevi and the subsequent development of hyperplasia, dysplasia, invasion and metastasis [13]. According to Clark's model, the first phenotypic change in melanocytes is the development of benign nevi. Histologically, such lesions have an increased number of nested melanocytes either restricted to the epidermis along the basal layer (junctional nevus), to the dermis (dermal nevus), or to both (compound nevus).

The next step toward melanoma is the occurrence of cytological atypia such as aberrant growth and dysplastic cells, which are characteristic for so called dysplastic nevi. These lesions may occur either at a new location or in a preexisting nevus. Nevi are generally benign, but can progress rarely into radial growth phase (RGP) melanoma [13]. RGP presents with a completely (in situ) or a predominantly intraepidermal proliferation of melanocytes with invasion of single cells or nests into the papillary dermis. RGP cells cannot form colonies in soft agar *in vitro*.

RGP cells can progress to vertical growth phase (VGP) melanoma, which is either confined to the papillary dermis with development of a tumor nodule or extends deeply into the dermis and/or the subcutaneous fat. These cells have the ability to form colonies in soft agar *in vitro* and tumor nodules when implanted into nude mice and have acquired metastatic potential. The distinction between RGP and VGP is the single most important pathologic observation in melanoma as most RGP melanomas are curable by surgical resection only, whereas VGP melanomas have the capacity to metastasize.

Not all melanomas strictly pass through each of these individual phases, but can directly develop from melanocytes and progress to metastatic melanoma [15].

THERAPY OF MALIGNANT MELANOMA AND ITS LIMITATIONS

Surgical excision is often curative in patients with thin primary tumors. However, the majority of patients with deeply infiltrating primary melanomas or tumors that metastasize to regional nodes will develop distant metastases later on.

Conventional Therapeutics

Over the last four decades only little, if any, progress has been made in the systemic treatment of metastatic melanoma. The alkylating agent dacarbazine (DTIC-Dome, Bayer, West Haven,

CT) has been approved by the Food and Drug Administration (FDA) in the 1970s and is considered to be the reference single agent for advanced disease. DTIC has been reported to induce objective clinical responses in about 15 to 20% of patients in older trials, but with response rates of below 10% in recent multicenter trials [16, 17]. Although complete clinical responses have been reported, most responses are partial and last for around 5–7 months [16, 17].

Interleukin-2 (IL-2) was approved by the FDA in 1998 based on a 6% complete response rate in phase II study data set of 270 patients [18]. If induced, clinical responses may be remarkably sustained (31 patients of 270 are still alive at a 10 years follow-up) [19]. In the absence of any phase III data demonstrating a clinical benefit of any dose of IL-2 in metastatic melanoma and with regard to its high and sometimes unacceptable toxicity, it is unlikely that IL-2 will be approved in Europe, particularly as long as it is not known which subgroup of patients will respond.

Targeted Therapy

Even though many if not most of the current effective therapies in cancer are targeted (*e.g.* taxanes are targeted to beta-tubulin, the fluoropyrimidines to thymidylate syntethase, and doxorubicin to topoisomerase-II), “targeted therapy” has become a well-worn password in clinical oncology today. This is the consequence of the remarkable efficacy of novel targeted agents such as small inhibitory molecules and antibodies in other cancers and led to a remarkable shift in the investigational paradigm for melanoma.

The concept of “oncogene addiction” argues that cancer cells rely more heavily on hyperactivated intracellular signaling pathways than do normal cells, and therefore rely critically on activated oncogenes that drive those pathways (reviewed in [21]). Not all oncogenes are tractable to therapeutic targeting, but intracellular enzymes such as kinases, proteases and phosphatases and extracellular antigens such as cell surface receptors are prime targets. In the following paragraph, we want to give three prominent examples of targeted therapies that have been evaluated in advanced melanoma patients.

Based on the observation that activating mutations of a serine/threonine-specific protein kinase BRAF occur in a high proportion of melanomas (for more details see later) clinical trials in advanced melanoma patients have been initiated with sorafenib, a tyrosine kinase inhibitor that targets mutant and wild-type BRAF, vascular endothelial growth-factor receptors (VEGF-R)-2 and -3, c-KIT, and platelet-derived growth factor receptor (PDGF-R)-β. Whereas sorafenib was well tolerated, it had only little or no antitumor activity [22].

It is also known that melanoma expresses a number of growth factor receptors at the cell surface and that ligands for these receptors may be present in the tumor milieu [23]. Imatinib mesylate is an oral tyrosine kinase inhibitor that targets BCR-ABL, c-KIT, PDGF-R-α and -β. As activating mutations and gene amplifications of c-*KIT*[24], as well as signal transduction through PDGF-R-α and -β have been described in melanoma, clinical trials have been initiated with imatinib mesylate in metastatic melanoma patients. Patients experienced significant toxicity, but no objective clinical responses [25, 26].

Another trial targeted epidermal growth factor receptor (EGFR), a type 1 receptor tyrosine kinase involved in cellular differentiation and proliferation of cancer cells including melanoma [27, 28]. A phase II trial in metastatic melanoma patients with erlotinib, a small molecule EGFR kinase inhibitor, revealed mild toxicity, but no objective responses [29].

All in all, no agent or combination of agents has been shown to have an impact on survival in patients suffering from metastatic melanoma and thus, no single agent can be regarded as

standard of care. The intractability of advanced melanoma painfully illustrates how much we still have to learn about the biology of this disease and the molecular changes associated with, or better, resulting in progression, metastasis and resistance to therapy. In the past, too many clinical trials have been conducted with a poor understanding of the mechanisms of action of the involved compounds and without an adequate consideration or knowledge of the biology of the disease. In other words, testing a targeted therapy when we don't know the expression pattern and don't understand the functional consequences of the expression of a target molecule in a disease-specific cellular context is a low-yield clinical research strategy.

THE KEY QUESTIONS

The etiology of transformation and progression of melanocytes to melanoma is not well understood. The most critical questions to melanoma cell biology that have to be answered are: (1) which genetic alterations are responsible for development, progression and maintenance of the established disease? (2) what genetic events underlie the propensity for metastasis and treatment resistance? (3) finally, what maintenance-essential biological or molecular signaling pathways/networks might prove amenable to preventive and/or therapeutic intervention in man?

Many studies conducted over the last decades on benign and malignant melanocytic lesions as well as melanoma cell lines have implicated numerous genes in melanoma development and progression. Here, we will focus on (1) validated genetic events in cell signaling pathways and growth factors, which include predisposing or somatic structural alterations in melanoma specimens on the DNA level, such as translocation, amplification/deletion and point mutations [30] and (2) how functional genomic information can contribute to the identification and validation of new candidate genes.

MELANOMA-RELEVANT CELL SIGNALING PATHWAYS

RAS, RAF and Other Activators of MAP Kinase Signaling

The RAS/RAF/MEK/ERK mitogen-activated protein (MAP) kinase pathway (Fig. 1) has been most directly linked to the development of melanoma due to its growth-promoting activities. Since self-sufficiency in growth signaling is a requisite capability acquired by all cancer cells [31], hyperactive extracellular signal-regulated kinases (ERKs) are common in many human cancers, including melanoma [32, 33]. In melanocytes, this pathway can be activated by growth factors such as stem cell factor (SCF), fibroblast growth factor (FGF) and hepatocyte growth factor/scatter factor (HGF) [34], but individually these growth factors induce only weak or transient ERK activation. A sustained hyperactive state can theoretically be achieved by activating mutations of any signaling mediator upstream of ERKs and there are clear tumor-type specific patterns of mutational activation. The small G protein RAS is perhaps the most frequently activated component of this signaling cascade with a reported incidence of 15 to 30% in all human cancers [35]. In the absence of gain-of-function mutations in RAS genes, BRAF alone is responsible for coupling RAS signals to MEK. However, as soon as melanoma cells acquire a mutation in RAS, cells switch their signaling from BRAF to CRAF which is accompanied by disruption of cAMP signaling, a prerequisite for CRAF signaling to MEK [36].

BRAF is also commonly targeted in human cancers with an overall mutation frequency of 7% [37], but a reported mutation frequency of up to 70% in metastatic melanoma [38–44]. ERK activation can induce transcription of genes involved in melanoma cell proliferation (*e.g. FGF-2*, *IL-8*, and *HIF-1a*), actin organization and cell motility (*e.g. PREX1, COTL1*), angiogenesis (*e.g. angiomotin-like 1*), metastasis (*TWIST1*), and immune response (*e.g. CD58*, *CD200*) [45]. However, ERK activation can also regulate differentiation, senescence and

survival. Therefore, genotype-phenotype correlation must take into account consequences other than growth promotion by activating mutations in components of ERK signaling.

The RAS Family of Proto-Oncogenes: H-, N- and K-RAS

In contrast to other solid tumors, activating mutations of *RAS* are not detected with high frequency in melanoma, ranging from low to 10–15% incidence [46] (reviewed in [47]). *N-RAS* is the most frequent *RAS* family member targeted in the melanocyte lineage, with activating mutations in as many as 81% of congenital nevi [48], up to 33% of primary and 26% of metastatic melanoma samples [49]. Activating *N-RAS* mutations have been correlated with nodular lesions and sun exposure [50, 51]. Interestingly, *N-RAS* mutations are rarely found in dysplastic nevi [50, 52, 53], which may imply their distinct evolutionary path to melanoma. *H-RAS* activation has occasionally been detected in melanoma, albeit more commonly associated with Spitz nevi, based on amplification of its genomic locus on 11p and oncogenic point mutations [54]. *K-RAS* mutations have not been described in human melanocytic lesions.

These mutation patterns point to distinct biological activities of the different RAS family members in melanocyte biology. The phenotypic impact of activated *H-RAS* versus *N-RAS* transgenic mice has reinforced this view. Specifically, an activated *H-RAS* transgene, together with inactivating mutations in *Ink4a, Arf* and/or *p53* promotes development of non-metastatic melanomas [55–57]. In contrast, when targeted to the melanocytic compartment, an activated *N-RAS* transgene and *Ink4a/Arf* deficiency drives cutaneous melanomas with high penetrance and short latency, as well as metastatic spread to lymph nodes and other distal sites (*e.g.* lung and liver) in a third of the cases [58].

BRAF, a Potent Activator of ERK Protein Kinases

The most commonly mutated member of the RAS/RAF/MEK/ERK pathway is *BRAF*, one the of the three *RAF* genes (together with *ARAF* and *CRAF*). Since its discovery through a genome-wide cancer re-sequencing effort [37], mutations in *BRAF* have been detected in a variety of tumor types, with the highest incidence in melanoma (ranging from 27 to 70%) [38–44]. *BRAF* mutations occur particularly in melanomas at body sites with intermittent UV exposure [59] and are associated with the occurrence of germline variants of the *MC1R* gene [60]. The point mutations cluster in specific regions of biochemical importance, with the predominant melanoma mutation being a single phosphomimetic substitution in the kinase activation domain (V600E), which confers constitutive activation [61]. $BRAF^{V600E}$ stimulates constitutive ERK signaling and directly and indirectly regulates expression and function of several genes critical to proliferation and survival of melanoma cells. These include transcription factors microphthalmia-associated transcription factor (*MITF*) [62], *NF-kB* [63], and the cell cycle regulators *Cyclin D1* [64], $p16^{INK4A}$ [65], and $p27^{Kip1}$ [66].

An intriguing observation is that *BRAF* mutations are also common in benign and dysplastic nevi [39, 42, 67, 68] suggesting a role in the earliest stages of neoplasia. It is notable, however, that most nevi remain growth-arrested for their whole lifetime and only rarely progress into melanoma. This raises the possibility that $BRAF^{V600E}$-induced checkpoint mechanisms exist operating to constrain malignant transformation. Indeed, a recent study showed that human congenital nevi are positive for both $p16^{INK4A}$ and senescence-associated acidic beta-galactosidase (SA-beta-Gal), the classical senescence-associated marker [65]. Furthermore, $BRAF^{V600E}$ expression alone is not sufficient to transform human melanocytes [69], but able to transform $p16^{INK4A}$-deficient murine melanocytes [70]. Thus, $BRAF^{V600E}$ alone seems not to be sufficient to induce melanoma, but induces cell cycle arrest with concomitant induction of both $p16^{INK4A}$ and markers of senescence [65]. This phenomenon is called oncogene-induced senescence (OIS), a mechanisms known to constrain progression of early premalignant lesions [71]. Together, these results argue for a model whereby $p16^{INK4A}$ serves as a brake to

BRAF^{V600E}-triggered melanocyte proliferation and p16^{INK4A} pathway inactivation is required for progression to melanoma. Notably, in melanocytic nevi expression of p16^{INK4A} is not in 100% concordance with SA-beta-Gal positivity, suggesting the presence of a non-p16^{INK4A} dependent pathway in mediating BRAF-induced OIS [65]. Therefore, it seems likely that OIS will lead to the discovery of another tumor suppressor whose importance in melanoma may rival that of p16^{INK4A}.

The notion that BRAF^{V600E} is not sufficient for transformation of melanocytes has also been demonstrated in other model systems. In zebrafish, it has been shown that BRAF activation leads only to development of benign nevi, while progression to frank melanoma requires p53 deficiency [72]. Similarly, *BRAF^{V600E}* mutation alone in TERT-immortalized *RB-p53* mutant human melanocytes was found to produce only junctional nevi in the human/mouse skin graft, in contrast to activated *NRAS* or *PI3K p110a* mutants which generated invasive melanoma lesions [73]. These biological outcomes indicate distinct roles for NRAS and BRAF activation in melanoma development. The mutually exclusive occurrence of either activated *NRAS* or *B-RAF* alleles in melanoma and other tumor types [37, 74, 75] may argue for some functional overlap of NRAS and BRAF activation, but may also be the result of a synthetic lethality between NRAS and BRAF activation at the single cell level [45].

PTEN, a Negative Regulator of the Phosphatidylinositol 3-Kinase (PI3K)-AKT Pathway

Another pathway promoting cell growth and survival in melanoma is the phosphoinositide-3-OH kinase (PI3K)-AKT pathway (Fig. 1). Phosphoinositides are membrane lipids that are converted to phosphatidylinositol phosphate (PIP_3) second messengers through hyperphosphorylation by one of the PI3K family members [76]. Integrins, extracellular matrix components such as fibronectin, and established growth factors, such as HGF and insulin-like growth factor (IGF)-1, act through this signaling pathway [77–79]. In the presence of growth factor signaling, the intracellular level of PIP_3 rises leading to phosphorylation of AKT, which is known to promote cell cycle progression and to inhibit apoptosis, and whose expression in its phosphorylated form is correlated adversely with patient survival [80]. PIP_3 second messengers are negatively regulated by the lipid and protein phosphatase phosphate and tensin homologue (PTEN) and inactivation of PTEN results in accumulation of PIP_3, AKT hyperphosphorylation, and induction of expression of genes involved in enhanced cell survival/proliferation, tumor growth and metastasis such as the cell cycle promoting kinase *Cyclin D3* [81] and the glycophosphoprotein *Osteopontin* [82]

Unlike the MAP kinase pathway, genetic alterations specifically targeting components of this signaling cascade do not occur at high frequency in melanoma [83]. Of those that do occur, the best-known culprit is the PTEN tumor suppressor. *PTEN* resides on chromosome 10q, a region known to sustain LOH in many human cancers, including melanoma [84, 85]. Allelic loss or altered expression of PTEN occurs in 20% and 40% of melanoma tumors, respectively [74, 86–88], although somatic point mutations and homozygous deletions are rarely observed. Functionally, ectopic expression of PTEN in PTEN-deficient melanoma cells can abolish phospho-AKT activity, induce apoptosis, and suppress growth, tumorigenicity and metastasis [89–91]; reviewed in [79]. Correspondingly, germline or somatic inactivation of Pten in the mouse strongly promotes tumor phenotypes in multiple cell lineages [92–95] including melanoma [96].

Most recently, additional ways of inactivation of PTEN activity have been described. PTEN can be inactivated by *poly*-ubiquitilation through NEDD4-1, which leads to PTEN degradation in the cytoplasm [97]. By contrast, *mono*-ubiquitylated PTEN localizes to the cell nucleus [97], where it antagonizes the (PI3K)-AKT survival pathway and maintains chromosomal stability through physical interaction with centromeres and control of DNA repair [98]. Whereas PTEN inactivation through NEDD4-1 alone is not sufficient to induce tumors, it significantly augments the efficiency of Ras-mediated transformation of mouse fibroblasts in the presence

of p53 deficiency [99]. This observation underlines the importance of the simultaneous activation of ERK and PI3K signaling for tumor induction, a phenomenon similarly important for melanoma development. In three-dimensional melanoma cultures as well as in the transgenic TPRas melanoma mouse model both signaling pathways must be inhibited to suppress cell growth [100, 101]. In melanoma specimens, *NRAS* and *PTEN* mutations are mutually exclusive, but *BRAF* and *PTEN* mutations have been shown to coincide in about 20% of cases [59, 102].

In line with the experimental evidence supporting a melanoma suppressive role of PTEN, constitutive activation of AKT has been shown to be a potent inducer of melanocyte transformation [73] and progression of RGP into VGP melanoma *in vivo* [103]. In addition, DNA copy gain involving the *AKT3* locus has recently been described in melanoma, and selective AKT3 activation may characterize 40–60% of sporadic tumors [104]. However, the complexity of this signaling cascade has not been fully understood till today. Recent data have suggested that activation of different AKT isotypes may elicit distinct effects on cell proliferation and survival. For example, one report found that targeted deletion of AKT3, whose expression correlated most strongly with melanoma tumor progression amongst the three AKT isotypes, triggered apoptotic signaling [104]. On the other hand, AKT1 activation was found to inhibit the migration and invasion of certain cancer cell lines [105], including MDA-MB435, a line previously believed to derive from breast cancer but subsequently shown through transcriptional and SNP array profiling studies to be a melanoma cell line [69, 106]. Thus, although the PI3K/AKT pathway clearly demonstrates enhanced activity in many melanomas, the extent to which this constitutes a critical melanoma dependency remains unresolved.

Activation of Receptor Tyrosine Kinases

Considering the prominent roles of RTKs in transmitting extracellular signals to intracellular effectors, and the importance of homotypic and heterotypic cell-cell interactions in cancers, it is not surprising that almost all of the direct signaling components of RTKs have been implicated in the development of human melanoma (Fig. 1). Several RTKs map to known regions of recurrent DNA copy number gain or amplification. Moreover, considering the example of c-KIT (see below), it is expected that systematic re-sequencing efforts will identify activating mutations in these and other RTKs in melanomas.

The *c-KIT* gene encodes a RTK that serves as the receptor for stem cell factor (SCF). The regulation of the KIT pathway is complex and tightly regulated. A number of isoforms are known for the receptor and its ligand, and the ligand can interact with the receptor in both soluble and membrane-bound forms [107]. Binding of soluble SCF leads to KIT receptor activation, internalization and degradation, whereas binding of membrane-bound SCF leads to prolonged KIT activation. The KIT receptor can interact with multiple downstream signaling pathways including RAS/RAF/MEK/ERK, PI3K/AKT, phospholipase C, and the SRC family. The mechanisms underlying the differential activation of these pathways are not understood till today. In melanocytes, KIT plays a critical role in migration, survival, proliferation and differentiation. Mice deficient in Kit activation loose melanocytes [108] and KIT inhibition appears to drive melanocyte cell loss in human skin [109], indicating that KIT is a critical survival factor for melanocytes. KIT is also responsible for melanocyte cell proliferation. *In vitro*, KIT activation induces proliferation of cells of the melanocytic lineage [110] and transgenic mice overexpressing the membrane-bound form of SCF in the epidermis develop melanocytic hyperplasia [111]. Most recently, the D814Y activating KIT mutation has shown to induce PI3K signaling and migration of melanocytes [112].

Numerous immunohistochemical studies have linked progressive loss of c-KIT expression with the transition from benign to primary and metastatic melanomas [113–115]. Reconstitution of c-KIT in metastatic melanoma cells apparently conferred sensitivity to SCF-induced apoptosis *in vitro* [116]. Thus, at first glance, KIT does not fit the profile of a RTK targeted for

activation in melanoma. However, a recurrent L576P mutation in c-KIT has recently been reported in melanoma. Among 153 cases examined, Holden and colleagues identified four metastatic melanomas with robust expression of c-KIT on IHC. High-resolution amplicon melting analyses followed by direct DNA sequencing revealed that three of them harbored a L576P mutation with selective loss of the normal allele [117, 118]. L576P is a known GIST-associated mutation that maps to the 5′ juxtamembrane domain where most activating KIT mutations cluster [119, 120]. Most recently, Bastian and colleagues reported in a chort of 102 primary melanoma samples on the presence of activating *KIT* mutations and gene amplifications at a frequency of 28 to 39% in particular subtypes of melanomas, *i.e.* acral melanoma, mucosal melanoma, and melanomas from chronically sun-damaged skin. 69% of the identified *KIT* mutations were predicted to affect the 5′ juxta-membrane domain and 19% the kinase domain [24]. The example of the *EGFR* mutational status as a predictor for therapeutic responses in NSCLC [121, 122] suggests the possibility of identifying a melanoma patient subpopulation that will respond to the c-KIT inhibitor imatinib mesylate based on c-KIT mutational status.

Activation of EGFR by its ligands EGF, transforming growth factor (TGF)-α, amphiregulin and heparin-binding EGF (HBEGF) has been shown to activate several downstream signaling pathways such as the MAPK pathways, the PI3K-AKT pathway, the stress-activated protein kinase C and the Janus kinase (Jak)- signal transducer and activator of transcription [16] pathway and thus critically regulates cell differentiation, proliferation, survival, and migration [123]. Late stage melanomas often exhibit EGFR over-expression in association with increased copies of chromosome 7 [124–126]. Enforced activation of EGFR has been associated in metastatic progression in a cell-based study [127, 128]. However, unlike glioblastomas or lung adenocarcinoma [129, 130], focal amplification and/or mutation of *EGFR* have not been reported in melanoma. The non-focal nature of chromosome 7 gains in melanoma renders it impossible to assign EGFR as a target of such genomic alterations. In an inducible HRAS-driven mouse melanoma model [131], transcriptome analysis revealed the existence of a RAS-dependent EGFR signaling loop mediated through upregulation of EGF family ligands (e.g. amphiregulin and epiregulin) [132]. This EGFR signaling pathway provides important survival signals involving PI3K-dependent activation of AKT, as sustained EGFR activity is able to prolong viability of established melanoma upon inactivation of RAS. Conversely, inactivation by dominant negative EGFR abolishes tumorigenicity of RAS-driven melanoma cells, consistent with observations in other cell systems (fibroblasts, keratinocytes, and intestinal epithelial cells) that autocrine EGFR signaling is required for transformation by activated RAS [133–135]. Thus, in addition to providing experimental evidence that EGFR activation is biologically relevant, the above-mentioned study in the inducible model also points out the possibility that EGFR or its ligands may constitute alternative point(s) of therapeutic intervention in RAS-activated melanoma. It should be mentioned that the contribution of EGFR signaling to melanoma development and possibly progression is evolutionarily conserved, as activating mutations in the EGFR homologue, Xmrk, increase melanoma susceptibility in *Xiphophorus* fish [136–138]; reviewed in [139]. It therefore remains possible that similar activating mutations exist in human melanoma, although systematic re-sequencing of large cohorts of melanomas from different ethnic and/or molecular subclasses will be required to uncover such examples.

The RTK c-MET is normally expressed on epithelial cells and melanocytes [140] and is activated by binding of its ligand, HGF, through a number of downstream signaling pathways including RAS/RAF/MEK/ERK, PI3K, phospolipase c-g and STAT [47, 141]. c-MET is a multifaceted regulator of growth, motility and invasion in a number of cell lineages. Whereas RAS/RAF/MEK/ERK signaling may be responsible for proliferation, PI3K signaling is required for scattering and these two pathways in combination with STAT signaling may be important in morphogenesis [142–144]. Although MET is normally activated in a paracrine manner, autocrine activation of HGF–MET has been described in melanoma progression [145]; reviewed in [146].

Accordingly, increased c-MET expression has been observed in metastatic melanoma [147], and copy number gain of the *c-MET* locus at 7q33 - qter seems to be a late event in melanoma progression [148]. However, similar to EGFR above, neither focal *MET* amplifications nor activating *MET* point mutations have been detected in melanoma, although both have been observed in other human cancers [149–152]. However, several lines of experimental and functional evidence support a causal role for MET signaling in human melanoma. For example, in explant models, it has been shown that elevated c-Met expression or Met receptor tyrosine kinase activity may correlate with metastasis [153]. In genetically engineered models, constitutive and ubiquitous HGF expression establishes an autocrine loop with c-Met, leading to step-wise development and progression of cutaneous and metastatic melanomas, which cooperates with UVB, Ink4a/Arf deficiency, and activated Cdk4 [154–156]. Correspondingly, while enforced expression of c-Met in melanocytes provides only weak cancer-initiating activity, this mutation drives the development of metastatic disease, and such tumor lesions show concomitant activation of HGF and establishment of HGF-Met signaling loop (LC, unpublished observations). Finally, *c-MET* was recently shown to be a direct transcriptional target of MITF [157], the melanocytic lineage transcription factor that can be activated by focal amplification in melanoma (see below).

THE MELANOMA GENOME

In probing the cancer genome for novel targets, technological advances have enabled the rapid production of genome-wide datasets informative for changes in gene expression, DNA copy number, and loss of heterozygosity. Such assays have revealed in melanomas an increasingly complex pantheon of genetic aberrations beyond the established ones described above. Novel tumor suppressors and oncogenes are continuing to be described, but many validation steps are required before any gene can be considered bona fide therapeutic targets. Effects on tumor initiation, growth, or invasion must be experimentally demonstrable both *in vitro* and *in vivo*.

Typically, experiments in cell culture make use of overexpression vectors, knockdown vectors such as siRNA or shRNA, drug inhibitors, or competitive antibodies to modulate gene expression or product levels. Cells can then be assayed for their ability to form colonies in soft agar, indicative of anchorage-independent growth and loss of contact inhibition. Growth curves can be measured, and invasion can be tested in Boyden chambers, which simulate the initial steps of metastatic invasion through the extracellular matrix. Three- dimensional cultures, including organotypic skin rafts and collagen matrices, may better portray the conditions of the *in vivo* environment. Culture preparations can also be injected subcutaneously into athymic mice, and the incidence, growth, and invasiveness of the resulting xenograft tumor is considered more clinically relevant than cell culture. Tail or portal vein injections and the observation of subsequent metastasis formation can measure the intravasation and seeding steps. Finally, genetically engineered mouse models can provide strong evidence for a gene's role in melanoma, as cell type-specific gene knockouts, knockins, and transgenics can recapitulate human mutations in the context of established mouse melanoma models (see above). A number of studies employing genome-wide assays are catalogued in Table 1, along with the extent of candidate gene validation performed in each.

As can be seen, the majority of gene discovery studies have utilized gene expression arrays. For example, comparisons of metastatic and non-metastatic cell lines, the latter with an engineered extra copy of chromosome 6, identified CRSP3 and TXNIP as correlated with the suppression of metastasis [158]. Although overexpression of either gene did not affect tumor growth *in vitro* or in xenografts, they significantly and independently suppressed the formation of metastases to the lung when the cells were injected either subcutaneously or intravenously. Preliminary evidence suggested that both genes operate through the KISS1 (Kisspeptin-1) pathway, which may affect cell motility [159]. Others [160] used a similar set of cell lines with

or without an extra chromosome 6, with the latter displaying anchorage-independent growth in soft agar and a downregulation of the connexin gene CX43. Overexpression of CX43 significantly suppressed the soft agar colony formation. As a final example, tenascin and fibronectin were upregulated in progressed tumors, and their co-localization confirmed by IHC [161]. Overexpression of either gene conferred growth and invasive behavior in three-dimensional collagen gels.

Combinations of genome-wide technologies – expression microarrays, array comparative genomic hybridization, and SNP microarrays – can together define a genomic atlas of recurrent DNA and gene regulatory aberrations. As the accumulation of genome-wide data continues to outpace the technology to validate individual genes, basic cancer research is faced with a need to isolate bona fide therapeutic targets. The brief survey of articles in Table 1 indicates that we are faced with a wealth of candidate melanoma modifiers, each with a long experimental process ahead of it (it should be noted, however, that many of the studies with a smaller extent of validation achieved goals other than gene discovery.) The extents to which these listed genes truly control melanoma behavior and thus represent promising therapeutic targets await further testing, as illustrated by the examples of NEDD9 (neural precursor cell expressed, developmentally down-regulated 9) and MITF below.

NEDD9

The power of array technology further benefits from comparisons among species, with the evolutionary conservation of genetic pathways providing a means to highlight significant changes; this is evidenced by the novel cross-species identification of Nedd9 as a regulator of melanoma metastasis. The Ink4a/Arf$^{-/-}$, inducible H-ras mouse melanoma model [55, 131] provided a tractable means by which to create isogenic metastatic and non-metastatic tumors. Selective pressure for “escaper” tumors no longer dependent on the doxycycline-induced H-ras signal was generated by alternating the signal over defined time periods. Two such lines proved metastatic to distant sites in xenografts. DNA copy number profiles of these lines and a non-metastatic sister line were compared by aCGH, pinpointing an 850kb common site of amplification on chromosome 13. Only one gene in this region, Nedd9, showed a significant upregulation in mouse melanomas but not in normal melanocytes. The syntenic human region, 6p24-25, undergoes copy number gain in 36% of human metastatic, but not non-metastatic, melanomas [124, 162], indicating a common route of genetic modification between human and mouse. Indeed, human melanoma tissue microarrays (TMAs) revealed a significant correlation of Nedd9 protein levels with tumor progression.

Illustrating the power of candidate gene validation, Nedd9 showed metastasis-modulating activity at multiple levels. In Boyden chamber assays, overexpression of Nedd9 enhanced the invasiveness and growth of non-metastatic Ink4a/Arf$^{-/-}$ cell lines expressing activated H-Ras or B-Raf, while knockdown by shRNA inhibited invasiveness in the original metastatic escaper line. Demonstrating this bidirectional behavior revealed the dependency of the cell line on Nedd9 for invasive potential. Nedd9 also conferred a capacity to metastasize to distant sites when overexpressing cells were injected subcutaneously into SCID mice, providing more clinically relevant *in vivo* evidence. Finally, the mechanism of Nedd9 action was dissected with biochemical and genetic assays, which showed focal adhesion kinase (Fak) to be a critical mediator of Nedd9-induced *in vitro* invasion, formation of dynamic focal contacts, and attachment to matrigel. These properties are considered hallmarks of cell motility and a capacity for extravasation into the bloodstream.

The identification of Nedd9 as a cross-species melanoma metastasis gene reinforced two major concepts: that the fundamental properties of cancer genetics are conserved and thus highlighted between mouse and human and that modulation of a single genetic pathway can greatly alter

tumor fate. The ongoing research into creating and testing mice genetically engineered to over- or underexpress Nedd9 moves it closer to a thorough conception for its clinical translation.

MITF

The identification of MITF as a critical melanoma survival gene took a cross-tissue approach, wherein the NCI-60 cell line panel representing nine tumor types was subjected to both gene expression and SNP array analysis [69]. A recurrent gain of 3p13-14 significantly segregated melanoma from other tumor classes and was confirmed by quantitative PCR (qPCR). The combined expression and qPCR data isolated MITF as the only gene in the region with maximal amplification and overexpression. Fluorescence in situ hybridization and immunohistochemistry on melanoma TMAs revealed a correlation of increased MITF gene dosage and protein levels with malignancy and decreased survival. Correlation, however, does not discriminate between MITF upregulation as a cause or bystander effect of malignant transformation. Therefore, the effect of exogenous MITF was determined in human melanocytes engineered to be immortalized but non-transformed. Only when MITF was expressed in a melanoma-relevant signaling context (*i.e.* activated BRAF) cells were able to grow in the absence of otherwise essential media factors and able to form colonies in soft agar, both results suggestive of full transformation. Conversely, inhibition of MITF in cell lines showing 3p13-14 amplification reduced growth and survival and conferred sensitivity to certain anticancer drugs. This careful validation of MITF in human cells sets the stage for *in vivo* mouse studies.

By comparing different tumor types, genetic changes specific to the relevant lineages were highlighted. The critical role of MITF in both normal melanocyte development and melanoma survival was therefore suggested to identify, along with androgen receptor, a novel class of oncogenes termed “lineage addiction” oncogenes [163]. The tumor may “hijack” extant lineage survival mechanisms in the presence of selective pressures; indeed, activated BRAF is known to target MITF for proteolytic degradation, which may select for refractory cellular variants with amplified MITF. Tellingly, MITF gene disruption leads to coat color graying in mice [164, 165] and pigmentation and hearing defects (melanocytes play a role in cochlear development) in humans, termed Waardenburg Syndrome Type 2A [166]. The demonstration that a gene involved specifically in melanocyte maintenance and differentiation is dysregulated in melanomas opens the possibility that anticancer drugs can be targeted not only to specific cellular pathways, but also to specific cell types.

Epigenetics, miRNAs, and proteomics

Even with a combination of the three main avenues of genome-wide interrogation – gene expression, DNA copy number, and LOH – a full picture of the changes that take place in tumorigenesis is lacking. The intracellular milieu has many levels of regulation, both at the DNA and protein levels. Thus, a number of other large-scale technologies will build towards a more comprehensive view of melanoma: epigenetic profiling, screening for non-coding but functional DNA, and proteomic profiling (Fig 2).

Epigenetic studies in cancer have focused on the role of both DNA methylation and histone modifications to understand how genes can be aberrantly regulated in the absence of disruptive mutations. The methylation of cytosines within CpG islands upstream of gene promoters results in gene silencing, while methylation at other sites may activate genes, such as at the differentially methylated region of *Igf2* (insulin-like growth factor 2) [167]. In conjunction, the “histone code” represents a complex combination of modifications of specific histone tail residues, with certain methylation and acetylation (and, perhaps to a lesser extent, phosphorylation, ubiquitination, and sumoylation) patterns governing chromatin structure and hence gene expression. Though the interaction of DNA and histone modifications continues

to be clarified, recent studies suggest that silencing via histone methylation may supercede the cytosine methylation status [168].

Hence, genome-wide epigenetic scans can be either DNA-specific – methylation-specific digital karyotyping [169], restriction landmark genome scanning [170], or ChIP-Chip [171] – or histone-specific, such as applying expression arrays to cells treated with histone deacetylase (HDAC) inhibitors [172]. One study made use of the DNA methylation inhibitor 5-Aza-2′-deoxycytidine to compare treated and untreated melanoma cell lines [173]. The genes SYN and HOXB13 were found to be significantly re-expressed upon treatment, and were also reduced in untreated cell lines compared to normal melanocytes. Overexpression of each gene individually reduced *in vitro* proliferation and *in vivo* xenograft tumor size, indicating a tumor suppressor role for both genes. Other current evidence points to a pivotal role for the epigenetic regulation of specific genes in melanoma progression: focal comparisons of primary and metastatic tumor specimens have confirmed differential methylation statuses of peroxiredoxin 2 [174], skeletrophin [175], and estrogen receptor (ER)-α [176]. Intriguingly, ER-α [176] and PTEN [177] methylation are increased in DNA circulating in the serum of patients with metastatic disease, suggesting that they may be useful as clinical indicators. Furthermore, HDAC inhibitors show some initial promise in cell lines and xenografts [178], supporting global epigenetic changes as relevant for tumor maintenance.

Long referred to as “junk DNA,” certain non-coding genetic elements have also recently taken a prominent spot in gene regulation. Of particular interest are miRNAs, which are short, 21-23bp RNAs that bind to homologous mRNA segments and mediate the translational blockage or argonaute-directed degradation of gene transcripts as well as initiate epigenetic gene silencing modifications. Multi-platform assays have identified an enrichment for miRNAs in various solid cancers that target known tumor suppressors and oncogenes [179]. The same group discovered that miR155 can function as an oncogene in B cell lineages, with Eμ-directed overexpression resulting in the development of leukemia and lymphoma [180]. Whether miRNAs play a significant functional role in melanoma remains to be seen, though initial studies on different tumor types demonstrate that melanoma can be differentiated from other tumor types based solely on miRNA expression signatures [181] and that each of 243/283 miRNAs exhibited copy number changes in at least seven of 45 melanoma cell lines [182].

Finally, gene expression levels do not necessarily correlate with protein levels, due to posttranslational modifications undetectable by gene-centric assays. Comprehensive analysis of the tumor proteome is a technically challenging process, requiring the use of low-throughput biochemical isolation techniques (2D-gel electrophoresis, protein chips) and specialized machinery for protein identification and quantification (MALDI-TOF). Nevertheless, such analysis of a mouse melanoma xenograft model identified associations of increased VEGF and cathepsin D levels with progression [183]. Proteomics also provides a plausible method for non-invasive prognostic tests: the MALDI-TOF analysis of patient sera identified a protein spectrum that could retrospectively classify 55 progressive and non-progressive stage III tumors with 80% specificity [184]. Such proteomic data awaits predictive testing. Continuing technology improvements will necessitate larger scale studies towards a more complete understanding of the melanoma cell.

STRATEGIES FOR TRANSLATION OF GENETIC INFORMATION INTO MELANOMA THERAPY

All in all, melanoma must be regarded as a genetically and biologically heterogeneous disease. This heterogeneity is exemplified in the clinics by the differential and until today unpredictable response to therapy. Rational drug design has significantly changed the daily practice of clinical oncology through introduction of small molecule inhibitors and antibodies, *e.g.* against

overexpression of HER2/NEU in breast cancer, activating c-KIT mutations in gastrointestinal stromal tumors, and EGFR mutations and overexpression in non-small cell lung cancer. What could be the prospects for the paradigm of genetic targets to melanoma?

Due to the high incidence of activating mutations in genes of the RAS/RAF/MEK/ERK signaling pathway, BRAF targeting has been predicted as a promising therapeutic strategy for melanoma, but failed to accomplish meaningful clinical activity [22]. A possible explanation for this could be that the agent itself may be insufficiently potent. However, with a more complete understanding of the genetic and functional data of this pathway -as outlined above- we favor explanations suggested by genetic and functional data: (1) $BRAF^{V600E}$ alone is not sufficient to cause melanoma, but induces cell cycle arrest with concomitant induction of oncogene induced senescence, (2) BRAF activity is modulated by the genetic background at the single cell level, *e.g.* the presence or absence of control mechanisms such as $p16^{INK4A}$, (3) RAS/RAF/MEK/ERK signaling switches from BRAF to CRAF signaling upon the acquisition of RAS mutation, (4) pathway redundancy or digression may occur: in contrast to targets successfully targeted in other cancers, the most frequent mutation of the RAS/RAF/MEK/ERK signaling pathway lies several steps downstream of the initial receptor-ligand interaction, thus favoring the recruitment of alternative pathways of cell signaling, (5) the development of resistance mechanisms: several melanoma cell lines survive MAPK inhibition by expression of the antiapoptotic factors Mcl-1, Bcl-x_L and Bcl-2 and suppression of tumor suppressor p53 through ROS [185].

Unlike the RAS/RAF/MEK/ERK pathway, genetic alterations specifically affecting components of other established signaling cascades do occur only in a small proportion of melanomas. This complex and heterogeneous genetic and biological background significantly challenges the identification of targets for drug development and should preclude the unselected enrollment of melanoma patients into clinical trials. In this context, strategies towards a genetic-based patient selection and individualized therapy have to be developed. In the last few years, we have been witnessing the establishment of first genotype-phenotype correlations. Examples are the reported association of activating *N-RAS* mutations with nodular lesions and sun exposure [50, 51], the occurrence of *BRAF* mutations particularly in melanomas from body sites with intermittent UV exposure [59] and their correlation with germline variants of the *MC1R* gene [60], the presence of *CDK4* amplifications preferentially in acral and mucosal melanomas [59], the presence of *Cyclin D1* amplifications particularly in melanomas from skin with chronic sun damage [59], and the presence of activating *KIT* mutations and gene amplifications in acral melanoma, mucosal melanoma, and melanomas from chronically sun-damaged skin [24].

However, these correlations can provide only a rough estimate on the presence of certain types of genetic alterations and are far from being predictive for the most appropriate avenue of personalized treatment. In this regard, individualized genomic information can provide significant contributions.

OUTLOOK: INDIVIDUAL GENETIC TYPING FOR GENOTYPE-PHENOTYPE CORRELATIONS

Success in identifying gene expression signatures predictive of survival in breast and other solid cancers [186, 187] establishes a real possibility that the molecular profiling of melanoma can inform clinical decisions. Recent descriptive, retrospective observations of gene expression in metastatic versus non metastatic tumors [188–190] provide a tantalizing glimpse into what these signatures may look like, but they must be functionally validated in order to have true clinical relevance.

Indeed, the ability to classify melanomas by particular genomic signatures suggests such a predictive capacity. For example, CGH profiling could distinguish among 126 acral, musocal, and chronically and non-chronically sun-damaged primary skin melanomas with 70% accuracy [59]. BRAF and NRAS mutations were significantly associated with the non-chronically sun damaged subtype, suggesting that knowledge of common gene mutations alone could provide a degree of classifying information. In fact, a microarray analysis of cell lines with and without CDKN2A deletions, coupled with confirmatory RT-PCR on 14 cell lines, identified eight genes consistently differing in expression between the two classes [191]. Conversely, although two studies supported an expression profile difference between NRAS and BRAF mutant tumors [192, 193], others employing stricter statistical parameters did not [188, 194]. Overall, genome-wide profiling may provide more specific classification than single-gene sequencing.

In the farther future, the feasibility of personal whole-genome sequencing comes ever closer as sequencing costs continue to drop and novel techniques continue to be rapidly developed. Recent initiatives to exhaustively sequence annotated genes in breast and colon cancers have unearthed only a fractional minority of putatively functional aberrations [195]. Given the rich diversity of cellular regulation illustrated above, any future clinically-oriented sequencing efforts may need a massive supporting network of data to have true prognostic value.

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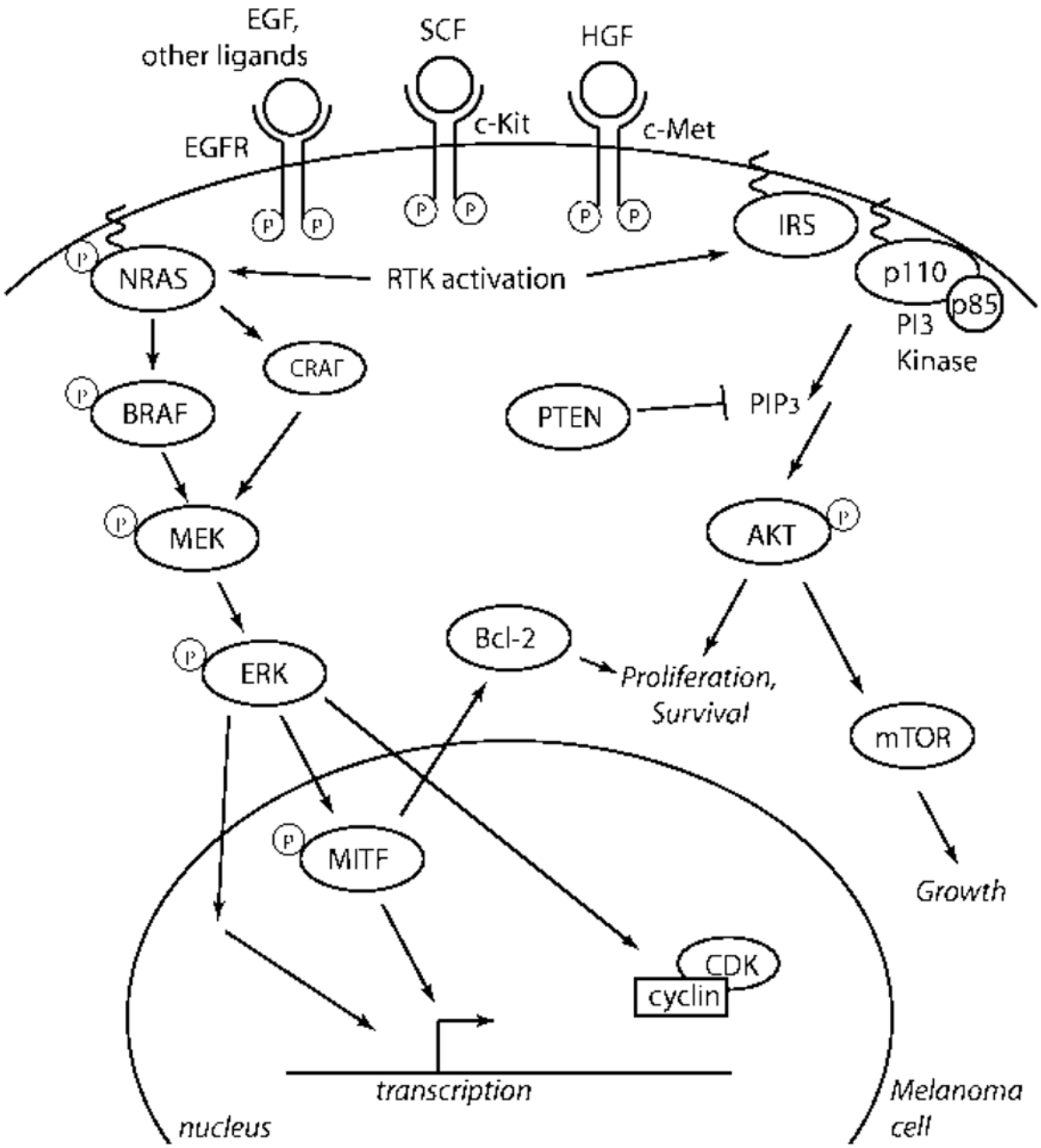

Figure 1.
Signaling pathways important in melanoma. RTKs activate both the MAPK and PI3K pathways, which together promote mitogenesis and survival.

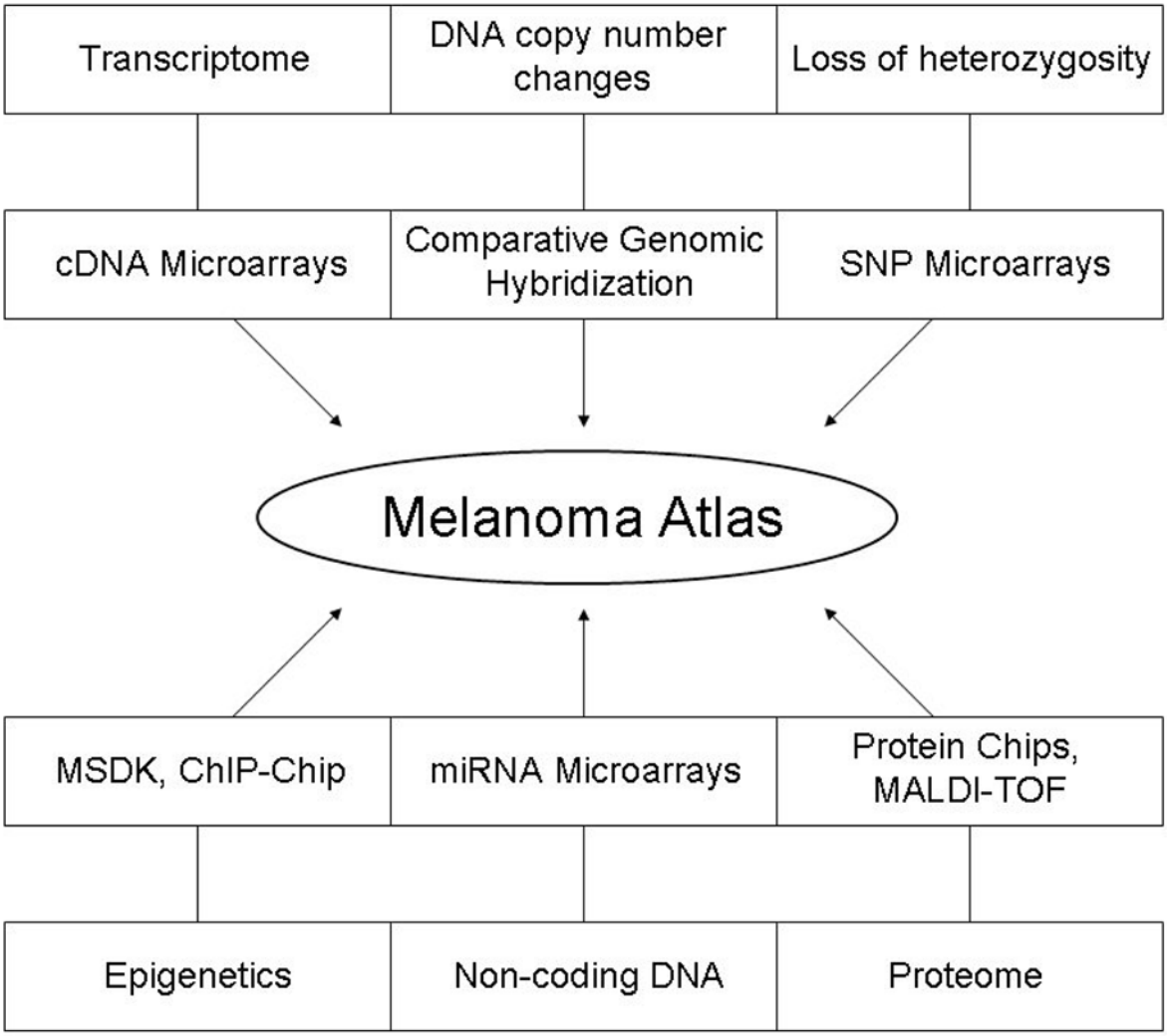

Figure 2.
Types of cellular data and some of the respective high-throughput technologies.

Table 1

The extent of validation for recently identified genes in melanoma. **E** (Expression validaton) is achieved by RT-PCR, northern, or western blots, while **I** (IHC or tissue microarrays) refers to histological protein analysis. **O** (Overexpression), **K** (Knockdown/dominant negative) and **X** (Xenograft) refer to manipulation of gene expression in cell lines. **M** (Mechanism) indicates studies of interactions with putative pathway members. **P** (Patient survival data) indicates a matched Kaplan-Meier analysis.

Sample[a]	Array	Genes	Proposed gene	Extent of validation							Ref
				E	I	O	K	X	M	P	
T[b]	aCGH	NEDD9	Metastasis	•	•	•	•	•	•		Kim, et al, 2006
C	SNP/cDNA	MITF	Oncogene	•	•	•	•			•	Garraway, et al, 2005
C	cDNA	CRSP3,	Metastasis	•		•		•	•		Goldberg, 2003
C	cDNA	SYN,	Tumor suppressor	•		•		•			Muthusamy, et al, 2006
C	cDNA	CX43	Oncogene	•		•					Su, et al, 2000
C, T	cDNA	TN-C, FN	Metastasis		•	•					Kaariainen, et al, 2006
T	cDNA	PTGDS,	Multiple	•	•					•	Winnepenninckx, et al, 2006
T	cDNA	HLA-DR,	Lymphocytic		•					•	Mandruzzato, et al, 2006
C	cDNA	TWIST1,	Metastasis	•	•						Shields, et al, 2007
T	cDNA	DSC1,	Multiple	•	•						Jaeger, et al, 2006
C	cDNA	Mitf/Tgfb-	Progression	•	•						Hoek, et al, 2006
T	aCGH	KIT	Oncogene		•						Curtin, et al, 2006
C	cDNA	TSPY	Marker,	•							Gallagher, et al, 2005
T	cDNA	PLAB,	Multiple	•							Talantov, et al, 2005
C	SNP	PTPRD[c]	Multiple								Stark and Hayward, 2007
T	cDNA	TNFSF10[c]	Proapoptotic								Jensen, et al, 2006

[a] T = tissue, C = cell lines; all human, but see footnote b

[b] Mouse and human tissues

[c] Sampling of identified genes