Anja K. Bosserhoff *Editor*

Melanoma Development

Molecular Biology, Genetics and Clinical Application

Second Edition

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ISBN 978-3-319-41317-4 ISBN 978-3-319-41319-8 (eBook)
DOI 10.1007/978-3-319-41319-8

Library of Congress Control Number: 2016959044

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The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

This book, *Melanoma Development: Molecular Biology, Genetics and Clinical Application*, in its second edition provides a comprehensive insight into the molecular changes of malignant melanoma and implications for therapeutic approaches. In this updated version, all chapters were revised and important new developments and findings of the recent 4 years were added.

The recent clinical success in treating melanoma by inhibitors to mutated BRAF or activated MEK or by stimulating the immune system by checkpoint blockage is very encouraging. After years without strong effects of therapeutical attempts, there is hope that melanoma is curable or at least can be shifted into chronic disease. Of course, more research is strongly needed for a comprehensive understanding of the molecular processes leading to melanoma and of ways to therapy resistance. Nevertheless, the first steps are made with possibilities leading to significantly prolonged survival of the patients with metastasized melanoma. This is a highly inspiring and promising development which should further boost melanoma research.

I am again very grateful to all the authors for their interesting, forward-looking contributions and for their support of this book project. I feel that this book, after the success on the first edition, will further enhance the development in the field of melanoma research. *Melanoma Development: Molecular Biology, Genetics and Clinical Application* aims to contribute to this body of knowledge.

Erlangen, Germany
June 2016

Anja K. Bosserhoff, PhD

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1 Clinicopathologic Overview of Melanoma

Anja Bosserhoff and Luigi Strizzi

Human melanoma is the most rapidly increasing malignant skin disease in Caucasians (Siegel et al. 2015). Once considered a rare disease, the lifetime risk for developing melanoma in the US has increased from approximately 1 in 1500 during the 1930s to its present risk of approximately 1 in 60 (Giblin and Thomas 2007). The American Cancer Society's recent cancer report estimates that 76,380 new cases of melanoma will be diagnosed and 10,130 deaths will result from melanoma during 2016 in the United States (American Cancer Society 2016). Important risk factors for developing melanoma include increased number of melanocytic nevi, a family history of melanoma, or a history of previous melanoma (Seykora and Elder 1996; Psaty et al. 2010). Prolonged sun exposure associated with increased outdoor activity has been suggested to play an important role in the epidemiologic increase in the incidence of melanoma (Leiter and Garbe 2008; Moan et al. 2008). Acute exposure of the skin to ultraviolet radiation (UVR) can induce varying degrees of erythema, pigmentation, and impairment of immune function (Matsumura and Ananthaswamy 2004). Increased numbers of melanocytic nevi associated with sunburn and intermittent or "holiday" sun exposure has been suggested as a major risk factor for developing melanoma in different studies (Elwood and Jopson 1997; Newton-Bishop et al. 2010). In fact, the Clark model for melanoma suggests a stepwise progression from hyperplastic and dysplastic nevi to melanoma (Clark et al. 1984).

From a clinical perspective, melanocytic nevi are benign proliferations that appear as flat or slightly raised pigmented growths generally found on sun-exposed skin. Histologically, these are formed by proliferating melanocytes that gradually

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_1

assume a more round or oval shape from their normal dendritic-like morphology, forming nests along the basal layer and growing towards the dermis as cords. As the nevus cells grow into the dermis, melanin production significantly decreases as tyrosinase activity is progressively lost in a process known as “maturation.” Dysplastic nevi can progress from preexisting benign nevi or form ex novo in a new location. Clinically, they are larger than most benign nevi, have irregular borders, and appear with varying degree of pigmentation. Dysplastic nevi are composed of irregularly shaped cells with hyperchromic nuclei. Discordance in the diagnosis of benign, dysplastic, and melanoma based on morphology alone, however, has continued to plague even experienced pathologists (Ackerman 1996; Lodha et al. 2008; Shoo et al. 2010). Different molecular biomarkers have been proposed to help to differentiate benign nevi from malignant melanomas and are still being validated. Recent studies, for example, have claimed high success rates in discriminating benign lesions from melanoma with the use of multiple tissue marker arrays (Kashani-Sabet et al. 2009) or different fluorescence in situ hybridization (FISH) DNA probes (Gerami et al. 2009). The ideal goal would be to develop high throughput analytical systems that would increase the feasibility of adopting such multimarker approaches at all diagnostic centers.

There remains the fact that some melanomas can also form in areas of the body not exposed to the sun, such as mucous membranes (DeMatos et al. 1998; Das et al. 2010) or arise independently of previous nevi suggesting that other factors, alone or in combination, are also involved in the pathogenesis of melanoma. Recent advances in the understanding of the different cellular signaling events in melanoma have shed some light on the identification of potential underlying molecular mechanisms. To this regard, downstream signaling events caused by mutations in NRAS and BRAF as well as PI3K/Akt, MAPK/ERK, and c-KIT activity have been found to play a role in melanoma signaling (Kyrgidis et al. 2010). Since exposure to UVR can lead to DNA damage, sun exposure may represent an obvious cause of these mutations. However, the identification of some of these genetic mutations in congenital nevi as well seems to argue against the fact that UVR is the sole culprit of these mutations. As mentioned previously, a family history of melanoma is an important predictor of melanoma risk (Psaty et al. 2010), indicating that genetic predisposing factor(s) must also play a role during melanomagenesis, as for instance, with CDKN2A, where up to 40 % of members of melanoma-prone families show germ-line mutations in this tumor suppressor gene (Hansson 2010).

As described in the Clark model for melanoma progression, early melanoma is characterized by localized growth referred to as “radial growth phase” or “thin melanoma.” During this phase, the melanoma cells tend to grow between the layers of the epidermis and superficial dermis with significantly low risk for metastasis. Surgical excision of these relatively flat lesions is associated with high cure rates. With time, the melanoma assumes a more nodular appearance as the relatively larger and irregular melanoma cells begin to penetrate vertically and invade the dermis (“vertical growth phase”). The depth of invasion of the melanoma related to the anatomical structures of the dermis and subcutaneous tissue or measured directly in millimeters is used to predict clinical outcome with deeper (or thicker) lesions associated with

reductions in overall patient survival (Balch et al. 2009). Other factors, such as the presence of tumor ulceration or increased mitotic rates, also negatively affect prognosis (Balch et al. 2009). The metastatic process of melanoma is facilitated once melanoma cells begin to invade vascular and lymphatic structures. At this point chemotherapy is the therapeutic option of choice. Current treatment strategies for advanced stage melanoma employing cytotoxic agents are often accompanied by important side effects and associated with relatively low percentages of objective response rates (Atallah and Flaherty 2005). Similarly, molecular redundancy and cross-talk between multiple signaling pathways appear to have undermined the efficacy hoped to achieve with targeted molecular biotherapy (Shain and Bastian 2016). Generally, anti-cancer drugs appear to have some initial effect usually due to killing of the majority of the cancer cells sensitive to the chemotherapeutic agent. However, increased signaling of cell survival pathways, enhanced DNA repair mechanisms or mutations of molecular targets in melanoma cells often lead to resistance to therapy. Attempts continue at identifying novel diagnostic markers and molecular targets important for melanomagenesis and disease progression. Work is also needed to detect and quantify chemoresistance and to better understand the molecular mechanisms that are involved in inducing drug resistance in melanoma. Results from these efforts could help to identify those patients most likely to present resistance to treatment and that would otherwise benefit from a combinatorial approach.

1.1 Chapters of the Book

In the individual chapters of this book, all aspects of basic biology of melanoma are addressed. Further, general mechanisms and therapeutic approaches based on this knowledge are described.

The second chapter by Zalpha Abdel-Malek, Viki B. Swope, and Arup Indra concentrates on melanocytes, the cellular origin of malignant melanoma. For a general understanding of the molecular processes in melanoma development, epidemiology, as illustrated in Chap. 3 by Marianne Berwick, is of major importance enhancing our knowledge of the tumor inducing stimuli. Basic genetic and genomic changes are summarized in Chap. 4 by Allen Ho, Göran Jönsson, and Hensin Tsao.

The following chapters are focusing on changes in basic molecular regulation. Here, chapters on transcriptional regulation by Satoru Yokoyama and David Fisher; on miRNAs by Maria Mione, Leonel Munoz, Janika Liebig, and Anja Bosserhoff; on cell signaling by Yann Cheli, Eric Lau, and Ze'ev A. Ronai; on proteases by Paola Zigrino and Cornelia Mauch; and on molecules in cell–cell and cell–matrix contacts by Silke Kuphal and Nicolas Haass give insight into molecular details.

The next chapters summarize characteristics of molecular processes in melanoma. The chapter by Jürgen Eberle Lothar F. Fecker describes changes in apoptotic processes in malignant melanoma, and Helen Rizos, Sebastian Haferkamp, and Lyndee L Scurr summarize the knowledge on the role of senescence.

As melanoma is a complex disease understanding is enforced by looking at general biological mechanisms and the analysis of other cell types than melanoma cells existing in a melanoma tumor. Chapters on melanoma stem cell by Markus Frank; on lessons from embryology by Alec Gramann, William Tyler Frantz, and Craig J. Ceol; and on the influence of the tumor microenvironment by Lawrence W. Wu, Meenhard Herlyn, and Gao Zhang are summarizing the available information.

A chapter by Birgit Schittek and Thomas Tüting concentrates on model systems which are available in melanoma research.

The last two chapters comment on the current status of melanoma therapy: Ryan J. Sullivan and Keith T. Flaherty summarize the new approaches targeting cellular signaling whereas the chapter of Alexander M.M. Eggermont, Caroline Robert, and Dirk Schadendorf focuses on the new era of immunotherapy in melanoma.

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2 Revisiting Epidermal Melanocytes: Regulation of Their Survival, Proliferation, and Function in Human Skin

Zalfa A. Abdel-Malek, Viki B. Swope, and Arup Indra

2.1 Introduction

Melanocytes are cells specialized in the synthesis of the pigment melanin, in the form of eumelanin, the brown/black, and pheomelanin, the red/yellow pigment (Ito and Wakamatsu 2003). Melanocytes reside in the cutaneous epidermis, within hair follicles, in the eye, the leptomeninges, the inner ear, and in the heart (Brito and Kos 2008; Goldgeier et al. 1984; Tachibana 1999; Yajima and Larue 2008). Melanin produced by melanocytes provides the skin, hair, and eyes with their distinctive coloration. In this chapter, we focus on epidermal melanocytes, since they have been the most thoroughly investigated due to their importance in photoprotection against sun-induced skin cancers, and for being the precursors for cutaneous melanoma, the deadliest form of skin cancer, and their involvement in pigmentary disorders, such as albinism and vitiligo. We, hereby, provide a brief summary of the properties of melanocytes, review how cutaneous pigmentation is regulated, and discuss the significance of paracrine and autocrine factors and their signaling pathways in modulating the survival, proliferation, and function of melanocytes,

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_2

constitutively, and in response to solar ultraviolet radiation (UV), a major environmental stressor and etiological factor for skin cancers, including melanoma (Gilchrest et al. 1999). We end by briefly describing how the knowledge gained about the regulation of melanocytes can be translated into preventative and therapeutic strategies for melanoma.

2.2 Unique Properties of Melanocytes

In the human epidermis, melanocytes reside on the basement membrane, at the epidermal–dermal junction (Jimbow and Fitzpatrick 1975). Melanocytes differ from keratinocytes, the main structural cells of the epidermis, in many respects. Unlike basal keratinocytes that are highly proliferative and capable of regenerating the epidermal layers, melanocytes have a low proliferation potential. Keratinocytes undergo a well-defined differentiation program that culminates in their death by an apoptosis-like process (reviewed by Eckert et al. 1997). On the other hand, most melanoblasts, the precursors for melanocytes, become fully differentiated upon reaching their final destination, the epidermis, after their migration during embryonic development from the neural crest (Bronner-Fraser 1993). Melanocytes are resistant to apoptosis, as they are endowed with anti-apoptotic mechanisms, exemplified by constitutive expression of the anti-apoptotic protein Bcl2 (Plettenberg et al. 1995). Contrary to keratinocytes, melanocytes have a very long life span and survive for decades in the epidermis (Quevedo et al. 1969). However, the longevity of melanocytes and their resistance to apoptosis is a double-edged sword, since these properties make them vulnerable to mutations that arise over the years, particularly due to repetitive sun exposure, and might culminate in melanoma formation in high-risk individuals. These properties also explain the resistance of melanoma tumors to various chemotherapeutic agents and to radiation therapy. Given the significance of melanocytes in protection of the skin from UV-induced skin cancers, it is critical to maintain genomic stability of these cells to insure their proper function and ability to preserve epidermal homeostasis.

2.3 Factors That Determine Cutaneous Pigmentation

Cutaneous pigmentation is determined by the rate of synthesis of melanins (eumelanin and pheomelanin) by melanocytes, the relative eumelanin and pheomelanin contents, and the rate of transfer of melanosomes, melanin-containing organelles, from melanocytes to keratinocytes (Pathak et al. 1980). These are the main factors that account for individual differences in skin pigmentation. Melanosomes contain enzymes that catalyze melanin synthesis, namely, tyrosinase, the rate-limiting enzyme for melanin synthesis, tyrosinase-related protein (TYRP-1), and dopachrome tautomerase (DCT), also known as tyrosinase-related protein 2 (TRP-2) (reviewed by Hearing 2005). The activity of tyrosinase and the protein levels of these three melanogenic enzymes correlate directly with melanin content of melanocytes (Abdel-Malek

et al. 1993; Wakamatsu et al. 2006). Melanosomes also express on their membrane OA1, a G-protein-coupled receptor that is activated by L-DOPA, an intermediate in the melanin synthetic pathway, and a substrate for tyrosinase (Hearing 2005). The number of melanocytes does not significantly differ among individuals with different pigmentary phenotypes (Szabo 1954). The difference in pigmentation lies primarily in the rate of melanin synthesis, which is determined by many genes expressed in melanocytes, and code for regulatory proteins, including melanogenic enzymes, growth factor receptors, and transcription factors, as well as structural proteins that make up the melanosome. The difference in constitutive pigmentation among individuals is primarily dictated by eumelanin, which correlates directly with the extent of pigmentation (Hennessy et al. 2005; Wakamatsu et al. 2006).

Melanocytes interact with keratinocytes by donating fully melanized (mature) melanosomes (Pathak et al. 1980). Melanocytes are dendritic cells, and their dendrites serve as conduits for the transport of melanosomes to surrounding keratinocytes. In turn, keratinocytes participate in regulating the transfer of melanosomes by expressing protease-activated receptor 2 (PAR-2), a G-protein-coupled receptor that is activated upon proteolytic cleavage by trypsin, or by binding of its agonist, Ser-Leu-Ile-Gly-Arg-Leu-NH_2 (SLIGRL), resulting in increased melanosome phagocytosis in a Rho-dependent manner (Scott et al. 2003; Seiberg et al. 2000). Expression of PAR-2 by keratinocytes is up-regulated by UV exposure in vitro and in vivo (Scott et al. 2001). In the epidermis, the ratio of melanocytes to keratinocytes is 1:34, and the interaction of these cells via transfer of melanosomes has been coined the epidermal melanin unit. Melanosome transfer is important for normal and uniform skin pigmentation, is increased upon stimulation of melanogenesis, and is critical for optimal photoprotection.

2.4 Pigmentation: The Main Photoprotective Mechanism in the Skin Against Solar UV

Solar UV is the main environmental factor that affects skin pigmentation and the main etiological factor for skin cancers, including melanoma (Epstein 1983; Gilchrest et al. 1999; Pathak 1991). Melanin synthesized by melanocytes is the main photoprotective mechanism in the skin (Gilchrest et al. 1999; Halder and Bridgeman-Shah 1995; Pathak et al. 1980). Melanosomes transferred to keratinocytes form supranuclear caps that protect the nucleus from impinging UV rays (Kobayashi et al. 1998). Melanin in the epidermis is also photoprotective for dermal fibroblasts, preventing photoaging caused by UV, particularly long wavelength UVA (Gilchrest and Rogers 1993). An interesting paradigm is that increased melanin synthesis is part of the DNA damage response of melanocytes, as treatment of human skin with DNA oligonucleotides that are homologous to the telomere 3′ overhang sequence (T-oligos) enhanced nucleotide excision repair and subsequently increased epidermal melanin content (Arad et al. 2006).

The photoprotective effects of melanin are mainly conferred by eumelanin, since pheomelanin seems to have detrimental rather than beneficial effects. Eumelanin acts as a scavenger of reactive oxygen species produced upon exposure to UV, and

thus reduces the oxidative damage to DNA, proteins, and lipids (Bustamante et al. 1993). On the other hand, pheomelanin might be a pro-oxidant, resulting in oxidative DNA damage that causes melanoma formation in melanoma-prone mice expressing the activating mutation in Braf, Braf ^{v600E}, in the absence of UV or any other carcinogen (Mitra et al. 2012). Additionally, pheomelanin seems to exacerbate the formation or the effects of peroxynitrite in UV-irradiated melanocytes, resulting in increased formation of cyclobutane pyrimidine dimers (CPDs), the major form of DNA photoproducts, even after cessation of UV exposure (Premi et al. 2015).

There is overwhelming clinical and epidemiological evidence for the role of melanin in prevention of sun-induced skin cancers (Epstein 1983; Halder and Bridgeman-Shah 1995; Newton Bishop and Bishop 2005). The incidence of these cancers is by far higher in individuals with fair skin and low melanin content, than in individuals with dark skin containing high levels of melanin, mainly eumelanin. Experimental evidence shows that exposure to UV results in less DNA photoproducts in dark-skinned individuals with high melanin (mainly eumelanin) content than in light-skinned individuals with low melanin content (Tadokoro et al. 2003). Similarly, an inverse relationship between eumelanin content and the induction of DNA photoproducts was found in cultured human melanocytes derived from donors with different pigmentary phenotypes, with CPDs being lowest in melanocytes with the highest eumelanin content, and highest in melanocytes that have least eumelanin content (Hauser et al. 2006; Smit et al. 2001).

2.5 Evidence for a Paracrine/Autocrine Network in Human Skin

A symbiotic relationship exists between cutaneous melanocytes, keratinocytes, and fibroblasts. It is well established that a complex and well-regulated paracrine/autocrine network is present in human skin and that this network is modulated in response to stress, such as in response to UV or inflammation. In turn, the paracrine/autocrine factors mediate many of the stress responses of epidermal cells. Many of the cytokines and growth factors synthesized by keratinocytes and fibroblasts play important roles in regulating melanocyte function and survival (summarized in Table 2.1 and Fig. 2.1). The first evidence for keratinocyte-derived paracrine factors that affect melanocytes came from the observation that medium conditioned by cultured human keratinocytes stimulated the proliferation and melanogenesis of cultured normal human melanocytes (Gordon et al. 1989). Additional evidence came from the observation that melanocytes co-cultured with keratinocytes exhibited a dose-dependent increase in melanogenesis following irradiation with very low doses of UVB, while melanocytes in monoculture required irradiation with at least a ten-fold higher dose of UVB in order to stimulate pigmentation (Duval et al. 2001). These latter results implicated keratinocyte-derived factors in the melanogenic response of melanocytes to UVB. Medium conditioned with human fibroblasts also stimulated the proliferation of cultured human melanocytes (Imokawa et al. 1998). Mutations in genes that code for paracrine factors or their receptors,

Table 2.1 Summary of known paracrine/autocrine factors, their cell of origin in the skin, and their effects on melanocytes

Factor	Synthesized by	Role in pigmentation	Melanocyte function
POMC derived: α-MSH, ACTH	KC, MC	↑	MC1R agonist (↑ cAMP)
POMC derived: β-endorphin	KC, MC	↑	Inhibits cAMP, unknown signaling pathway
Agouti signaling protein (ASIP)	Skin, cell of origin Unknown	↓	MC1R antagonist
Human β defensin 3 (HBD3)	KC	↑ In dog and mouse; ? In human	MC1R antagonist
Bone morphogenetic factor (BMP-4)	KC, MC	↓	↓ Tyrosinase activity, ↓ TRP-1, ↓ MC1R
Noggin	Cell of origin in human skin unknown	↑	Antagonist of BMP-4
Interleukin-1 α/β	KC, MC	↓	↓ Tyrosinase activity, ↓ proliferation
Tumor necrosis factor-α (TNF-α)	KC	↓	↓ Tyrosinase activity, ↓ proliferation
Endothelin-1 (ET-1)	KC	↑	↑ Tyrosinase activity, ↑ proliferation, ↑ survival
Stem cell factor (SCF)	KC, FB	↑	↑ Proliferation, ↑ survival, ↑ dendricity
Hepatocyte growth factor (HGF)	KC, FB	↑	↑ Proliferation, ↑migration
Basic fibroblast growth factor (bFGF)	KC, FB	↑	↑ Proliferation
Prostaglandins PGE_2 and $PGF_{2\alpha}$	KC, MC	↑	↑ Dendricity, ↑ tyrosinase activity
Leukotrienes LTC4 and LTD4	KC	↑	↑ Proliferation
Corticotropin releasing hormone	KC, MC	↑	↑ cAMP, ↑ *POMC* gene (↑ACTH)
Nerve growth factor (NGF)	KC	–	↑ Dendricity, ↑ migration, ↑ survival
Neurotrophin 3 (NT-3)	KC, FB	–	↑ Survival
Semaphorin 7a	KC, FB	–	↑ Spreading, ↑ dendricity
Neuregulin-1 (NRG-1)	KC, FB	↑	↑ Pigmentation, ↑ dendricity, ↑ MC size
Nitric oxide	KC, MC	↑	↑ Melanogenesis

such as stem cell factor or its receptor c-kit, or endothelin (ET)-3 and the endothelin-B receptor (ENDBR), result in pigmentary abnormalities (piebaldism or Hirschprung's disease, respectively) due to reduced melanoblast survival and migration during embryonic development (Giebel and Spritz 1991; Puffenberger et al.

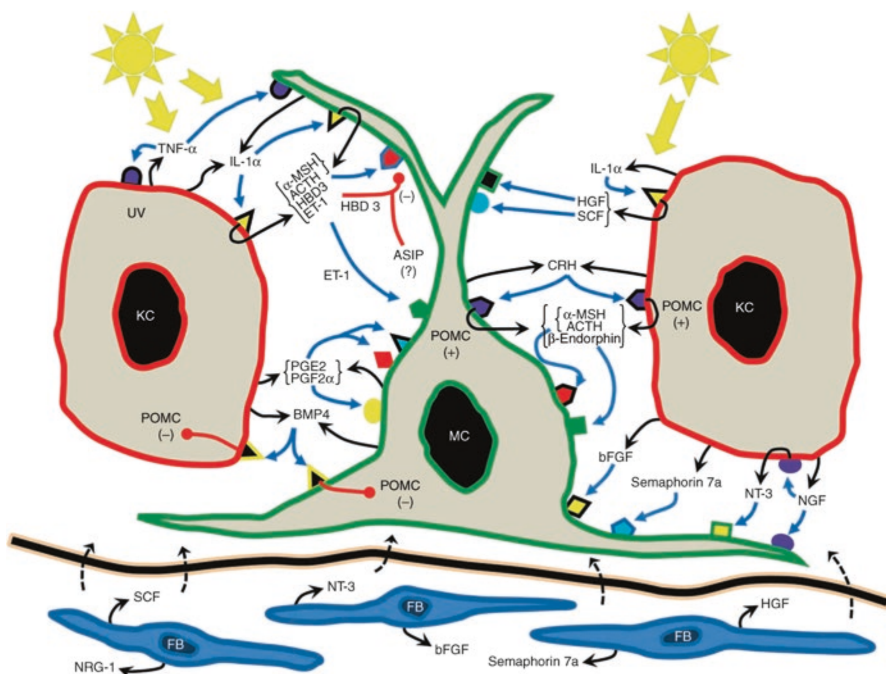

Fig. 2.1 Summary of the major participants in the paracrine/autocrine network that regulates melanocyte function, survival, and proliferation and the regulation of this network by UV. Irradiation of the skin with UV up-regulates the expression of the primary cytokines TNF-α and IL-1α by keratinocytes, both of which directly affect melanocytes. In turn, IL-1α increases the production of α-MSH and ACTH by keratinocytes and melanocytes, as well as HBD3, ET-1, HGF, and SCF by keratinocytes. In response to UV, CRH production is increased by both keratinocytes and melanocytes. CRH affects melanocytes directly and indirectly by increasing the expression of POMC, and hence its derivatives α-MSH, ACTH, and β-endorphin. Melanocytes and keratinocytes synthesize BMP-4, which affects melanocytes directly, and might also inhibit POMC production by keratinocytes and melanocytes. Additionally, both keratinocytes and melanocytes synthesize PGE_2 and $PGF_{2\alpha}$. Keratinocytes synthesize bFGF and semaphorin 7a, and also NGF, which affects melanocytes directly, as well as indirectly by enhancing the production of NT-3 by keratinocytes. Fibroblasts contribute to the paracrine network by synthesizing HGF, SCF, NT-3, semaphorin 7a, and NRG-1

1994). Collectively, these results provide evidence for the existence of a paracrine network in human skin that modulates melanocyte function, proliferation, and survival under constitutive conditions and in response to UV.

2.5.1 The Melanocortin 1 Receptor Physiological Agonists and Antagonists Are Epidermal-Derived Factors That Regulate Eumelanin and Pheomelanin Synthesis

There has been particular interest in understanding the regulation of eumelanin and pheomelanin synthesis by melanocytes, given the significance of eumelanin in photoprotection, and to elucidate the underlying causes for the diversity of human

pigmentation. Stimulation of eumelanin synthesis results mainly from activation of the melanocortin 1 receptor (MC1R), a G_s-protein-coupled receptor expressed on melanocytes (Chhajlani and Wikberg 1992; Hunt et al. 1995; Mountjoy et al. 1992; Suzuki et al. 1996). The physiological agonists for the human MC1R are α-melanocyte-stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), which bind the MC1R with the same affinity (Suzuki et al. 1996). Pro-opiomelanocortin, the precursor for ACTH and α-MSH, is synthesized and processed by cultured keratinocytes and melanocytes, and its expression in the skin is increased by UV exposure (Chakraborty et al. 1996; Corre et al. 2006; Suzuki et al. 2002; Wakamatsu et al. 1997). Actually, the presence of MSH peptides in human skin was reported decades ago, long before a physiological role for these peptides in human pigmentation was identified (Thody et al. 1983). Therefore, MC1R agonists are paracrine/autocrine factors that are expected to participate in the UV response of melanocytes (as described later in this review). This is supported by the observation that activation of the cAMP pathway by α-MSH is critical for UV-induced melanogenesis (i.e., tanning response), and enables human melanocytes to overcome the UV-induced growth arrest (Im et al. 1998a).

Agouti signaling protein (ASIP) is the physiological antagonist of the MC1R that acts as an inverse agonist, competing with α-MSH for receptor binding, and abrogating the activation of the cAMP pathway, the main signaling pathway of the activated MC1R (Suzuki et al. 1997). Concomitant treatment of cultured human melanocytes with α-MSH and ASIP blocked the mitogenic and melanogenic effects of α-MSH. In mice, the recessive yellow mutation that causes loss of function of the mc1r results in a yellow coat color (Robbins et al. 1993). Similarly, overexpression of *agouti* results in the same pigmentary phenotype, due to inhibition of eumelanin synthesis, in addition to other pleiotropic effects resulting from interaction of ASIP with other melanocortin receptors, which include obesity, diabetes, and increased susceptibility to develop tumors (Siracusa 1994). Pheomelanin synthesis is considered to be the default pathway, which takes place in the absence of MC1R signaling, unlike eumelanin synthesis that has stringent requirements, including high concentrations of tyrosine, the substrate for tyrosinase, and activation of the MC1R, which leads to stimulation of cAMP formation and increase in activities and protein levels of the melanogenic enzymes tyrosinase, TYRP-1, and TRP-2 (Abdel-Malek et al. 1995; Sakai et al. 1997).

Another factor that affects eumelanin/pheomelanin synthesis is human β-defensin 3 (HBD3), an antimicrobial peptide which was cloned from human keratinocytes and is best known for its role in innate immunity (Candille et al. 2007; Harder et al. 2001). Genetic studies on dogs revealed that mutation in the *HBD3* gene resulted in black coat color, an effect that was postulated to be due to inhibition of ASIP binding to the MC1R (Candille et al. 2007). Receptor binding assays revealed that HBD3 acts as a competitive inhibitor of α-MSH binding to the MC1R. HBD3 acts as an antagonist of the MC1R expressed on human melanocytes, abrogating the effects of α-MSH on cAMP formation (Swope et al. 2012). Brief pretreatment with either HBD3 or ASIP prevented cultured human melanocytes from responding to α-MSH with stimulation of cAMP synthesis. This effect might be due to MC1R desensitization, another mechanism of limiting the response to α-MSH.

2.5.2 Antagonistic Effects of Bone Morphogenetic Protein and Noggin on Pigmentation and Their Potential Regulation of MC1R Agonists and Antagonists

Two interesting modulators of melanogenesis are bone morphogenetic protein-4 (BMP-4) and noggin, which modulate melanogenesis directly, and possibly indirectly, by regulating the expression of the MC1R agonists and ASIP. BMP-4 is a member of the TGF-β superfamily and has been shown to be produced by both human melanocytes and keratinocytes and to inhibit melanogenesis in human melanocytes (Yaar et al. 2006). The inhibitory effect of BMP-4 involved reduction in the levels of the melanogenic enzymes tyrosinase, and TYRP-1, as well as MC1R (Park et al. 2009; Yaar et al. 2006). The BMP-4 receptors-1A, -1B, and -2 are expressed by human melanocytes, confirming the role of BMP-4 as a paracrine/autocrine factor (Yaar et al. 2006). Irradiation of melanocytes with UV down-regulated the expression of BMP-4 receptor-1B, which might be one mechanism by which UV stimulates melanogenesis (i.e., tanning), which requires activation of MC1R and its cAMP signaling pathway (Im et al. 1998b). Studies on mouse coat color showed that noggin acts as an antagonist of BMP-4, and noggin overexpression reduced the expression of ASIP (Sharov et al. 2005). On the other hand, BMP-4 enhanced the expression of ASIP by primary mouse keratinocytes and fibroblasts. Moreover, in the pituitary gland, BMP-4 repressed the expression of *Pro-opiomelanocortin (POMC)* by corticotrophs (Nudi et al. 2005). In addition to increasing ASIP, potential inhibition of POMC production in the skin and reduction of MC1R expression in melanocytes might be a mechanism by which BMP-4 inhibits pigmentation, and reversal of these effects by noggin might be a mechanism to stimulate melanogenesis. The effects of BMP-4 and noggin add another layer of complexity to the regulation of melanogenesis upstream of POMC and ASIP, and the modulation of MC1R expression.

2.5.3 Identification of the Nature of Paracrine Factors for Melanocytes in the Skin

Basic fibroblast growth factor (bFGF), basic fibroblast growth factor (bFGF), the cytokines interleukin (IL)-1α and tumor necrosis factor (TNF)-α, as well as hepatocyte growth factor (HGF) and stem cell factor (SCF) that are induced by these cytokines (Imokawa et al. 1998). It has long been known that production of primary cytokines, namely, IL-1 α and TNF-α, by keratinocytes is up-regulated by UV (Kock et al. 1990; Kupper et al. 1987) and that these cytokines regulate the synthesis of potent mitogenic and melanogenic factors by keratinocytes, such as α-MSH and endothelin-1 (ET-1) (Chakraborty et al. 1996; Imokawa et al. 1992). Human melanocytes were also found to synthesize IL-1α and β and to respond to IL-1α and TNF-α with inhibition of proliferation and melanogenesis, suggesting that these cytokines directly modulate melanocyte function and proliferation (Swope et al. 1991, 1994). Using whole human genome microarray analysis, it was shown that repetitive irradiation of human skin in situ by UVB resulted in altered expression of

genes that encode for paracrine factors or their receptors. These genes included those that encode for HGF, bFGF, IL-1 α and β, and GM-CSF, and for the PAR-2 receptor, the SCF receptor c-kit, the endothelin-1 B receptor ENDBR, and MC1R. These in vitro and in vivo findings provide compelling evidence that many factors that regulate melanocytes are synthesized locally in the skin.

The first keratinocyte-derived paracrine factor for melanocytes to be identified was bFGF, an essential mitogen for melanocytes (Halaban et al. 1988). Basic FGF stimulates melanocyte proliferation by binding and activating a specific tyrosine kinase receptor (Pittelkow and Shipley 1989). Another important keratinocyte-derived paracrine factor is ET-1, which induces melanocyte proliferation, melanogenesis, and migration (Horikawa et al. 1995; Tada et al. 1998b; Yada et al. 1991; Yohn et al. 1993). Human melanocytes predominantly express ENDBR, a G_q-coupled receptor, which when bound by either ET-1 or ET-3, activates PKC, intracellular calcium mobilization, and nonreceptor tyrosine kinases (Imokawa et al. 1992; Tada et al. 1998b). Mutations in either the gene for endothelin-3 (which during embryonic development, mimics ET-1 in its effects and mechanism of action) or ENDBR result in Hirschprung's disease Type II, which is characterized by hypopigmentation due to inefficient migration of melanoblasts from the embryonic neural crest and their reduced survival, and by aganglionic megacolon due to absence of neural crest-derived ganglia (Puffenberger et al. 1994). Treatment of cultured human keratinocytes with IL-1α or irradiation of human skin in vivo induced the production of ET-1 (Imokawa et al. 1992). These results suggest a role for ET-1 in the UV response of melanocytes, which is described later in this review.

Two important paracrine factors that are synthesized by both keratinocytes and fibroblasts are SCF and HGF (Imokawa et al. 1998; Matsumoto et al. 1991). Stem cell factor elicits its mitogenic and survival effects on melanocytes by activating a specific tyrosine kinase receptor, c-kit, and mutations in the *Kit* gene result in piebaldism, which is characterized by depigmented skin patches as a consequence of death of melanocytes during their migratory route during embryonic development to populate the epidermis (Giebel and Spritz 1991). In adult skin, SCF is required for melanocyte maintenance, since injection of c-kit antibody resulted in loss of pigmentation due to melanocyte death (Grichnik et al. 1998). Hepatocyte growth factor, which activates the tyrosine kinase receptor c-Met, allows for melanocyte homing to the epidermis during embryonic development and stimulates human melanocyte proliferation (Matsumoto et al. 1991). The observation that HGF transgenic mice have extensive skin melanosis provides genetic evidence for the significance of HGF in directing the migration of melanocytes to the epidermis (Otsuka et al. 1998).

2.5.4 Eicosanoids as Paracrine/Autocrine Factors for Melanocytes

The eicosanoids, prostaglandins (PGs), and leukotrienes (LTs) are lipid-signaling intermediates that are derived from arachidonic acid via the cyclooxygenase and lipooxygenase pathway, respectively. Although the major source of PGs in the skin

is the keratinocytes, experimental evidence showed that melanocytes also synthesize the major two forms of PGs, PGE_2 and $PGF_{2\alpha}$, in response to UV irradiation (Scott et al. 2005). Human melanocytes express cyclooxygenase (COX)-1 and −2, the latter of which is the inducible form, and the rate-limiting enzyme for the synthesis of PGs (Nicolaou et al. 2004). Si-RNA mediated knock-down of COX-2 in melanocytes decreased the expression of tyrosinase, TYRP-1, TRP-2, gp100, and MITF and also reduced tyrosinase enzyme activity, suggesting utility of COX2 inhibitors against hyperpigmentary disorder such as melasma, postinflammatory hyperpigmentation, and solar lentigo (Kim et al. 2012). Human melanocytes express FP receptor, the receptor for $PGF_{2\alpha}$ in vitro and in vivo, and expression of this receptor is up-regulated upon UV exposure (Scott et al. 2005; Starner et al. 2010). Melanocytes responded to $PGF_{2\alpha}$ with stimulation of dendricity and melanogenesis that was evidenced by increased activity and protein levels of tyrosinase. Multiple irradiations of cultured melanocytes with moderate noncytotoxic doses of UV induced the synthesis of PGE_2 via activation of cytoplasmic phospholipase A_2 ($cPLA_2$), the rate-limiting enzyme in eicosanoid synthesis (Starner et al. 2010). Epidermal melanocytes were found to produce PGE_2 under basal conditions, which further increased after arachidonic acid stimulation (Gledhill et al. 2010). Human melanocytes express two of the four PGE_2 G-protein-coupled receptors, EP2 and EP4, and treatment with PGE_2 increased cAMP formation and stimulated proliferation and tyrosinase activity (Starner et al. 2010). Recently, PGE_2 has been shown to drive melanosome transfer by promoting filopodia formation in melanocytes (Ma et al. 2014). The leukotrienes $(LT)C_4$ and D_4 were found to be potent mitogens for cultured human melanocytes (Morelli et al. 1989). These results demonstrate the participation of eicosanoids and their receptors in the autocrine/paracrine network that regulates melanocyte proliferation and functions and the response to UV.

2.5.5 An Equivalent of the Hypothalamic/Pituitary/Adrenal Axis Is Present in Human Skin

Studies from various laboratories provided evidence that the skin is a "neuroendocrine organ," which contains an equivalent of the systemic stress-induced hypothalamic/pituitary/adrenal axis. Skin cells, including melanocytes, express corticotropin-releasing hormone (CRH) mRNA and protein, and also its receptor CRH-R1 (Funasaka et al. 1999; Slominski 1998; Slominski et al. 1995). Treatment of human melanocytes with CRH resulted in increased cAMP levels and up-regulated the expression of *POMC* gene. The latter effect led to increased production of the POMC derivative ACTH, which contains within its structure the entire amino acid sequence of α-MSH. These two bioactive peptides are produced upon processing of POMC by the enzymes pro-convertase 1 and 2, respectively. The significance of POMC in human pigmentation was supported by the observation that mutations in the human *POMC* gene that affected its expression resulted in red hair phenotype, in addition to metabolic abnormalities, such as adrenal insufficiency and obesity (Krude et al. 1998). In melanocytes, ACTH induced the production of

cortisol and corticosterone, which feedback negatively to inhibit the production of ACTH and thus terminate the response to stress (Slominski et al. 2005). These series of events recapitulate the functional hierarchy in the hypothalamic/pituitary/adrenal axis and provide further evidence for the participation of the melanocyte in the cutaneous stress response by producing and responding to stress-induced factors.

In addition to α-MSH and ACTH, β-endorphin, a third POMC-derived bioactive peptide, is produced in vitro and in situ by keratinocytes and melanocytes upon the cleavage of β-lipotropic hormone (β-LPH) (Kauser et al. 2003; Slominski 1998). Production of β-endorphin by melanocytes correlated with their differentiation status, as determined by their dendricity and pigmentation, indicating that β-endorphin functions as an autocrine factor (Kauser et al. 2003). Melanocytes and keratinocytes express functional μ-opiate receptors, and treatment of cultured human melanocytes with β-endorphin stimulated proliferation, dendricity, and melanogenesis (Kauser et al. 2003). In melanocytes, both β-endorphin and μ-opiate receptors co-localized in premelanosomes and stage II melanosomes, suggesting that the ligand and receptor internalize into immature melanosomes where they induce melanogenesis. Unlike melanocortins, which elicit their effects by activating the cAMP pathway, β-endorphin inhibited adenylate cyclase, thus reduced cAMP levels. The signaling pathway(s) that mediates the effects of β-endorphin on melanocytes is yet to be determined. The increase in β-endorphin production in response to UV might explain the "addictive" behavior of sun worshippers and the feeling of wellness associated with sun exposure.

2.5.6 Neurotrophins as Members of the Cutaneous Neuroendocrine Paracrine Network

Given that the cutaneous epidermis and the nervous system arise from a common ectodermal origin, it was postulated that growth factors that regulate the survival and function of neurons play a role in the maintenance of epidermal homeostasis. Neutrophins (NTs) are family of neuronal growth factors comprised of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4, two of which, namely, NGF and NT-3, are synthesized in the skin (reviewed by Botchkarev et al. 2006). Human keratinocytes, particularly basal keratinocytes with the highest proliferation capacity, were found to synthesize and release NGF, which in turn enhanced the secretion of NT-3, also synthesized by dermal fibroblasts (Marconi et al. 2003; Yaar et al. 1994). Neurotrophins share about 50 % amino acid sequence homology and interact with tyrosine kinase receptors (Trk) A, B, and C, the high affinity receptors for NGF, BDNF, and NT-3, respectively. All NTs interact with the low-affinity p75 NT receptor, a member of the TNF-α family of receptors. Depending on the intracellular adaptor molecules that interact with p75 receptor, its signaling may be linked to the JNK-p53-Bax pro-apoptotic pathway, or to the NF-kB survival pathway. Human melanocytes responded to NGF with increased migration and dendricity, and additionally, with inhibition of apoptosis after UV irradiation via

increasing the levels of the anti-apoptotic Bcl2 (Stefanato et al. 2003; Yaar et al. 1991; Zhai et al. 1996). Both NGF and NT-3 increase the survival of human melanocytes maintained in growth factor-depleted culture medium (Yaar et al. 1994; Zhai et al. 1996). Human melanocytes express p75NT receptor, and this is increased upon UV irradiation (Yaar et al. 1994). Melanocytes also express low levels of TrkC, the receptor for NT-3, and TrkA expression is induced upon stimulation of protein kinase C. Based on these studies, it was concluded that NGF, which is the most prevalent NT that is constitutively produced by keratinocytes, insures the survival of melanocytes by inhibiting UV-induced apoptosis, and NT-3 that is strongly expressed in fibroblasts might also contribute to melanocyte maintenance.

2.5.7 Semaphorin 7a: A Neuronal Factor Synthesized in Human Skin

Sempahorin 7 is a member of the secreted and membrane-bound semaphorin family of proteins that function in neuronal pathfinding and axonal guidance (Yazdani and Terman 2006). It was the first glycosylphosphatidylinositol-linked semaphorin to be identified and shown to bind plexin C1 and β1-integrin receptor (Pasterkamp et al. 2003; Sato and Takahashi 1998; Tamagnone et al. 1999). Semaphorin 7a was found to be expressed in the skin in vivo and in keratinocytes and fibroblasts, as demonstrated in vitro (Scott et al. 2008). In response to UV, fibroblasts exhibited a marked increase in semaphorin 7a expression. The role of semaphorin 7a as a paracrine factor for melanocytes was demonstrated by the finding that co-culturing of melanocytes with cells expressing semaphorin 7a led to increased spreading and dendricity and that melanocytes responded directly to treatment with exogenous semaphorin 7a. These effects were mediated by binding the β1-integrin receptor and were inhibited by plexin C1. These results identify a novel neuronal factor expressed in the skin, which regulates melanocyte spreading and dendricity positively via β1-integrin receptor, or negatively by interacting with Plexin C1. These effects might have significant implications on melanosome transfer, an important determinant of cutaneous pigmentation.

2.5.8 Neuregulin-1: A Neuroendocrine Factor Synthesized by Human Epidermal and Dermal Cells

The neuroendocrine factor Neuregulin-1 (NRG-1) was identified as a fibroblast-derived factor that regulates constitutive human pigmentation (Choi et al. 2010). Neuregulin-1 is a secreted growth factor that is expressed in the central nervous system and is critical for neuronal differentiation, migration, and dendrite formation (Krivosheya et al. 2008). The effects of NRG-1 are mediated by binding to ErbB3 and ErbB4 receptors, tyrosine kinase receptors that belong to the family of epidermal growth factor receptors, which dimer upon ligand binding. NRG1/ErbB3 signaling has been shown to inhibit later stage differentiation of melanoblasts derived

from neural tubes of mouse embryos but is dispensable for melanoblast specification and melanocyte maturation and promotes undifferentiated, migratory, and proliferative features (Buac et al. 2009). Neuregulin-1 treatment increased melanocyte proliferation, invasion, and altered morphology together with decreased levels of differentiation genes (Buac et al. 2009). Cultured human fibroblasts derived from skin type VI donors expressed higher levels of NRG-1 than fibroblasts derived from skin type II donors (Choi et al. 2010). In vivo, NRG-1 was highly expressed in the epidermis as well as the dermis of skin type VI donors but was expressed at very low levels only in the dermis of skin type II donors (Choi et al. 2010). Furthermore, treatment with exogenous NRG-1 gave rise to increase in pigmentation, melanocyte size, and dendricity in cultured skin substitutes, and these effects were more pronounced in skin substitutes representative of skin type VI than in their counterparts representative of skin types IV or II. The bioactive motif of NRG1 that is involved in modulating melanin production in human melanocytes has been characterized and was found to increase melanin production without affecting proliferation (Choi et al. 2012). ErbB3 expression was higher in melanocytes derived from dark skin than in melanocytes cultured from light skin, suggesting that activation of this receptor is responsible for the melanogenic effects of NRG-1 (Choi et al. 2012). On the other hand, ErbB4 expression was expressed at higher levels in melanocytes derived from light skin, compared to melanocytes from dark skin. These findings implicate NRG-1 and its receptors in regulating constitutive pigmentation.

2.6 Role of Melanocyte- and Keratinocyte-Derived Nitric Oxide in Regulating Pigmentation

In addition to enhancing the production of a large panel of paracrine and autocrine growth factors, UV stimulates the production of nitric oxide (NO) by both keratinocytes and melanocytes (Romero-Graillet et al. 1996, 1997). In keratinocytes, this effect was mediated by increased constitutive NO synthase (Romero-Graillet et al. 1997) and might involve the activation of Akt (Dimmeler et al. 1999). In lipopolysaccharide (LPS)-treated human melanocytes, the inducible NO synthase (iNOS) gene expression was suppressed by activin, which also inhibited toll-like receptors (TLR) and cytokine expression (Kim et al. 2015). The melanogenic effect of UV was abrogated to a large extent upon treatment with NO scavengers, while treatment of melanocytes with exogenous NO donors stimulated melanogenesis and dendricity, lending direct evidence for the melanogenic effect of NO (Romero-Graillet et al. 1997). This effect of NO was mediated by increasing the levels of cGMP, and treatment of melanocytes with guanylate cyclase inhibitors blocked the UV-induced melanogenesis (Romero-Graillet et al. 1996). It is possible that the melanogenic effect of cGMP is indirect, resulting from inhibition of cAMP phosphodiesterase, which leads to increased cAMP levels, the principle mechanism for stimulation of melanogenesis. In B16 and human melanoma cells, α-MSH increased the UV-induced NO levels, and as in normal human melanocytes, NO stimulated melanogenesis (Tsatmali et al. 2000). This melanogenic effect was abrogated by

inhibition of iNOS. The findings that α-MSH modulates the production of NO raises the question whether NO functions as an autocrine factor or as a second messenger that mediates the effects of α-MSH.

Abnormal levels of NO can have detrimental effects on melanocytes. It has been recently shown that variants of *iNOS* gene, −954 G/C and Ex 16+14 C/T, might be genetic susceptibility markers for nonsegmental vitiligo among Egyptians (Zayed et al. 2015). Exposure of human melanocytes to UV induced the formation of superoxide and NO, which combine to form peroxynitrite that excites an electron in fragments of the pigment melanin leading to the formation of CPDs even after cessation of UV exposure (Premi et al. 2015). Peroxynitrite solubilizes melanin into fragments or releases pre-melanin monomers from melanosomes, leading to migration of these fragments or monomers to the nucleus, where they induce CPDS. Not only the initial induction of CPDs but also latent formation of CPDs, coined "dark CPDs," was found to be twice as high in the skin of UVA-irradiated *K14-Kitle/e* mice (homozygous for the loss of function recessive yellow mutation in *Mc1r*) than in the skin of their counterparts with black coat color. These results suggest that pheomelanin sensitizes melanocytes to excessive CPDs, which might be attributed to either increased peroxynitrite formation or sensitivity of pheomelanin to peroxynitrite. Further investigation of the role of NO in the UV response of human melanocytes should shed light on its effects on UV-induced DNA damage in individuals with different pigmentary phenotypes, and on the risk for melanoma.

2.7 Signaling Pathways Responsible for Regulating Melanocyte Proliferation, Survival, and Function

The first melanocyte growth medium was based on the use of phorbol esters that activate protein kinase C, and cAMP inducers, such as cholera toxin (Eisinger and Marko 1982). This underscored the significance of these signaling pathways in sustaining the survival, proliferation, and function of human melanocytes. Later, it was shown that paracrine growth factors for melanocytes that activate tyrosine kinase receptors, such as bFGF, SCF, and HGF, as well as ET-1, can substitute for phorbol esters in the culture medium and stimulate proliferation by activating the MAP kinases ERK1/2 (Bohm et al. 1995; Swope et al. 1995a; Tada et al. 1998b). Activation of ERK1/2 resulted in phosphorylation, hence activation of the transcription factor cyclic AMP response element binding protein (CREB), upstream from the melanocyte master regulator, the transcription factor MITF (Bohm et al. 1995; Tada et al. 2002). Signaling pathways that were involved in ERK1/2 activation included protein kinasec (PKC), tyrosine kinases, and intracellular calcium mobilization. The cAMP pathway has long been known to be essential for stimulating melanogenesis in pigment cells (Hirobe and Takeuchi 1977; Pawelek et al. 1973). The main signaling pathway for α-MSH is the cAMP-dependent pathway, and α-MSH is primarily a melanogenic factor for human melanocytes (Abdel-Malek et al. 1992). In contrast to other growth factors, such as bFGF or ET-1, α-MSH and other cAMP inducers are poor activators of the ERK1/2 pathway, yet α-MSH

interacts synergistically with factors that stimulate PKC, tyrosine kinases, or intracellular calcium mobilization to activate these MAP kinases and their downstream targets, leading to increased melanocyte proliferation and melanogenesis (Herraiz et al. 2011; Tada et al. 2002). Based on these findings, a melanocyte growth medium supplemented with bFGF, ET-1, and α-MSH was described and found to support the long-term proliferation of human melanocytes (Swope et al. 1995b).

The transcription factor Mitf is a member of the basic helix–loop–helix leucine zipper transcription factors and is considered the "master regulator" in melanocytes (reviewed by Steingrimsson et al. 2004). Germline mutations in *Mitf* result in Waardenburg Syndrome type 2, characterized by congenital white forelock, sensoneural deafness, and asymmetric iris color. The significance of Mitf lies in its ability to regulate the expression of many target genes that are involved in melanin synthesis, proliferation, and survival of melanocytes (reviewed by Cheli et al. 2009). Direct targets for Mitf are the genes for the melanogenic enzymes *Tyrosinase*, *TYRP1*, *DCT*, and the melanosomal genes PMel 17, OA1, as well as Rab27A that is involved in melanosome transfer. Other Mitf target genes are Bcl2, BIRC7, and DICER that are involved in regulating melanocyte survival, KIT, NGFR, and ENDBR genes that encode for receptors for melanocyte growth and survival factors (SCF, NGF, and ET-1, respectively), and APE/Ref1 that regulates the redox state, as well as HIF1α, which is induced by hypoxia. Additional targets for Mitf include the cell cycle regulatory genes TBX2 and CDK2, as well as CDKN1A (p21) and CDKN2A.

It is well-established that Mitf is regulated by the ERK1/2 pathway. Treatment of melanoma cells with SCF activated ERK, which resulted in the phosphorylation of Mitf on Ser 73 and Ser 409 (Wu et al. 2000). The SCF-induced phosphorylation of Mitf on Ser 73 transiently increased its transcriptional activity, as evidenced by increasing tyrosinase expression, and subsequently targeted Mitf for ubiquitination (Hemesath et al. 1998; Wu et al. 2000). Activation of the cAMP pathway in mouse and human melanoma cells by α-MSH or forskolin also resulted in the phosphorylation of Mitf (Price et al. 1998). Treatment of cultured human melanocytes with α-MSH and/or ET-1 in the presence of bFGF increased total protein levels of Mitf, as well as its phosphorylated form (Kadekaro et al. 2005). Mitf was also regulated transcriptionally, as shown by global gene analysis of melanocytes treated with α-MSH (Kadekaro et al. 2010). Loss of Zinc Finger E-box binding protein 2 (ZEB1) in melanocytes has been shown to induce melanoblast migration defects and melanocyte differentiation defects in vivo, accompanied by down-regulation of MITF (Denecker et al. 2014). Results identified a signaling network in which transcription factor ZEB2 regulates MITF to control melanocyte differentiation. Phosphodiesterase 4D3 has been identified as a direct target of the MSH/cAMP/MITF pathway, which creates a negative feedback loop inducing refractoriness to sustained stimulation of the cAMP pathway in melanocytes (Khaled et al. 2010). Wnt3a has been shown to play an important role in melanocyte homeostasis by up-regulating the expression of MITFA and its downstream targets in vitro (Guo et al. 2012a). In vivo, Wnt3a signaling is activated in mouse follicular melanocytes during anagen stage of the hair cycling and promotes melanin synthesis through induction of MITF (Guo et al. 2012b).

Exposure to UV activated the stress MAP kinases p38 and JNK/SAPK, which regulate the activity of downstream transcription factors that mediate the stress response (Ono and Han 2000; Rosette and Karin 1996). Increase in pigmentation, i.e., tanning, is considered part of the stress response, which is mediated by the paracrine/autocrine network that is activated by UV. The transcription factor Upstream Stimulating Factor-1 (USF-1) was activated by p38 and proved to be an important regulator of *MC1R* and *POMC* expression in melanocytes exposed to UV (Corre et al. 2004). In addition, USF-1 up-regulated the expression of *Tyrosinase*, *TYRP-1*, and *DCT*. Phosphorylation of USF-1 resulted in its activation and enhanced its ability to bind DNA (Galibert et al. 1997). Another transcription factor, ATF2, known to regulate genes involved in DNA repair, such as *XPC* and *ERCC1*, apoptosis, such as *Bcl2*, and the cell cycle, such as *CDK4*, was also activated by p38, as well as by JNK (Fuchs et al. 2000; Hayakawa et al. 2004), and thus is expected to play an important role in the DNA damage response of melanocytes to UV. Global gene analysis of human melanocytes irradiated with UV or treated with α-MSH revealed that ATF2 and its target genes *Bcl2*, *CDK4*, and *ERCC1* were reduced in expression by UV, and up-regulated in expression by α-MSH (Kadekaro et al. 2010). The modulation of ATF2 and Bcl2 expression by UV and α-MSH was confirmed by Western blotting, indicating that ATF2 participates in the UV response of human melanocytes. Recently, it was reported that exposure of human melanocytes to UV induced the phosphorylation of ATF2 on Tyrosine 69 and 71, and pretreatment with ET-1 augmented this effect (von Koschembahr et al. 2015). Interestingly, ET-1 induced ATF2 phosphorylation even without any UV exposure, suggesting that this potent paracrine factor primes melanocytes to respond avidly in order to counteract the genotoxic effects of UV. The impact of phospho-ATF2 on repair of UV-induced DNA damage remains to be determined.

The p53 transcription factor is considered a universal sensor of genotoxic stress (Chouinard et al. 2002; Huang et al. 1999; Liu et al. 1996). It is known to accumulate following UV exposure due to its stabilization and induces growth arrest in order to allow for DNA repair, and this was shown to occur in human melanocytes (Liu et al. 1996; Marrot et al. 2005; Medrano et al. 1995). P53 was regulated by p38, and in turn, it regulated the expression of *Tyrosinase* and *TYRP-1* (Chouinard et al. 2002; Khlgatian et al. 2002; Nylander et al. 2000). In mouse skin, p53 was found to up-regulate the expression of POMC, the precursor for melanocortins, and β-endorphin that stimulate melanogenesis (Cui et al. 2007). Another study found that mutations in the ribosomal protein s (Rps) genes, *Rps6*, *Rps19*, and *Rps20* gave rise to dark skin, due to epidermal melanocytosis (McGowan et al. 2008). The dark skin phenotype was dependent on increased p53 in keratinocytes, which led to increased expression of *SCF* that encodes for a potent mitogen and melanogenic factor for melanocytes. These intriguing results observed in mouse skin implicate p53 in regulating the expression of important paracrine factors known to have significant impact on melanocytes.

Recently, a positive feedback loop between p53, SCF, and ET-1 was described (Murase et al. 2009). Inducing high levels of p53 in cultured human keratinocytes resulted in increased production of SCF and ET-1, and treatment of cultured human

melanocytes with either SCF or ET-1 increased phosphorylation of p53 on Ser 15, which led to its stabilization. In contrast, silencing or inhibition of p53 in melanocytes resulted in decreased Kit expression, inhibition of Mitf, as well as reduced tyrosinase levels and melanin content. In cultured skin substitutes, inhibition of p53 suppressed melanogenesis and led to reduced pigmentation, and silencing of p53 in vivo inhibited pigmentation of UV-irradiated mouse ears. These effects of p53 confirm its significance in regulating pigmentation, particularly the tanning response to UV. It was recently shown that p53 directly regulates ET-1 expression in human epidermal keratinocytes, as well as mouse epidermis, and controls UV-induced melanocyte homeostasis in the skin of adult mice (Hyter et al. 2013). These results underscore the significance of p53 in the maintenance of melanocyte homeostasis via regulating expression of paracrine factors that regulate melanocyte proliferation, pigmentation, and survival.

The type II nuclear hormone receptor and transcription factor Retinoid-X-Receptor α (RXRα) has been shown to regulate mouse keratinocyte and melanocyte homeostasis following acute UV irradiation (Wang et al. 2011). Activation of RXRα expressed in keratinocytes regulated in a cell-autonomous manner their proliferation through secretion of heparin-binding EGF-like growth factor, GM-CSF, IL-1α, and cyclooxygenase-2 and activation of mitogen-activated protein kinase pathways. Expression of several keratinocyte-derived paracrine growth factors such as ET-1, hepatocyte growth factor, α-MSH, stem cell factor, and fibroblast growth factor-2 was elevated/altered in skin of mice lacking RXRα in epidermal keratinocytes, which in a non-cell-autonomous manner modulated melanocyte proliferation and activation after UV exposure (Wang et al. 2011).

In comparison, in vivo melanocyte-specific ablation of RXRα and RXRβ in mice led to altered expression of pro- and anti-apoptotic genes following UV exposure and increased survival of these melanocytes, as compared to their wild-type counterpart (Coleman et al. 2014). These results underscore a “cell autonomous” role of melanocytic RXRs in controlling melanocyte survival post-UV exposure.

2.8 The *MC1R*: A Main Determinant of the Diversity of Human Pigmentation and a Melanoma Susceptibility Gene

The *MC1R* is a highly polymorphic gene, with about 200 allelic variants expressed in different human populations (reviewed by Garcia-Borron et al. 2005, 2014). The *MC1R* is considered a main determinant of the diversity of human pigmentation, with the wild-type *MC1R* predominantly expressed in Africa, where high eumelanin content in the skin is critical for optimal photoprotection. A few of the *MC1R* variants, mainly R151C, R160W, and D294H, are strongly associated with red hair phenotype (Box et al. 1997; Smith et al. 1998). Expression of any two of these variants in the homozygous or compound heterozygous state results in loss of function of the receptor, disrupting its ability to signal when bound by its agonists (Kadekaro et al. 2010; Scott et al. 2002). Epidemiological studies from different populations in

different geographical locations demonstrated that these allelic variants are also associated with poor tanning ability and increased risk for melanoma and nonmelanoma skin cancers (Box et al. 2001; Kadekaro et al. 2010; Kennedy et al. 2001; Palmer et al. 2000). The *MC1R* gene is considered a low penetrance melanoma susceptibility gene. However, co-expression of one of the *MC1R* red hair-associated variants with a mutation in the highly penetrant *p16* gene significantly increases the risk for melanoma above that caused by the p16 mutation alone (Demenais et al. 2010). Stimulation of eumelanin synthesis by activation of the MC1R confers photoprotection; however, the effect of *MC1R* genotype on melanoma risk is independent of the effect on pigmentation, suggesting that MC1R determines the risk for melanoma by other mechanisms (Kennedy et al. 2001; Landi et al. 2005; Palmer et al. 2000; Stratigos et al. 2006). That the MC1R plays a central role in regulating human pigmentation is further supported by the findings that its expression is up-regulated by its agonist α-MSH and by ET-1 (Kadekaro et al. 2010; Swope et al. 2012; Tada et al. 1998a).

2.9 Role of ET-1 and Melanocortins in the DNA Damage Response of Melanocytes

In 2005, novel roles for melanocortins and ET-1 were discovered. In addition to the well-known effects of these factors on melanogenesis and proliferation, they increased the survival of UV-irradiated human melanocytes, enhanced nucleotide excision repair, and reduced oxidative damage by inhibiting the generation of hydrogen peroxide (Kadekaro et al. 2005). The global effects of ET-1 and α-MSH are shown in Fig. 2.2. Genetic deletion of ET-1 in murine epidermis decreased dermal melanocytes in adult skin without altering melanocyte homeostasis in newborn skin (Hyter et al. 2013). Topical treatment with the EDNRB antagonist BQ788 abrogated UV-induced melanocyte activation and recapitulated the phenotype of ET-1 deletion in mice. Endothelin-1 from epidermal keratinocytes in a non-cell autonomous manner controlled melanocyte proliferation, DNA damage, and apoptosis following UVB irradiation (Hyter et al. 2013). Treatment of cultured human melanocytes with ET-1 reduced the induction and enhanced the repair of CPDs (von Koschembahr et al. 2015). These effects were mediated by increased phosphorylation of p38 and JNK, mainly due to increased intracellular Ca^{2+} mobilization, a signaling pathway activated by ENDBR. Treatment with ET-1 also increased the phosphorylation of ATF-2, known to be involved in DNA repair.

Enhancement of nucleotide excision repair capacity by α-MSH was reported independently by other investigative teams (Bohm et al. 2005; Smith et al. 2008). Treatment of human melanocytes with the potent α-MSH analog NDP-MSH increased the transcription of NR4A subfamily of orphan nuclear receptors, which played a role in the MC1R-mediated repair of DNA photoproducts (Bohm et al. 2005; Smith et al. 2008). Furthermore, treatment of cultured human melanocytes with α-MSH reduced the induction of 8-oxo-guanosine, thus confirming reduction of oxidative DNA damage (Song et al. 2009). The survival effects of α-MSH and

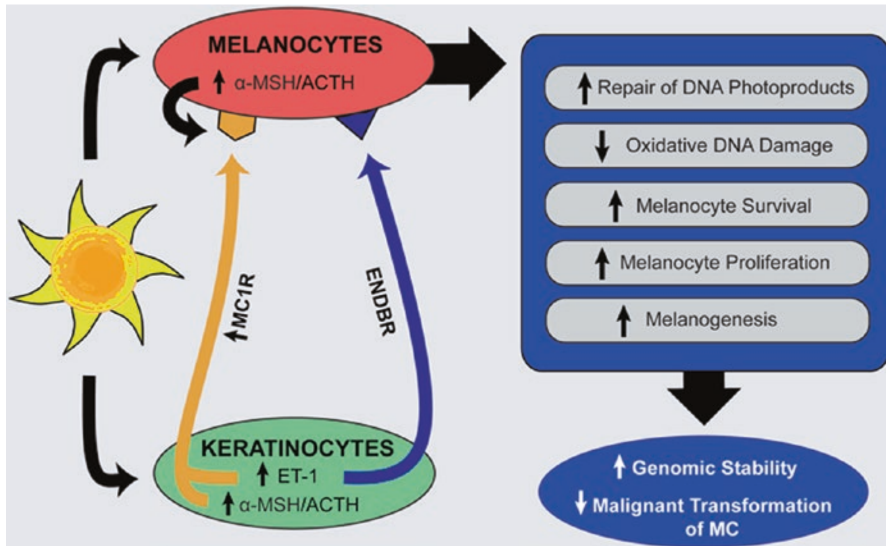

Fig. 2.2 The global effects of α-MSH and ACTH, and ET-1 on melanocytes and their response to UV exposure. Exposure to UV increases the production of ET-1 by keratinocytes, and α-MSH and ACTH by keratinocytes and melanocytes. The melanocortins α-MSH and ACTH, as well as ET-1, up-regulate the expression of the MC1R. Activation of MC1R and ENDBR results in enhanced repair of DNA photoproducts, reduction in generation of reactive oxygen species that lead to oxidative DNA damage, increased melanocyte survival and proliferation, as well as melanogenesis. The effects of these factors on UV-induced DNA damage are expected to maintain genomic stability of melanocytes and reduce the chance for malignant transformation to melanoma

ET-1 on UV-irradiated human melanocytes were mediated by activating the MC1R and ENDBR, respectively, and were independent of increased melanogenesis, since they were observed in tyrosinase-negative albino melanocytes (Kadekaro et al. 2005). These survival effects were mediated by activating Akt and Mitf, and maintaining high levels of Bcl2, a known Mitf target (Kadekaro et al. 2005; McGill et al. 2002). The recent finding that Mitf activation by α-MSH up-regulated the expression of DICER, a RNase II endonuclease that digests premature miRNA to yield mature miRNA, which reduced the expression of the pro-apoptotic BIM (Levy et al. 2010), suggested that decreased BIM expression is involved in the survival effects of α-MSH and ET-1.

The effects of α-MSH on nucleotide excision repair, generation of reactive oxygen species, and oxidative DNA damage received considerable attention, given the high polymorphism of the *MC1R* that results in the differential responses of melanocytes derived from donors with different *MC1R* genotypes to α-MSH and UV (Kadekaro et al. 2010; Scott et al. 2002). These effects required functional MC1R as they were absent in melanocytes that express loss of function receptors. The cAMP pathway mediated the effects of α-MSH on DNA damage, since these effects were also induced by forskolin, a direct activator of adenylate cyclase. Further evidence for the significance of MC1R in reducing the burden of UV-induced DNA

damage was provided by the finding that transfection of melanocytes expressing loss-of-function MC1R with the wild-type gene restored the ability to respond to α-MSH by enhanced repair of DNA photoproducts, reduced generation of reactive oxygen species, and increased survival (Kadekaro et al. 2010). These findings provide a molecular mechanism for the increased melanoma susceptibility associated with expression of loss of function variants of the *MC1R*, as melanocytes expressing these variants have compromised DNA repair capacity and sustained oxidative stress.

Oxidative DNA damage seems to play an important role in melanocyte transformation to melanoma. Unlike basal or squamous cell carcinomas that originate from keratinocytes, melanoma tumors rarely have UV signature mutations that result from unrepaired pyrimidine dimers, as in the *p53* gene (Brash et al. 1991; Lubbe et al. 1994). This suggests that other forms of DNA damage are causative for melanoma. Treatment with α-MSH immediately (within minutes) reduced the generation of hydrogen peroxide in UV-irradiated melanocytes (Kadekaro et al. 2005; Kadekaro et al. 2010; Song et al. 2009). This effect absolutely required functional MC1R was inhibited by ASIP and was absent in melanocytes that express loss-of-function receptor. Additionally, α-MSH increased the activity of catalase, a first-line-of-defense antioxidant enzyme, and counteracted the inhibitory effect of UV on catalase activity and protein levels in melanocytes expressing functional MC1R (Song et al. 2009). Recently, it was reported that catalase was transported with melanosomes to keratinocytes (Maresca et al. 2010). This intriguing finding suggests that melanocytes protect keratinocytes not only by transferring melanin contained within melanosomes that reduce the penetration of UV rays to nuclear DNA but also by providing additional catalase, which might further reduce reactive oxygen species, and prevent oxidative damage. Besides increasing the activity and levels of catalase, α-MSH also up-regulated the protein levels of ferritin, an iron sequestrant in melanocytes (Song et al. 2009). Treatment with α-MSH also activated the transcription factor Nrf-2, which regulates the expression of phase II detoxifying enzymes that contain antioxidant response element (ARE) in their promoter (Kokot et al. 2009). Examples of such enzymes are hemeoxygenase-1 (HO-1), γ-glutamylcysteine-synthase, γ-glutathione S-transferase, which were up-regulated by α-MSH in UV-irradiated melanocytes (Kokot et al. 2009).

Microarray analysis of genes altered in expression by α-MSH and/or UV in melanocytes expressing functional versus nonfunctional MC1R revealed that the former responded to α-MSH by altered expression of many genes, particularly those that regulate melanogenesis (e.g., melanogenic enzymes, melanosome biogenesis, transcription factors, growth factor receptors), survival, cell cycle, DNA repair, and oxidative stress, while the latter showed no changes in gene expression, further confirming the refractoriness of these cells to α-MSH (Kadekaro et al. 2010). In general, α-MSH up-regulated, while UV down-regulated transcription. Importantly, α-uMSH reversed the effects of UV on many genes, including some that are involved in the DNA damage response to UV, particularly melanogenesis, DNA repair, cell cycle, oxidative stress, and apoptosis. These effects of α-MSH were only evident in melanocytes expressing functional MC1R and were absent in melanocytes

expressing loss-of-function receptor, lending further explanation for why certain *MC1R* genotypes that cause loss of function of MC1R are associated with increased susceptibility to mutagenesis and melanoma formation.

Further evidence for the involvement of the α-MSH/MC1R axis in the DNA damage response of melanocytes was provided by the following findings. Treatment of human melanocytes with α-MSH was shown to phosphorylate the DNA damage sensors ataxia telangiectasia Rad3-related (ATR) and ataxia telangiectasia mutated (ATM) and their downstream kinases Chk1 and Chk2 (Swope et al. 2014). These phosphorylation events led to increased formation of γ-H2AX, the phosphorylated form of histone 2AX (H2AX) known to be critical for recruitment of DNA repair enzymes involved in nucleotide excision repair, the main pathway for repair of DNA photoproducts, to the sites of DNA damage. An increase in the protein levels of XPC, the DNA damage recognition enzyme, was also observed in response to α-MSH treatment. These effects were recapitulated by treatment with forskolin, providing evidence that they are mediated by activating the cAMP pathway, and were absent in melanocytes expressing loss-of-function MC1R. Subsequently, it was reported that activation of the cAMP-dependent protein kinase A (PKA) by α-MSH or forskolin resulted in the phosphorylation of ATR on Ser 435 and the binding of ATR to XPA and their co-localization to DNA photoproducts (Jarrett et al. 2014). As expected, these events that lead to activation of nucleotide excision repair by α-MSH were absent in melanocytes expressing nonfunctional MC1R. The same authors showed that these effects of α-MSH were negated by the MC1R antagonists ASIP and HBD3 (Jarrett et al. 2015), thus providing unequivocal evidence for the significance of the activated MC1R in modulating nucleotide excision repair in melanocytes. The findings that reactive oxygen and nitrogen species can contribute to the formation of CPDs in melanin-containing cells, i.e., melanocytes as well as keratinocytes, following cessation of UV exposure points to the importance of repair of DNA photoproducts for maintenance of genomic stability in these cells, and for prevention of melanoma as well as nonmelanoma skin cancers (Premi et al. 2015).

2.10 Targeting the MC1R by Melanocortin Analogs

Since decades, there has been interest in targeting the MC1R to increase skin pigmentation (tanning). This strategy was initially based on utilizing potent melanocortin analogs for sunless safe tanning that is photoprotective. Injecting human subjects with the potent and best known α-MSH analog NDP-MSH was found to be effective in inducing tanning in the absence of sun exposure (Levine et al. 1991). Later, this analog was found to reduce the induction of DNA photoproducts in sun-exposed human skin (Barnetson et al. 2006). Recently, clinical trials demonstrated the efficacy of NDP-MSH in repigmentation of vitiligo skin (Lim et al. 2015), which may be attributed to the known mitogenic and survival effects of α-MSH (Abdel-Malek et al. 1995; Kadekaro et al. 2005). Given that vitiligo skin is under oxidative stress due to high levels of hydrogen peroxide (Rokos et al. 2002), treatment with

NDP-MSH is expected to have antioxidant effects, thus inhibiting melanocyte death. However, despite the effectiveness of NDP-MSH, it is not specific to the MC1R, as it can bind the other melanocortin receptors, MC3, 4, and 5R, which account for its off-target effects. For the goal of developing small analogs of α-MSH for topical application to prevent skin cancers, including melanoma, tetrapeptide analogs of α-MSH were developed, and shown to surpass α-MSH in their potency to stimulate melanogenesis, and to reduce UV-induced DNA damage and apoptosis (Abdel-Malek et al. 2006). More recently, tripeptide analogs were developed and shown to be capable of activating the MC1R and reducing UV-induced DNA damage (Abdel-Malek et al. 2009). Developing MC1R-selective analogs of α-MSH will reduce the off-target effects, and developing small analogs will facilitate their topical delivery, which should reduce systemic effects. These analogs require functional MC1R and are expected to confer photoprotection for individuals with wild type *MC1R*, or who are heterozygous for *MC1R* variants that reduce receptor function, or mutant for other melanoma susceptibility genes, such as the highly penetrant *p16* or *PTEN* (Demenais et al. 2010; Sosman and Margolin 2009). Others have proposed the use of forskolin, an activator of adenylate cyclase, for melanoma prevention (D'Orazio et al. 2006). Since forskolin activates the cAMP pathway, it has similar photoprotective effects as α-MSH (Kadekaro et al. 2010). However, forskolin is nonspecific, and its target, adenylate cyclase, is ubiquitously expressed in all cell types, which precludes its selective use for photoprotection.

The effects of ET-1 on repair of DNA photoproducts was evident in human melanocytes regardless of their *MC1R* genotype, as these effects were observed in melanocytes expressing loss-of-function MC1R (von Koschembahr et al. 2015). These results suggest that the ET-1/ENDBR axis might be an attractive target for photoprotective strategies. Given that ET-1 and its receptors are ubiquitously expressed, they cannot be targeted directly to activate the DNA damage response of melanocytes. However, the downstream effectors of ENDBR that are involved in the DNA damage response in melanocytes might be targeted, and this would have global benefit against UV-induced genotoxicity and melanomagenesis, independent of *MC1R* genotype.

2.11 What Normal Melanocytes Teach About Melanoma: Revise

Normally, melanocytes in the skin are quiescent, and their homeostasis is maintained via their interaction with keratinocytes and with their microenvironment. During the early stages of melanomagenesis, melanocytes acquire the ability to proliferate and escape from cell cycle regulation by uncoupling from keratinocytes (reviewed by Haass and Herlyn 2005). This is achieved by down-regulating the expression of the adhesion molecules E-cadherin, P-cadherin, and desmoglein in response to binding of the paracrine factor HGF to c-Met and activation of ERK1/2 and IP3 kinase (Li et al. 2001). Similarly, ET-1 can down-regulate E-cadherin (Jamal and Schneider 2002). Further studies showed that overexpression of bFGF in a human xenograft model

followed by UVB irradiation gave rise to hyperplastic melanocytic cells with high-grade atypia, reminiscent of lentiginous melanoma (Berking et al. 2001). Overexpression of bFGF concomitantly with ET-3 and SCF, followed by UVB exposure, led to the formation of nests of atypical melanocytes representing melanoma in situ, some of which progressed into invasive melanoma (Berking et al. 2004). These paracrine factors are up-regulated in expression by UV, and sun exposure might lead in vivo to deregulation of their expression, or expression of their receptors, e.g., ENDBR (Demunter et al. 2001). It is not known how the UV-induced levels of these factors compare to their levels when they are overexpressed in xenograft models. Until this is determined, the role of bFGF, ET-3 (or ET-1), and SCF in melanomagenesis remains unclear. As discussed earlier, some paracrine factors for melanocytes are synthesized by fibroblasts. Given the importance of tumor-associated fibroblasts in melanoma progression and invasion, it is critical to understand how these factors might be deregulated and their role in melanomagenesis.

The MAP kinase ERK1/2 and IP3 kinase pathways are important regulators of melanocyte homeostasis. Mutations that disrupt these pathways, such as the activating $BRAF^{V900E}$ mutation, upstream of ERK1/2, and loss of PTEN that causes continuous activation of AKT, thus increased proliferation and survival, are common in melanoma (Davies et al. 2002; Sosman and Margolin 2009). Therefore, understanding the regulation of normal melanocytes provides insight into the pathways that lead to melanoma formation, and the opportunity to target components of these pathways for melanoma therapy, as in the currently used BRAF inhibitors that hold promise for melanoma treatment.

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3 Melanoma Epidemiology

Marianne Berwick

3.1 Introduction

The incidence of cutaneous melanoma is steadily increasing, mainly in populations of European origin and is thus an important public health issue throughout the world. The pattern of mortality varies by country, age, and sex. In general, older males continue to have an increase in mortality, while younger males and females have a downward trend. This chapter reviews these trends and suggests a perspective. The chapter covers worldwide incidence and mortality patterns, the relationship of host characteristics to incidence and mortality, and the relationship of environmental factors to risk.

3.2 Rates and Trends

Incidence rates for melanoma have continued to increase since 1960 through 2016 and are highest among the developed countries with some potential increases among developing countries (Table 3.1), pointing to either a change in behavior, a change in screening, or a combination of both. Among non-Caucasian populations, incidence rates are relatively low and quite variable (see Fig. 3.1). World rates vary between a low of 0.2 per 100,000 among females in India to 55.8 per 100,000 among males in Queensland, Australia. Note that rates in this chapter are standardized to the world population, which is generally younger than the populations in developed countries, and so when evaluating rates, it is important to understand the population used for standardization. Thus, for the same time period, the Queensland Cancer Council cites melanoma rates in Queensland among males as 76.4, but this is standardized to the Australian population.

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_3

Table 3.1 Rates for age-standardized[a] melanoma incidence (per 100,000; IARC, 2012, Cancer Incidence in Five Continents, Volume X), both sexes, by level of development

Population	Number	Age-standardized rate
World population	232,130	3.0
More developed regions	191,066	9.6
Less developed regions	41,064	0.8

[a]Standardized to the anticipated world population, 2012

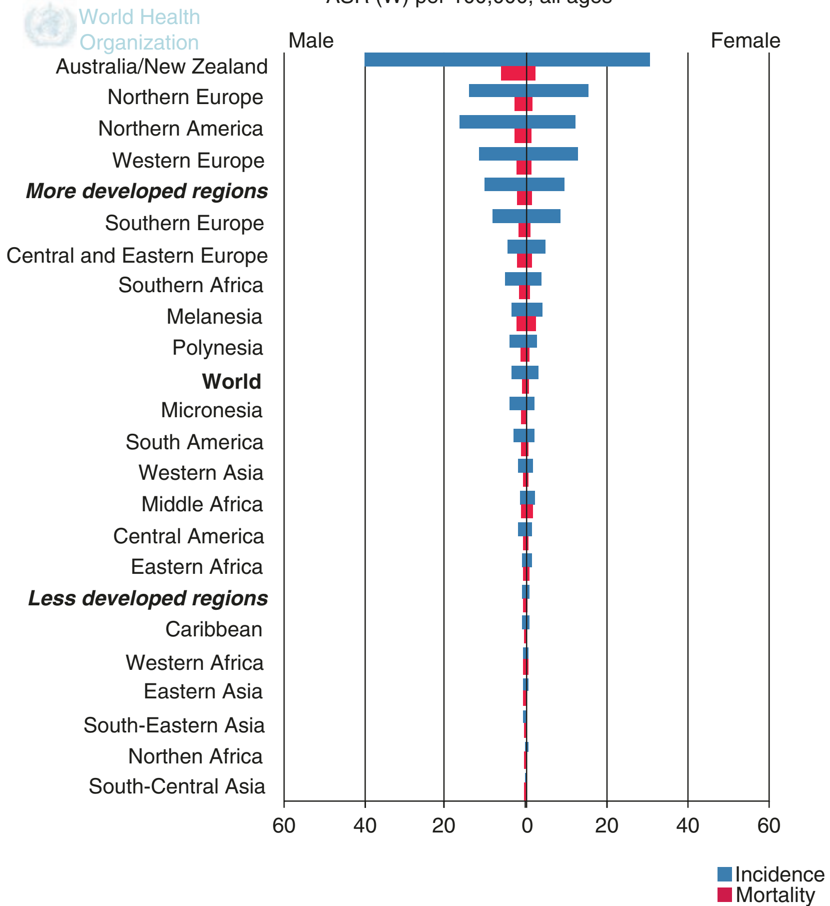

Fig. 3.1 Age standardized incidence and mortality rates of melanoma throughout the world (standardized to the world population)

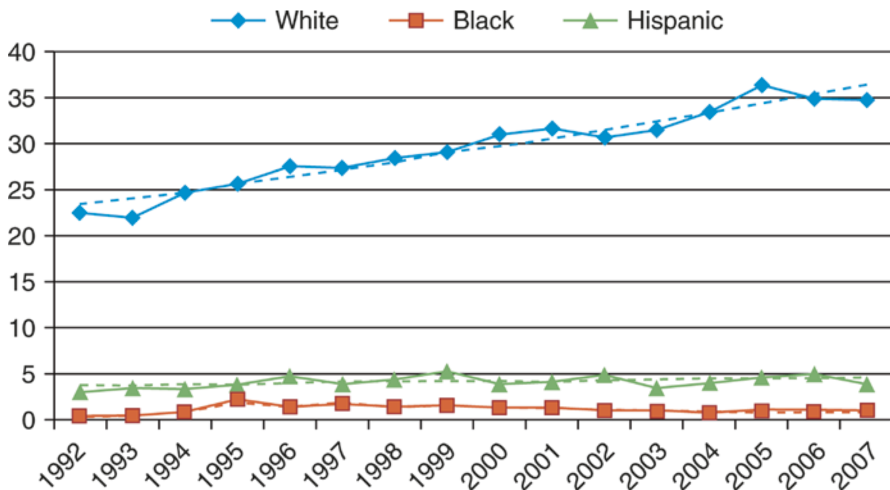

Fig. 3.2 Incidence rates for melanoma among males in the US SEER sites

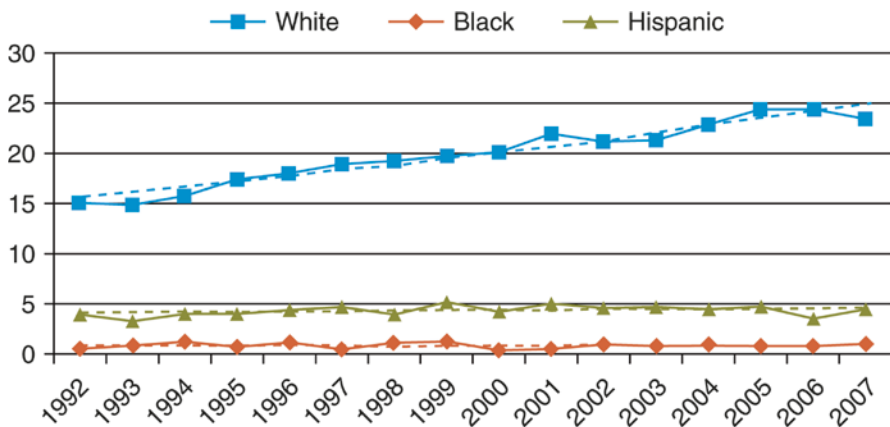

Fig. 3.3 Incidence rates for melanoma among females in the US SEER sites

Melanoma is notable for higher rates among non-Hispanic whites and this is noted in the USA Surveillance, Epidemiology, and End Results (SEER) registries, where the rates for white males are 28/100,000 and white females 17.8/100,000 compared to rates for Hispanic white males of 4.4/100,000 and 4.2/100,000 for Hispanic white females (Guy et al. 2015) (see Figs. 3.2 and 3.3).

Several recent analyses have reported on increasing incidence rates among Hispanics in California and Florida (Cockburn et al. 2006; Rouhani et al. 2010; Clairwood et al. 2014). However, although both groups represent a large proportion of the NCI SEER (Surveillance, Epidemiology, and End Results) analytic group, the overall the age-adjusted melanoma incidence rates do not reflect an increase overall among those who are identified as Hispanic, in either males or females.

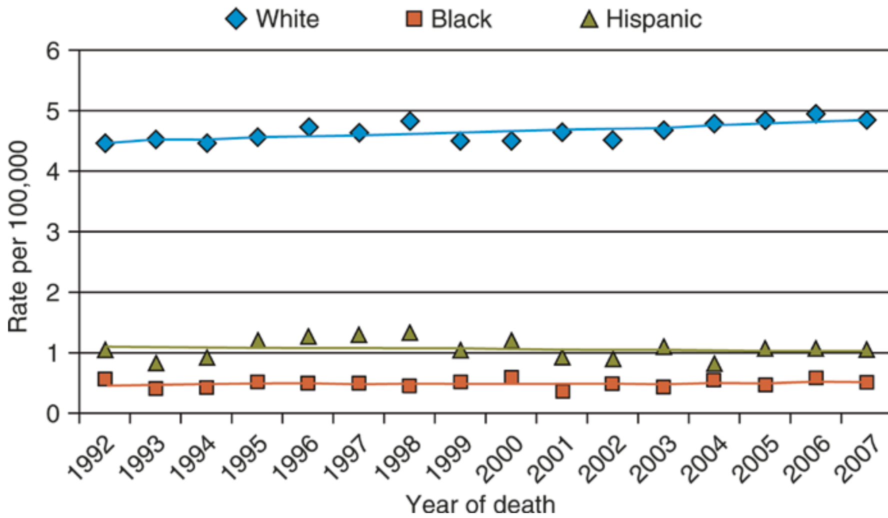

Fig. 3.4 Mortality rates for melanoma among males in the US SEER sites

When melanoma is identified among racial/ethnic groups other than whites, it is often at a deeper Breslow thickness and more advanced stage. This may be due to a lack of awareness of melanoma risk among these groups. Unfortunately, but logically, most risk models (Olsen et al. 2015) and melanoma awareness campaigns have been developed for white subjects, those at highest risk.

Mortality due to melanoma has continued to increase among white males worldwide; however, this may be focused on males over the age of 65. Over three decades (1969–1999), mortality rates from melanoma increased 157 % in men aged 65 and older (Geller et al. 2002) (Fig. 3.4). There has been a trend toward a plateau among females as noted in both the Australian data and the US data (Fig. 3.5). Unfortunately, this trend is not evident worldwide. In the UK, for example, the increase in mortality among women is continuing at a slower rate.

A thoughtful analysis by Erickson and Driscoll (2010) suggests that the discrepancy between a rapid increase in incidence and slower increase in mortality in combination with the increase in thick minimally invasive melanomas relative to thicker melanomas may be the result of intensive surveillance and a concomitantly increased biopsy rate, or "over-diagnosis" (suggested by Welch and Black 2010). Over-diagnosis can be noted when the curve for incidence is relatively steep and that for mortality is flat or relatively so. This situation exists in Australia and the United States, although mortality is not actually flat among the older age groups but is continuing to increase. Such an analysis is supported by Cho et al. (2014) but not by Shaikh et al. (2016) or Criscione and Weinstock (2010). The difference appears to lie in an emphasis by Shaikh et al. on tumor thickness, whereas Cho et al. focus on the pattern of increased 5-year survival (as with kidney and thyroid cancer) accompanied by increased incidence, but no change in mortality. Other projections suggest that incidence and mortality is declining from

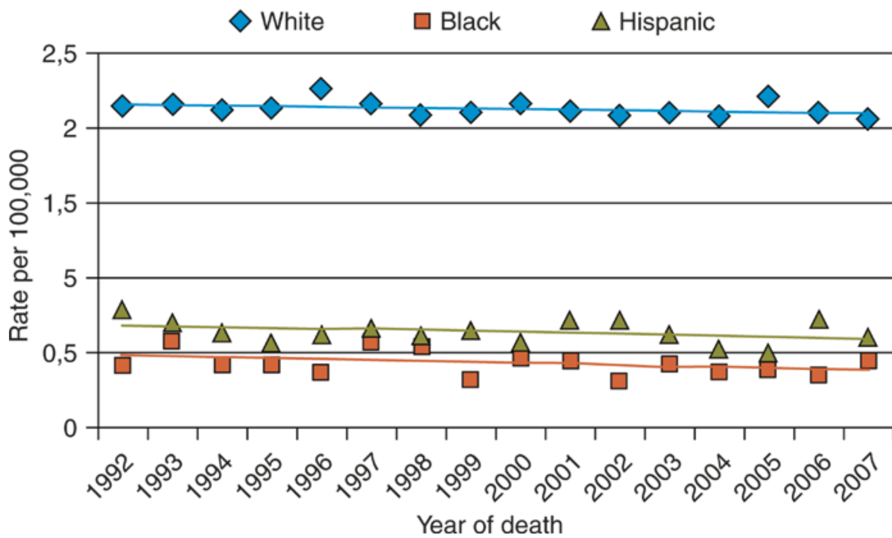

Fig. 3.5 Mortality rates for melanoma among females in the US SEER sites

melanoma (Guy et al. 2015 [mortality]; Autier et al. 2015; and among children and adolescents, Campbell et al. 2015).

In addition, there has been an approximately doubling of in situ tumors between 1988 and 2006. Until we are able to distinguish the faster growing, more aggressive tumors that are likely to lead to death from the more slowly growing tumor that may never cause problems, we will not be able to address this issue in a way to improve public health. Several investigators (Liu et al. 2006; Grob et al. 2002) have made attempts at evaluating these aggressive tumors from more slowly growing tumors; however, these methods are not yet standard and are unlikely to be applied globally.

Years of life lost (YLL) is a particularly salient method for estimating the burden of melanoma on a population. Thiam et al. (2015) estimated the highest YLL in Australia (19.9 years for males, 22.7 years for females) while Brazil appeared to have the lowest estimate (17.2 YLL for males and 19.0 for females).

3.3 Host Factors

3.3.1 Age and Sex

Lachiewicz and colleagues (2008) describe melanoma as a heterogeneous cancer with tumors with different biological mechanisms having different survival patterns. In an analysis of age-specific incidence rates, it is clear that there are two peaks of incidence in melanoma, one at 54 years and one at 74 years, with truncal melanoma peaking earlier and melanoma of the face and ears peaking later. The

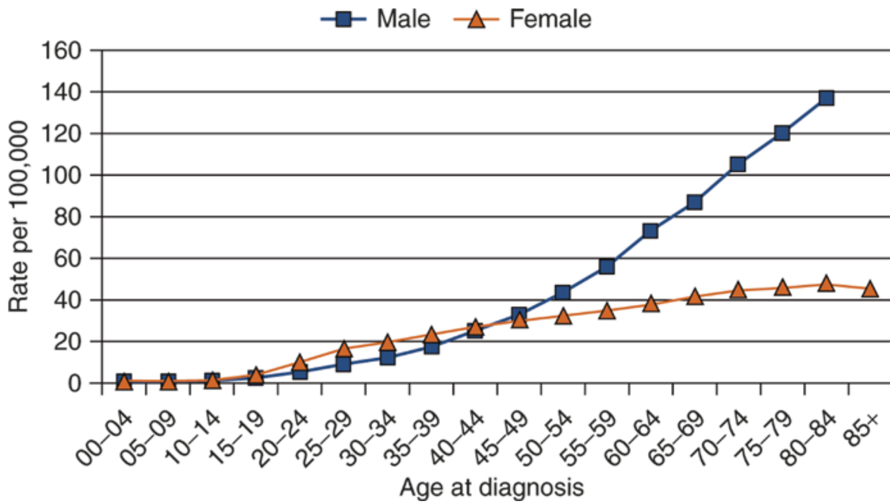

Fig. 3.6 Age at diagnosis for males (*blue squares*) and females (*red triangles*) in the US

median age of melanoma diagnosis overall ranges between 57 and 62 years. Unfortunately, melanoma among young women has been recently increasing in the US and is currently the cancer with the highest incidence among young women aged 15–24 (Purdue et al. 2008), and the site with the highest incidence is the trunk (Bradford et al. 2010). In the US, the age-specific incidence of melanoma among young women has generally been higher than that of males under the age of 40.

This pattern of the age-specific melanoma incidence is quite distinct for males and females, with a higher incidence among females up to the age of 45–50 when the age-specific rate for males climbs steeply while that for females continues to increase at a far slower rate. A "Clemmensen's hook" has been described for melanoma incidence, a similar pattern as seen in breast cancer, where there is a change in the slope of incidence near menopause, indicating a potential role for female hormones in the etiology of the disease (Fig. 3.6). It should be pointed out that while Australia's melanoma incidence rates are approximately three times those in the US, they are approximately nine times those in Ireland (Table 3.1). This statistic is rather startling and underlines the critical importance of the mixture of phenotype and intense solar exposure in Australia where much of the population migrated from Ireland and the UK. Given the great difference in incidence rates, the similarity of the age-specific rates between the US and Australia is all the more striking.

While sex differences in incidence are obvious, there are also differences in survival which are less clear (Crocetti et al. 2015) with women having a 34 % reduced risk of dying from melanoma compared to men. Biological, environmental, and behavioral factors underlie the differences (Roh et al. 2015), but more specifically, there is little understood as to precisely how Roh et al. summarize selected studies of the gender difference and find that the adjusted risk estimate for females compared to males ranges from 0.53 (deVries et al. 2008) to 0.84 (Balch et al. 2001).

Overall, female survival is generally better than males at all stages (Khosrotehrani et al 2015) even when controlling for risk factors such as age, thickness, nodal metastasis, distant metastasis, and ulceration.

3.3.2 Melanoma and Pregnancy

Given the survival advantage of females and the presence of a Clemmensen's hook in the incidence rates as well as the consistent pattern of age-specific incidence rates between an area of high incidence (Australia) and moderate incidence (the US), much speculation has focused on the hormonal aspects of melanoma. The role of pregnancy and melanoma incidence and survival has received the most attention. The bulk of evidence amassed over the past half century, however, suggests that pregnancy does not significantly affect the risk of developing malignant melanoma (Kjems and Krag 1993). Further, pregnancy does not appear to adversely influence overall survival from the disease. Results from some studies suggested that pregnant women with melanoma were more likely than their nonpregnant counterparts to exhibit adverse prognostic indicators, specifically, thicker lesions and shorter time to recurrence. Nonetheless, most studies found no difference in overall survival between pregnant and nonpregnant women with melanoma (Byrom et al. 2015). Recent reports from large-scale, population-based studies support these conclusions. Newly discovered estrogen receptors have led to new hypotheses about the role of estrogen in melanoma and these are under intense investigation (de Giorgi et al. 2009).

3.3.3 Body Site

In addition to the distinctly different pattern of incidence between males and females, the anatomic site for the development of melanoma varies distinctively among males and females. Many observers feel that this is due to specific patterns of sun exposure, but a minority think that there may be a sex-linked genetic factor that influences the distribution of melanomas by anatomic site. This conjecture is yet to be proven. In almost every registry in the world, women have a preponderance of melanomas on the leg while males have a majority of melanomas on the trunk. This difference has been modifying over time as women develop more melanomas on the trunk. Lachiewicz et al. (2008) demonstrated, as others have previously, that males and females have different incidences of melanoma on the trunk and head and neck, with the male excess on the trunk occurring at approximately age 54, while the head and neck incidence among males peaks at age 77.

These data are consistent with Whiteman's "divergent pathway" model (Whiteman et al. 2003) where those with an inherently low propensity for melanocyte proliferation require chronic sun exposure to habitually exposed sites, such as the face, to develop melanoma, whereas those with a high propensity for melanocyte proliferation develop melanomas on sites with unstable melanocytes – or aberrant melanogenesis, such as the trunk – with intermittent solar damage.

3.3.4 Pigmentation

As can be noted in Table 3.1, melanoma occurs most often among light-skinned individuals. Melanin type and content of both melanocytes and keratinocytes are critical for determining skin phenotype. Individual photoprotection of the skin is based primarily on the level of constitutive, or genetically determined, pigmentation afforded by the types and amounts of melanin synthesized and distributed in the skin. Constitutive pigmentation of the skin with higher melanin content protects the epidermis from DNA damage (Yamaguchi et al. 2006). The melanocortin 1 receptor (*MC1R*) seems to regulate the activity of melanocytes and thus is a critical genetic factor in melanin synthesis and is discussed in Chap. 2. Other genes, such as *OCA2*, for example, are clearly involved as well (Barón et al. 2014).

Those with light hair, light eyes, and skin that burns easily are at most risk for developing melanoma from ultraviolet (UV) exposure whether it is from the sun or from artificial tanning devices.

Genetics plays a role in risk to any UV and all the factors involved have not yet been determined. Recent publications show that those exposed to solar UV have a wide variety of responses to UV (see Tran et al. 2008) in terms of cellular response to DNA damage, DNA repair capacity (Wei et al. 2003), and vitamin D synthesis due to vitamin D receptor polymorphisms (Orlow et al. 2012), among others.

It is critical to note that individuals with similar levels of constitutive pigmentation may have different responses to UV (Bykov et al. 2000; Wagner et al. 2002). These differences may be due to variation in pigmentation genes. Data from the University of Pennsylvania (Kanetsky et al. 2010) and Australia (Palmer et al. 2000) demonstrate that pigmentary phenotype alone is not an adequate indicator of melanoma risk. Individuals who display darker phenotype characteristics (dark hair, brown eyes, and ability to tan) and who carry any variant *MC1R* alleles show an increased risk for melanoma (Pasquali et al. 2015).

3.3.5 Nevi

An important pigmentary-related factor is nevus density; individuals with many nevi are at consistently higher risk for developing melanoma. In fact, a large number of nevi have been shown to be the strongest known risk factor for developing melanoma among Caucasians (Armstrong and Kricker 2001). Even in the absence of clinically atypical nevi, a very high number of nevi (e.g., more than 100) have been shown to significantly increase risk for melanoma (Huynh et al. 2003). Patients with great many nevi may be missing a genetic checkpoint, the absence of which may permit the development of a higher number of nevi and increase melanoma risk. Nevus density is a simple characteristic that is likely to be useful in determining risk for melanoma among all persons and measuring genetic factors underlying nevus density should improve risk estimation.

There is an apparent interaction between sun exposure and nevus density with regard to the site of the melanoma (Karlsson et al. 2015). For example, in Australia,

sex differences in nevus density on the back and lower extremities are similar to sex differences for melanoma – men having higher rates on the back, women having higher rates on the legs – areas that are not chronically exposed to the sun (Green 1992). A similar distribution of nevi and melanoma has been noted (Juhl et al. 2009). Whiteman et al. (2003) have proposed a model for cutaneous melanoma in which two pathways – chronic exposure to the sun and melanocyte instability – represent divergent pathways for developing melanoma. Under this model, people with an inherently low propensity for melanocyte proliferation require chronic sun exposure to drive clonal expansion of transformed epidermal melanocytes. Melanomas arising in this group of people would occur on habitually sun-exposed body sites, such as the face. In contrast, the model would predict that in individuals with an inherently high propensity for melanocyte proliferation (e.g., high nevus counts), exposure to sunlight early in life would be required to start the process of carcinogenesis. These individuals would be expected to develop tumors on body sites with unstable melanocyte populations such as the trunk.

3.3.6 Family History

First-degree relatives of melanoma patients have a higher risk of the disease than individuals without positive family history (Greene and Fraumeni 1979), suggesting that a distinct hereditary component exists. Familial melanoma accounts for an estimated 5–10 % of all cases of melanoma, and characteristics that distinguish the familial from the nonfamilial form of the disease include younger age at first diagnosis, better survival, thinner lesions, multiple primary lesions, and increased occurrence of non-melanoma cancers (Begg et al. 2004; Aguilera et al. 2014). Ford pooled data from eight case–control studies and found that an individual's risk of melanoma increases by about twofold if he has an affected first-degree relative (Ford et al. 1995), and this effect was independent of host factors such as age, nevus count, hair and eye color, and freckling. Familial relative risk remained similar in all of the studies, even though melanoma incidence varied by about ten-fold in the study areas.

3.3.7 Immunologic Factors

A major enigma is that the host immune system is clearly associated with the development and progression of melanoma, but the mechanism by which it does so is not established. In some studies, melanoma incidence increased in frequency and aggressiveness after organ transplantation and immunosuppressive therapy (Vajdic et al. 2009; Dinh and Chong 2007). There is also evidence that immune reactions are altered after UV exposure in the skin (locally) and perhaps throughout the body (systemically) (Murphy et al. 1993; Hersey et al. 1983; Norval 2006). Thus, melanoma represents a unique model for studying the human immune system and the role of vitamin D in coordinating important changes in cancer development. Clearly,

the new more effective therapies that target the immune system underscore the importance of the immune system in the development and progression of melanoma (Naidoo et al. 2014).

Some data are available as to how the human immune system may alter during or as a result of UVB radiation exposure (Bechetoille et al. 2007; Muller et al. 2008; Seité et al. 2003; Berthier-Vergnes et al. 2001). However, very little is known about how and why the immune surveillance mechanism actually fails to destroy melanoma precursor lesions. Melanomas are extremely antigenic because melanoma cells produce high amounts of melanoma-specific proteins (Gould Rothberg and Rimm 2010). Even though certain T cell clones can recognize those proteins, they remain in a suppressed status; therefore, they cannot actively eradicate cancerous melanocyte growths. Lymphocytic infiltrates, mostly CD8+ or CD4+ CD25+ Fox3+ T regulator cells, have been detected in and around tumor sites in several cancers (Nedergaard et al. 2007). It is generally thought that UV exposure can induce direct immunosuppression; however, there are no strong data relating systemic and local immune reactions to UV with the etiology of melanoma. Finally, tumor-infiltrating lymphocyte status of the tumor is strongly associated with survival, indicating that immunological factors are critical for the development and prognosis of melanoma (Thomas et al. 2014).

3.4 Environmental Factors

3.4.1 Sun Exposure

Sun exposure is generally equated with ultraviolet radiation exposure, although the evidence does not rule out other unmeasured exposures associated with the sun. In the public mind, a major correlation exists between increased outdoor activity and increased skin cancer rates. In fact, there are no data available to substantiate such a relationship; that is, although there has been a dramatic increase in melanoma incidence over the last 50 years, no data show that there has been an increase in outdoor activity during the past 50 or so years although the trend toward wearing less clothing is self-evident (Chang et al. 2014).

The data to support an association between sun exposure and the development of melanoma are indirect. There has been a latitude gradient for the incidence of melanoma among Caucasians, such that the highest rates are nearest the equator. In Europe, this gradient has been confounded by the fact that those with darker pigmentary phenotype live in the Southern areas of Europe and those with lighter phenotype in the Northern, so that the gradient in Italy, for example, was actually reversed. However, this does not explain the higher melanoma rates in Norway than in Sweden. Furthermore, new data suggest that trends for incidence and mortality are “evening out” in terms of latitude. Armstrong and Kricker (1993) estimate that between 68 % and 90 % of all melanomas are caused by sun exposure. Most would not dispute this estimate; however, the major point here is that it is likely intermittent sun exposure among susceptible individuals that leads to melanoma as noted

among the UK migrants to Australia. Noticing that personal annual UV doses are low and melanoma is increasing rapidly, Merrill et al. (2015) suggest that low vitamin D and possibly viral infections may play a role in this paradox.

3.4.1.1 Patterns of Sun Exposure: Intermittent, Chronic, and Cumulative Sun Exposure

Although there is no standard measure of sun exposure in research, sun exposure can be generally classified as "Intermittent" or "Chronic," and the effects may be considered as acute or cumulative. *Intermittent sun exposure* is that obtained sporadically, during recreational activities usually, and particularly by indoor workers who have only weekends or vacations to be outdoors and have not adapted to the sun. *Chronic sun exposure* is incurred by consistent sun exposure, usually by outdoor work, but also among those people who are outdoors a great deal. *Cumulative sun exposure* is the additive amount of sun exposure that one receives over a lifetime. Cumulative sun exposure may reflect the additive effects of intermittent sun exposure or chronic sun exposure or both. Thomas et al. (2010) recently demonstrated that solar elastosis, a breakdown of collagen and elastin in the epidermis, when located near the site of a melanoma is directly linked to high levels of ultraviolet radiation at the site. Surprisingly, Berwick et al. (2005) demonstrated that solar elastosis is associated with better survival from melanoma, independent of age or histologic subtype of the tumor.

Indeed, different patterns of sun exposure appear to lead to different types of skin cancer among susceptible individuals. In Europe, Rosso et al. (1996) quantified suggestions by Kricker et al. (1995) that basal cell carcinoma and squamous cell carcinoma have different patterns such that squamous cell carcinoma appears to have a threshold at approximately 70,000 h of exposure to sun after which incidence increases sharply, regardless of whether it is chronic sun exposure or intermittent sun exposure. This is highly consistent with the molecular genetic evidence (Kraemer et al. 1994) where combined analysis of skin cancer mutations from several laboratories found the p53 tumor suppressor gene mutated in 90 % of human squamous cell carcinomas and approximately 50 % of basal cell carcinoma. Approximately 70 % of tumors exhibited the characteristic UVB footprint, a C to T or a CC to TT mutation at specific codons.

It is a surprise to many that analytic epidemiologic studies have shown only modest risks at best for the role of sun exposure in the development of melanoma incidence, and three systematic reviews have demonstrated extremely similar estimates of effect for the role of intermittent sun exposure, an odds ratio of 1.6 (Gandini et al. 2005; Nelemans et al. 1995; Elwood and Jopson 1997). It is important to note that chronic sun exposure, as in those occupationally exposed to sunlight, is protective for the development of melanoma, with an odds ratio of 0.7–0.9, equivocal for the development of basal cell carcinoma, and a risk factor for squamous cell carcinoma. As Elwood and Jopson point out, the measurement of sun exposure is complex and any discrepancies among studies could be sorted out by conducting new studies using compatible protocols in different populations with different levels of sun exposure.

A clearer explanation for the rise in melanoma incidence that takes into account the different effects of chronic and intermittent sun exposure, proposed by Gallagher et al. (1989), is that as people have replaced outdoor occupations with indoor, they have engaged in more intermittent sun exposure. Gallagher showed that the decrease in outdoor occupations, or chronic exposure which is not a risk factor for melanoma, could explain the increase in melanoma incidence in Canada.

The evidence for cumulative exposure comes from two sources to date: migrant studies and studies of lifetime exposure, controlling for intermittent and occupational exposure. Data from Australia and Italy show that individuals who migrate at a young age (less than 10 years) from areas of low exposure, such as the UK, to areas of high exposure, such as Australia or Israel, have a lifetime risk of developing melanoma that is similar to that of the new country. On the other hand, individuals who migrate later in life, adolescence or older, from areas of low solar exposure to areas of high solar exposure, have a risk that is quite reduced (Whiteman et al. 2001; see Table 3.1). These data have often been cited to indicate that childhood sun exposure is more important than adult sun exposure in the development of melanoma. However, they can also be interpreted to indicate that the length of exposure is critical rather than the time of exposure; that is, those who migrate early in life have a longer period for intense exposure compared to those who migrate later in life.

3.4.1.2 Effect Varies by Skin Type

The pattern of sun exposure that appears to induce melanoma development is complex and is clearly different by skin type (i.e., propensity to burn, ability to tan). Armstrong et al. (1997) have proposed a model consistent with data from other epidemiologic studies (White et al. 1994, among others) where risk for melanoma increases with increasing sun exposure among those who tan easily, but only with a small amount after which risk decreases with increasing exposure. Among subjects who are intermediate in their ability to tan, risk continues to increase slowly and then at some point declines with increasing exposure. On the other hand, those subjects who have great difficulty tanning have an almost linear increase in risk with increasing sun exposure. This model recognizes that individuals are differentially susceptible to sun exposure and have different levels of risk based on skin type. Moreover, it suggests that different types or patterns of sun exposure are associated with different levels of risk for melanoma.

It is worthwhile looking at the estimates of effect of sun exposure on the development of melanoma in tandem with the other major risk factors for the development of melanoma – nevi number and pigmentary phenotype. Work is ongoing to determine the interrelationship of genetic susceptibility and these phenotypic characteristics (Begg and Berwick 1997). In unpublished data from a population-based case control study in Connecticut (Berwick et al. 1996), the investigators estimated the risk for developing melanoma for nevus number, pigmentary phenotype, and sun exposure in early life as well as sun exposure 10 years prior to the diagnosis of melanoma, adjusting for age and sex. The risk for melanoma with numerous nevi in this study is six times that of someone with few nevi. The risk for melanoma with the most sensitive pigmentary phenotype is almost six times that of someone with

the least sensitive phenotype. However, the risk for melanoma with increasing early life sun exposure or increasing later life sun exposure is only two times that of someone with the least sun exposure. Clearly, genetically determined characteristics such as nevi and pigmentary phenotype are more powerful determinants of melanoma risk than is sun exposure.

3.4.1.3 Sunburn

The role of sunburns in the development of melanoma is a critical issue. This aspect of sun exposure is the one most often cited as key to determining melanoma risk. In fact, it can be forcefully argued that sunburn itself is not on the pathway to the development of melanoma, but that it is an important marker for the combination of genetically susceptible phenotype and excessive sun exposure. Numerous articles in the lay media as well as dermatology journals stress the importance of a specific number of sunburns in increasing risk for melanoma. However, a critical look at these studies will show that the relative risk for developing melanoma, when adjusted for host characteristics, is often not statistically significant and is not always impressive. Data from Europe (Autier and Dore 1998) support the concept that childhood sun exposure is not fully represented by sunburning episodes.

While sunburn is the most visible and immediate effect of overexposure to UV, it is also the one that the public is most likely to associate with the development of melanoma. The emerging consensus, however, is that it is unlikely that sunburn is causally associated with melanoma; it is more likely that sunburn is a clear indicator of the interaction between too much sun exposure and a susceptible phenotype, that is severe solar exposure to skin unaccustomed to it.

3.4.1.4 Suberythemic Exposure

Indeed, a great deal of research is currently being focused on suberythemic exposures, that is, those doses of ultraviolet radiation that do not cause an actual burn but that may have biologic significance. Certainly, exposures to the UVA portion of the UV spectrum may lead to the development of melanoma (Moan et al. 1999; Vogel et al. 2014).

Measurement error is a more serious problem in evaluating sunburn history than other sun-associated variables (English et al. 1998; Westerdahl et al. 1996; Berwick and Chen 1995). At least three studies have conducted test–retest reliability studies and concluded that sunburn history is poorly recalled with only a little over half the subjects giving the same answer at two points in time to the question: “Have you ever been sunburned severely enough to cause pain or blisters for 2 days or more?” Other sun-associated variables, such as time spent outdoors during recreation, for example, appear to be more reliably remembered (English et al. 1998; Petersen et al. 2013).

3.4.1.5 Timing of Sun Exposure

Much has been made of the critical time of sun exposure in the development of melanoma. This concept has not yet been proven. In fact, it is likely that all times in life are important in the development of melanoma. Data from a case–control study

of melanoma conducted in Connecticut show that intermittent exposure in the 10 years prior to the diagnosis of melanoma is just as important as intermittent exposure in early life. The argument that 70 % of one's sun exposure is likely obtained before the age of 20 may be true; however, this often-quoted statistic is merely an estimate (Stern 2005). With the changes in lifestyle of the 1990s and the early twenty-first century, it is quite possible that individuals in the latter half of life receive a very substantial amount of sun exposure as a result of early retirement and flexible work schedules. At the same time, there are numerous forces at work to diminish the outdoor experiences of young people: the tremendous increase in video games and computers as well as the increasing atomization of neighborhoods, so that "pick up" games of kick the can are no longer as easy to organize.

The preponderance of data shows that excessive intermittent sun exposure at any age increases risk for melanoma. Although the public and many researchers feel that sun exposure during early childhood is the critical period for melanoma induction, there are no empirical data to support this view, attractive though it is.

Autier and Dore (1998) attempted to address the issue as to whether early life or later life sun exposure was the critical factor in determining melanoma risk. They found that both time periods were important. An interesting comparison shows the joint effects of sun exposure during childhood and adulthood. They find, as one might expect, that the highest risk among adults is for those who had high intermittent sun exposure as children. Conversely, those who had low sun exposure during childhood and high sun exposure in adulthood had a similar risk to those who had high exposure during childhood and low exposure during adulthood.

Other data support the idea that intermittent sun exposure leads to increased risk at any age. Holly et al. (1995) showed that more than seven painful sunburns during elementary school increased risk twofold ($OR = 2.0$, 95 % $CI = 1.4$, 2.9) and that more than seven sunburns after the age of 30 (the age of women in this study ranged from 18 to 59) increased risk twofold ($OR = 2.0$, 95 % $CI = 1.1$, 3.8). In sum, data from very different setting seem to suggest that intermittent sun exposure is critical to the risk for developing melanoma. In the published studies that looked at both early life and adult sun exposure, there is very little difference between the effects of sun exposure at either stage, but that lifelong intermittent sun exposure is indeed cumulative.

3.4.1.6 Occupational or Chronic Sun Exposure

The reasons for the differing trends in melanoma risk between occupational and intermittent sun exposure are not well understood. Analyses of melanoma time trends from Canada (Bulliard and Cox 1999), New Zealand (Bulliard and Cox 2000), Germany (Garbe et al. 1994), Australia (Garbe et al. 2000; Marrett et al. 2001), and Denmark indicate that changes in lifestyle factors, such as sun exposure behaviors and fashion, correlate (Osterlind et al. 1988) strongly with increases in melanoma on skin areas exposed intermittently to the sun (trunk, upper arms, and upper legs). With regard to chronic occupational sun exposure, it is also possible that additional phenotypic differences among workforce members may be influencing the direction and intensity of melanoma risk. In a study of occupational melanoma from Spain (Espinosa Arranz et al. 1999), higher melanoma risk was observed

among construction workers than among farmers. The melanoma risk in construction workers became more significant when adjusted for skin type, age, freckle count (odds ratio (OR) 4.3; 95 % confidence interval (CI) 1.8, 9.9), and number of nevi (OR 2.8; 95 % CI 1.4, 5.8), while the risk in farmers remained protective even with these adjustments.

3.4.2 Tanning Beds

Tanning beds have been increasing in number and popularity throughout the world, particularly but not exclusively in more northern latitude. Great concern has been expressed by the International Agency on Cancer (IARC) that this increase will lead to increases in melanoma risk. IARC convened an expert panel of epidemiologists (IARC 2007) who performed a meta-analysis of 19 studies that have evaluated the association between sunbed exposure and melanoma and other skin cancers to that time. This analysis showed a significant summary, or overall, relative risk for melanoma of 1.8 (95 % CI 1.4, 2.3) for "first exposure under the age of 35"; a relative risk of 1.2 that was statistically significant (95 % CI 1.0, 1.3) for "ever use." Controversy has, however, continued over the carcinogenic properties of tanning beds. The tanning industry "sells" tanning beds as a safe alternative to UV exposure for both tanning as well as vitamin D biosynthesis. As most tanning beds are not regulated (Nilsen et al. 2016), the amount of UV exposure from using tanning beds is far higher than the same amount of time spent in the sun. Usage of tanning beds have been subject to regulations, with Brazil in 2009 and Australia in 2015 banning all tanning beds and the EU and many states in the US banning them for use by minors (Sinclair et al. 2014; Bulger et al 2015).

A recent study conducted in Minnesota has published high and significant risks for developing melanoma among tanning bed users. Among 1,167 cases and 1,101 controls, 62.9 % of cases and 51.1 % of controls had tanned indoors (adjusted OR 1.7; 95 % CI 1.4, 2.1). Melanoma risk was pronounced among users of both UVB-enhanced (adjusted OR 2.9; 95 % CI 2.0, 4.0) and primarily UVA-emitting devices (adjusted OR 4.4; 95 % CI 2.5, 8.0). Risk increased with use: years ($P<0.006$), hours ($P<0.0001$), or sessions ($P=0.0002$). ORs were elevated within each initiation age category; among indoor tanners, years used was more relevant for melanoma development (Lazovich et al. 2010). Thus, epidemiologic data suggest that tanning beds are not safer than solar ultraviolet radiation and that they may have independent effects from solar exposure that increase risk for melanoma.

A major problem in evaluating the risk from tanning beds and sunlamps is that they have changed over time in terms of their usage and their spectral output. In addition, the dosage of UV is extremely difficult to obtain as most tanning parlors do not calibrate their equipment or measure their output. When comparing dosage of tanning lamps to solar radiation, it is important to estimate the proportion of the body irradiated. From 15 % to 50 % of the total body is uncovered during outdoor activities, but up to 95–100 % of the total body is uncovered during indoor tanning. Therefore, the dosage is likely to be far greater than from a similar amount of outdoor solar exposure.

Females tend to use sunbeds more than males, particularly young women. Recent data from the United States National Cancer Institute show that the incidence of melanoma is growing among young females (Purdue et al. 2008). In addition, sunbed usage in the US is most prevalent among young women (Lazovich and Forster 2005). These statistics point up the fact that sunbed usage is an area for serious concern. In fact, Veierød et al.'s (2003) evaluation of use of sunbeds found that those who used sunbeds at ages 20–29 years once or more per month had a statistically significant relative risk of developing melanoma of 2.6 (95 % CI 1.5, 4.5). This fact will be crucial to guide prevention in the future.

Most studies have shown an increased risk for melanoma associated with sunbed use, but there are multiple qualifications that need to be taken into account. In the first place, it is difficult to disentangle the use of artificial UV from natural UV exposure. Many authors, for example, Wester et al. (1999), have found frequent tanning in sunlight correlated with sunbed use.

Gallagher et al. (2005) asks the critical question: "If there is a causal relationship, how important is the risk?" This is a difficult question to respond to at this point in time for a number of reasons: (1) Assessment of sunbed use needs improvement as well as assessment of spectral output. Although we see good agreement for individual's recall of sunbed usage, it is likely that the timing and the exposure are not all the same for all individuals. (2) It is unclear whether one can compare sunbed use and sunbathing; is there the same biological mechanism? (3) Most studies have taken place in higher latitudes in North America and Europe where the background ambient ultraviolet radiation is low; it would be useful to have more data from lower latitudes with higher levels of ambient UV, such as Australia and Southern US. (4) Ecological studies are inconsistent – even at similar latitudes with very good data. In Denmark Faurschou and Wulf (2007) concluded that sunbed risk for BCC is important but not CMM. However, in the UK, Diffey (2007) estimated that sunbed risk for CMM in women is dramatic and may have caused as many as 182 cases of CMM in women and 49 in males in the last 8 years.

3.4.3 Other Environmental Factors

Other environmental factors are receiving new attention.

3.4.3.1 Pesticides and Herbicides

Dennis' group evaluated data from the Agricultural Health Workers' Study and found significant associations between cutaneous melanoma and maneb/mancozeb (trend $P=0.006$), parathion (trend $P=0.003$), and carbaryl (trend $P=0.013$). Other associations with benomyl and ever use of arsenical pesticides were also suggested (Dennis et al. 2010). Seggato et al. (2015) found that indoor domestic use of pesticides may be an independent environmental risk factor for melanoma. Lerro et al. (2015) reported borderline associations for the use of acetochlor and melanoma incidence among acetochlor users in the Agricultural Health Workers' Study.

3.4.3.2 Arsenic

Arsenic has been suspected of involvement, since it is a known cocarcinogen with UV in exacerbating development of non-melanoma skin cancer (Rossman et al. 2004). However, most elevated levels of As are found in areas with populations who are highly resistant to UV-induced melanoma (Taiwan, Bangladesh) and so even a significant increase in malignant melanoma skin cancer rate would not be detected in epidemiology studies in those populations. A significant positive association has been shown between body arsenic levels (toenail arsenic) and melanoma risk in a (predominantly) Caucasian Iowa population, demonstrating that interactions between sunlight and arsenic may contribute to melanoma in these populations (Beane Freeman et al. 2004). Yager et al. (2016) found an interaction between DNA damage and UV among cases with melanoma, suggesting genetic predisposition

3.4.3.3 Polychlorinated Biphenyls (PCBs)

Some epidemiologic studies have suggested that exposure to organochlorine compounds might increase the risk of melanoma, but these studies have lacked biological measures of exposure and have not been able to control for the major environmental risk factor for melanoma – sun exposure. Gallagher and co-workers (2011) were able to conduct a pilot study with the ability to adjust for sun sensitivity and sun exposure. Plasma was collected from 80 cases and 310 controls in British Columbia. Assays for 14 PCB congeners and 11 organochlorine pesticide residues were conducted: risk of melanoma and plasma levels of non-dioxin-like PCBs (adjusted OR 7.0, 95% CI 2.3, 21.4 among those in the highest quartile) as well as several PCB congeners, organochlorine pesticides, or metabolites. This study suggests that other environmental factors in addition to UV radiation may play an important role in the etiology of melanoma.

3.4.3.4 Diet

Although dietary behaviors have not previously been shown to be associated with the development of melanoma, there is current interest in the role of coffee consumption. A meta-analysis and systematic review (Yew et al. 2016) of coffee consumption shows that the pooled relative risk for coffee drinkers for melanoma was 0.75 (95% CI 0.63–0.89, $P = 0.001$), although there is some suggestion of publication bias. This conclusion was supported by another meta-analysis by Wang et al. (2016) which reached similar conclusions. A study evaluating the quality of the diet and risk of melanoma in an Italian population (Malagoli et al. 2015) found that the DASH diet (Dietary Approaches to Stop Hypertension) was significantly inversely associated with melanoma in women. Data on alcohol's relationship to melanoma have been mixed. A thorough pooled analysis by Miura et al. (2015) finds no strong support for such an hypothesis.

3.5 Summary

Melanoma is clearly a complex disease for which we do not have simple answers. This chapter has attempted to delineate some of the challenges in understanding the trends for melanoma incidence and mortality. Although we do not yet understand

fully the etiology of melanoma, there seems to be a relatively clear message for the public: “Be cautious all your life. Enjoy the sun in moderation. Stay away from large bursts of sun exposure, particularly on untanned skin.”

Acknowledgments I acknowledge assistance with the SEER and IARC data from Angela Meisner and Dr. Charles Wiggins. This work was supported by NCI R01 CA 112425 and by the University of New Mexico Cancer Center and the University of New Mexico Department of Internal Medicine.

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Melanoma Genetics and Genomics

4

Allen Ho, Göran Jönsson, and Hensin Tsao

4.1 MAPK and PI3K Pathways and Melanoma

There is substantial evidence that two of the major pathways central for melanoma development are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways. Indeed, nearly 25 % of melanomas have activating mutations of *NRAS*, an additional 15 % have loss of Nf1 function, and a small fraction of melanomas have either *KIT* activating changes or translocations/fusions of *ALK* or *ROS* with other genes, which result in increased MAPK signaling (Bastian 2014). Moreover, the clinical success of targeting the MAPK pathway in the treatment of metastatic melanoma underscores the central role this pathway plays in melanoma pathogenesis. The first oncogene to be identified in melanoma was *NRAS* (Padua et al. 1984), which is known to be mutated not only in melanoma but also in other cancers. It would take an additional 20 years before the next established melanoma oncogene was discovered. In a comprehensive mutation screen of protein kinases in human cancer conducted by the Wellcome Trust Sanger Institute, *BRAF*, the immediate downstream effector of *NRAS*, was found to be somatically mutated

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_4

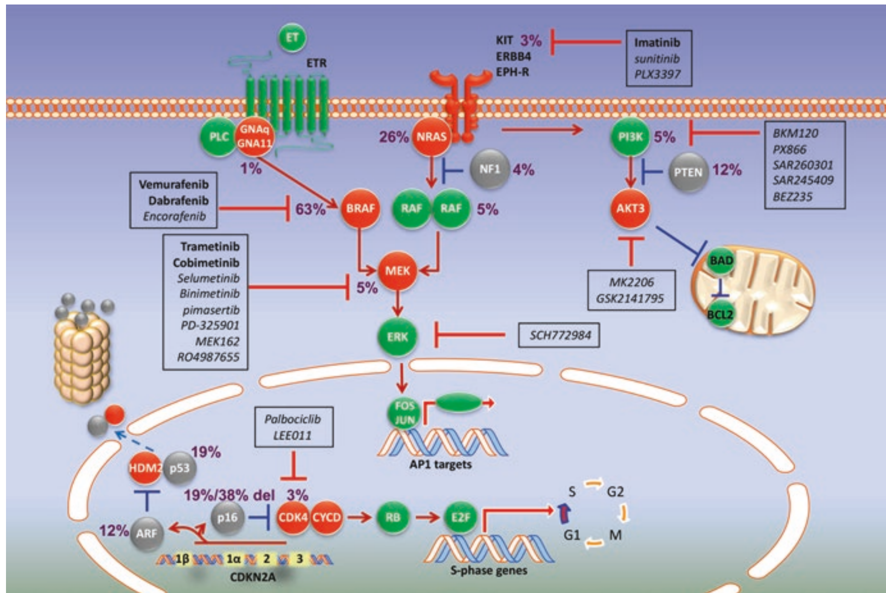

Fig. 4.1 Molecular lesions in melanoma signaling genes. Many components of the melanoma growth signaling cascade are mutated and activated. *Red* activated, *gray* inactivated, *green* normal function. Drugs (*shown in boxes*) that have been approved (*bold*), or in trial (*non-bold, italics*), are indicated

in >60 % of metastatic melanomas (Davies et al. 2002). The dual role of Nras in activating both the MAPK and PI3K pathways and Braf in activating the MAPK pathway underscores the importance and therapeutic opportunities the defects in these pathways presents. Here, we summarize the genetic alterations found in these pathways and their consequences.

4.1.1 MAPK Pathway

Braf is a serine threonine kinase and a member of the "classical" MAPK pathway, which regulates cell growth, survival, and differentiation. Braf is highly expressed in melanocytes and neuronal tissue, both of which are of neural crest origin. MAPK pathway activation is mediated by receptor tyrosine kinases and G-protein-coupled receptors, which subsequently activates the Ras family members and then the Raf kinase family (Araf, Braf, and Craf). The Raf kinases activate Mek1/2, which in turn stimulates Erk1/2, thereby triggering the eventual expression of genes involved in proliferation or differentiation (Fig. 4.1). It was demonstrated early on that mutations in *BRAF* was a common genetic event in nevi, suggesting that it is an early event in melanoma progression (Pollock et al. 2003). This led several groups to investigate whether *BRAF* germline mutations were present in melanoma kindreds not accounted for by *CDKN2A* mutation; however, no germline mutations were identified thereby excluding the role of *BRAF* as a melanoma susceptibility gene

(Casula et al. 2004; Jackson et al. 2005; Laud et al. 2003). The single most common mutation found in *BRAF* is the T1799A point mutation leading to an amino acid substitution of glutamic acid to valine at position 600 located in the kinase domain of the *BRAF* gene and in total >80 % of all *BRAF* mutations occur at this locus (Hocker and Tsao 2007). Other putative oncogenic mutations occur at other codons in exon 15 or in exon 11, both of which correspond to the kinase domain as well (Hocker and Tsao 2007). The frequency of mutations in melanoma varies from 30 to 70 % depending on the study; in the largest study on primary cutaneous melanomas, a *BRAF* mutation rate of 48.9 % was reported (Ellerhorst et al. 2011). Studies have demonstrated that *BRAF/RAS* mutant melanomas are driven, at least in part, by MAPK signaling (Hodis et al. 2012; Krauthammer et al. 2012). Noteworthy, *BRAF* mutation frequency differs in melanoma depending on certain clinical and pathological subsets. In melanomas arising from chronically sun-exposed body sites as well as in melanomas not related to sun exposure (mucosal and acral melanomas), the rate of *BRAF* mutagenesis is quite low, whereas the opposite is observed for melanomas arising on intermittently sun-exposed areas (such as trunk and back) (Curtin et al. 2005; Maldonado et al. 2003). Whether the difference is actually based on body site or histological subtype is difficult to tease apart since melanomas arising on chronically sun-exposed sites often are lentigo maligna melanomas (LMM) and melanomas on intermittently sun-exposed sites often are superficial spreading (SSM) or nodular melanomas (NMM) (Curtin et al. 2005). *NRAS*, the second most commonly mutated oncogene in cutaneous melanoma (~15–25 %), is known to activate both the MAPK and the PI3K pathways (Ellerhorst et al. 2011; Bastian 2014). Although some associate *NRAS* mutations with chronic actinic damage and nodular melanomas, *NRAS* mutations were not found to be specific for a melanoma subtype and were noted among melanomas from non-sun-damaged skin (Curtin et al. 2005). Most importantly, *BRAF* and *NRAS* mutations occur in a mutually exclusive manner and in all about 75 % of all melanomas harbor mutations in either of the genes (Hocker and Tsao 2007; Curtin et al. 2005). Additional *RAS* genes seem to have only a minor impact in melanoma. In recent studies, a few somatic mutations were detected in *KRAS* (Brose et al. 2002; Reifenberger et al. 2004). Likewise, *HRAS* mutations are rare in melanomas; however, 12 % of Spitz nevi harbor a genomic gain of the *HRAS* locus and simultaneously 67 % of nevi harboring a gain concomitantly carry a mutation in the *HRAS* gene (Bastian et al. 2000). In some rarer forms of melanoma, such as uveal melanoma, *BRAF*, *KIT*, and *NRAS* mutations are extremely rare. Instead, mutations in the heterotrimeric *GNAQ* gene were identified in 46 % of uveal melanomas. Mutations occurred at a single locus (Gnaq^{Q209L}) located in the Ras-like domain, and in vitro studies verified that the Q209L mutation activated the MAPK pathway suggesting an alternative route for *BRAF* and *NRAS* mutation (Onken et al. 2008; Van Raamsdonk et al. 2010). Indeed, pre-clinical studies examining inhibition of Mek and Akt pathways in uveal melanoma cells demonstrate decreased cell viability in a synergistic manner (Ambrosini et al. 2013). *GNAQ* mutations are also frequently detected in blue nevi, suggesting that it is an early genetic event similar to the findings of *BRAF* mutations in nevi (Van Raamsdonk et al. 2009). Additionally, in vivo analysis indicates that mutation of *GNAQ* is not sufficient for full progression to melanoma. This is also true for *BRAF*

and *NRAS* mutations suggesting overlapping functional properties of these three proteins. The discovery of somatic mutations of *GNAQ* in uveal melanoma led research groups to investigate mutation patterns of heterotrimeric G proteins in melanoma. The group of Yardena Samuels examined the mutation spectrum of 35 genes in melanoma samples. Somatic changes were detected in 17 % of all samples with *GNG10* and *GNAZ* having highest frequency of mutations (Cardenas-Navia et al. 2010). Mutations in *GNA11* were found in 32 % of uveal melanomas in a mutually exclusive pattern with *GNAQ* mutations (Van Raamsdonk et al. 2010).

Patients with high-risk ocular melanomas in the context of COMMON (*c*utaneous/*o*cular *m*elanoma, atypical *m*elanocytic proliferations and *o*ther *n*eoplasms) syndrome have somatic-inactivating mutations of *BAP1* (Njauw et al. 2012). Furthermore, a germline mutation of *BAP1* has been associated with uveal melanoma, suggesting a tumor suppression function (Höiom et al. 2013). However, the role of Bap1 in cutaneous melanoma may be more nuanced as Bap1 has been shown to have a growth-sustaining role in cutaneous melanoma cells (Kumar et al. 2015).

Genome-wide screens to discern whether molecular patterns associated to *BRAF* mutation exist have been performed. Pavey et al. used microarray expression profiling to distinguish *BRAF* and *NRAS* mutant and wild-type cell lines. A set of 83 genes was identified to clearly discriminate *BRAF* mutant and *BRAF* wild-type cells (Pavey et al. 2004), and the approach was later verified in additional data sets (Johansson et al. 2007). It has also been demonstrated that distinct genomic changes in *BRAF*-mutated cells where chromosomes 7 (harboring *BRAF*), 10q (harboring *PTEN*), 11q, 14q, and 20q were identified are discriminatory (Jonsson et al. 2007). The observation of concomitant *BRAFV600* mutation and *PTEN* loss is further enforced by a significant association between *BRAFV600* mutation and *PTEN* somatic mutation (Jonsson et al. 2007; Tsao et al. 2004). These genetic data are corroborated by a recent study where a mouse model with conditional melanocyte-specific expression of *BRAFV600E* developed melanocytic hyperplasias. Moreover, when *PTEN* was abrogated, mice developed melanomas with 100 % penetrance and subsequently induced metastases in the lymph nodes and lungs (Dankort et al. 2009). These data emphasize the cooperativity between the PI3K and the MAPK pathways for melanoma tumorigenesis, and thus activating mutations of *BRAF* as well as inactivating changes in *PTEN* represent one molecular partnership to attain the desired effect. Taken together, these results suggest that *BRAF* mutant melanomas develop via a distinct genetic pathway.

The success of therapeutic targeting of Braf in metastatic melanoma underscores the importance of understanding the genetics underlying melanoma pathogenesis (see Chap. 16). The first drug tested that targets Braf was Sorafenib, which was later found to be ineffective in the clinical setting (Eisen et al. 2006). Vemurafenib, a potent Braf inhibitor, is approved for patients with Braf^{V600E}-mutated melanomas that are unresectable or metastatic (Chapman et al. 2011). The phase III trial, BRIM-3, comparing vemurafenib versus dacarbazine as a first-line therapy for Braf^{V600E}-mutated metastatic melanoma demonstrated improved median progression-free survival (PFS; 5.3 versus 1.6 months) and better overall survival (OS; 84 % versus 64 %) at 6 months in the vemurafenib versus dacarbazine groups, respectively. The early positive results of BRIM-3 led to unblinding, allowing patients on the

dacarbazine arm to cross over to vemurafenib. Dabrafenib is another selective Braf inhibitor recently approved for the treatment of Braf^{V600E} mutation positive, unresectable, or metastatic melanoma. The phase III trial, BREAK-3, comparing dabrafenib versus dacarbazine demonstrated improved median PFS when compared to those in the dacarbazine arm, 5.1 versus 2.7 months, respectively, with a hazard ratio (HR) for progression of 0.30 (95 % CI 0.18–0.51; $p<0.0001$) (Hauschild et al. 2012). The true difference in overall survival cannot be assessed due to the predefined crossover of progressing dacarbazine patients into the dabrafenib arm.

The discovery of *BRAF* mutations suggested that other members of the MAPK pathway may prove to be attractive therapeutic targets. Mek 1/2 are effector proteins downstream of Braf. Moreover, exome sequencing of metastatic melanoma specimens identified somatic mutations in *MEK1* and *MEK2* as potential clinically significant aberrations, characterizing *MEK1* and *MEK2* mutations in 8 % of melanomas (Nikolaev et al. 2011). Trametinib is a selective Mek 1/2 inhibitor recently approved for the treatment of patients with Braf$^{V600E/K}$—mutant metastatic melanoma. The phase III trial, METRIC, comparing trametinib to chemotherapy (dacarbazine or paclitaxel) in the treatment of patients with Braf$^{V600E/K}$ mutant-positive metastatic melanoma demonstrated significant improvement in median PFS (1.5 versus 4.8 months; HR = 0.45; 95 % CI 0.33–0.63; $p<0.001$) and 6-month OS (67 % versus 81 %; HR = 0.54; 95 % CI 0.32–0.92; $p=0.01$), despite being permitted to crossover to trametinib (Flaherty et al. 2012a).

Notwithstanding these promising results with single agent selective Braf inhibitor use, clinical responses are typically short-lived with a PFS of approximately 7 months (Chapman et al. 2011; Hauschild et al. 2012). This led to several approaches to improve the durability of response, including using combination strategies and intermittent dosing schedules to delay selection of resistant tumor cells (Das Thakur et al. 2013; Flaherty et al. 2012b; Long et al. 2014; Robert et al. 2015). A phase III trial, COMBI-v, comparing first-line trametinib plus dabrafenib to dabrafenib alone in patients with Braf$^{V600E/K}$ mutant metastatic melanoma demonstrated improvements in the median PFS (9.3 months versus 8.8 months; HR = 0.75; 95 % CI 0.57–0.99; $p=0.03$) in the combination group (Long et al. 2014). Furthermore, an open-label phase III trial comparing first line dabrafenib plus trametinib to vemurafenib alone in patients with Braf$^{V600E/K}$ mutant metastatic melanoma showed a significant improvement in OS with combination therapy versus vemurafenib (HR = 0.69; 95 % CI 0.53–0.89; $p=0.005$); median PFS was 11.4 versus 7.3 months, respectively (HR = 0.56; 95 % CI 0.46–0.69; $p<0.001$) (Robert et al. 2015). A phase III study demonstrated the combination of vemurafenib and the MEK inhibitor cobimetinib improved PFS (9.9 versus 6.2 months; HR = 0.51; 95 % CI 0.39–0.68; $p<0.001$) and OR (68 % versus 45 %; $p<0.001$) when compared to vemurafenib alone (Larkin et al 2014). Taken together, these results suggest that combination therapy can attenuate resistance to Braf inhibition and has replaced single agent targeted therapy.

Genetic studies into the molecular mechanisms underlying resistance to Braf-targeted therapy can provide future avenues to prolong responses to therapy. Potential mechanisms of resistance include intrinsic and acquired modes of resistance. There are a number of proposed intrinsic mechanisms of resistance related to

cell cycle regulation. The amplification of cyclin D1, observed in 15–20 % of Braf mutant melanomas, has been associated with high rates of resistance to Braf inhibitors by allowing for cell cycle entry when MAPK signaling is abrogated (Smalley et al. 2008). Additionally, one study methodically screened kinase-coding genes for any that conferred resistance to a Braf inhibitor and identified Cot overexpression as a mediator of Braf inhibitor resistance. COT activation, which functions upstream of Mek, results in Raf-independent MEK and Erk activation (Johannessen et al. 2010). Loss or inactivation of Pten has also been associated with resistance to Braf inhibitors (Paraiso et al. 2011). However, Pten expression has been correlated with shorter PFS in patients treated with dabrafenib (Nathanson et al. 2013). Moreover, the deletion of the retinoblastoma protein (Rb) attenuates Raf dependence in $Braf^{V600E}$-mutated melanoma cells (Xing et al. 2012).

Acquired Braf inhibitor resistance in melanoma cells is mediated through a number of different proposed mechanisms. The reactivation of the MAPK pathway by bypassing mutant Braf is the most frequently described mechanism of acquired resistance to mutant Braf inhibition. Nras activating mutations promote enhanced Raf dimerization. Upon binding of the Braf inhibitor to one member of the dimer, allosteric transactivation of the drug-free Raf protein results in the downstream signaling (Nazarian et al. 2010). Furthermore, constitutive signaling by receptor tyrosine kinases results in signal transduction either through Craf or through the PI3K pathway (Nazarian et al. 2010; Sun et al. 2014). A number of different resistance mechanisms within the PI3K pathway that results in MAPK reactivation have been described including mutations in *AKT* and regulatory members of the PI3K pathway (Rizos et al. 2014). Alternative splicing of mutant Braf and copy number amplification of mutant Braf have also been described (Poulikakos et al. 2011; Shi et al. 2012). These results suggest that understanding the genetic alterations in melanoma cells pre- and post-exposure to targeted therapies can shed light onto the development of more effective therapeutic strategies.

4.1.2 PI3K Pathway

As in the MAPK pathway, Nras is also an essential part of the PI3K pathway. Here, Ras induces membrane translocation and activates PI3K, which in turn leads to phosphorylation and activation of one of the major targets of the PI3K pathway, AKT. Mutations in members of the PI3K pathway have been extensively studied in several cancers. Although *PIK3CA* (p110α subunit of PI3K) is a common oncogene in breast (~30 %) and colon (~15 %) cancers (Ogino et al. 2009; Saal et al. 2005), it is rarely altered in melanoma (Omholt et al. 2006). It appears that melanomas preferentially activate the PI3K pathway through inactivation of *PTEN*—another key component of the PI3K pathway. *PTEN*, located on chromosome 10, is deleted in 30–50 % of melanomas and is a major tumor suppressor gene commonly abrogated in human tumors. Somatic mutations of *PTEN* are rare in primary melanomas (Reifenberger et al. 2004; Tsao et al. 2004), whereas the frequency increases in metastases and melanoma cell line cultures (Guldberg et al. 1997; Pollock et al. 2002; Tsao et al. 2004); this may indicate a selective advantage for cells with a

PTEN deficiency. There are three isoforms of Akt—Akt1, 2, and 3; however, there are several lines of evidence suggesting that Akt3 is the main player in melanoma. It has been shown that Akt3 is the predominant active form in melanoma cells and the *AKT3* gene is located on chromosome 1q a region commonly gained in primary melanoma. At least one metastatic tumor has been described to harbor the focal amplification of the *AKT3* locus (Jonsson et al. 2007). Moreover, a rare point mutation (E17K) in the pleckstrin homology domain, which results in constitutive activation, was identified in melanoma cells (Davies et al. 2008).

Regulators of the PI3K pathway also impact melanoma pathogenesis. Prex2 is a PTEN-regulating protein that was demonstrated to accelerate tumor formation of in vivo immortalized human melanocytes (Berger et al. 2012). *PREX2* alterations were initially identified on sequencing of genomes of 25 metastatic melanomas and matched germline DNA. It was then confirmed in 14 % of an independent extension cohort of 107 human melanomas. Other attractive targets in the PI3K pathway have been identified including *MTOR*, *IRS4*, *PIK3R4*, *PIK3R5*, and *NFKB1* (Shull et al. 2012).

Therapeutic targeting of the PI3K pathway in melanoma has shown early promise in pre-clinical studies. The dual inhibition of Braf and the PI3K pathway demonstrated induction of melanoma cell apoptosis via a mechanism independent of Mek signaling (Sánchez-Hernández et al. 2012). Furthermore, combinations of Braf, Mek, and PI3K inhibitors overcame acquired resistance to the Braf inhibitor dabrafenib (Greger et al. 2012). However, phase II trials examining the combinations of everolimus with temozolomide and temsirolimus or tipifarnib with sorafenib failed to demonstrate promising clinical activity for metastatic melanoma (Margolin et al. 2012; Dronca et al. 2014). The reason for this appears to be interruption of negative feedback loops mediated by mTorc1, causing activation of PI3K, Akt, and Erk (Carracedo et al. 2008).

As mentioned above, the activation of the PI3K pathway is one proposed mechanism of resistance to Braf and MEK inhibitors. Furthermore, vemurafenib and selumetinib, a selective MEK inhibitor, co-resistant Braf mutant melanoma cell lines have been shown to be dependent on AKT induction for survival (Atefi et al. 2011). Therefore, efforts to target the PI3K pathway for inhibition as a means of combating Braf and MEK inhibitor resistance are underway (Greger et al. 2012). AKT inhibition overcame acquired resistance to both Braf and MEK inhibition with the exception of vemurafenib-resistant melanoma cells that acquired a *NRAS* mutation (Atefi et al. 2011). $BRAF^{V600E}$ mutant melanoma cells harboring *MEK* or *NRAS* mutations demonstrated resistance to dabrafenib and trametinib monotherapy. However, the addition of a PI3K inhibitor to dabrafenib resulted in improved growth inhibition (Greger et al. 2012). These studies suggest that combination therapy that includes concomitant inhibition of the PI3K pathway may prolong clinical responses in Braf mutant melanoma.

4.1.3 The CDKN2A Network

The chromosome 9p21 locus is commonly targeted by loss-of-heterozygosity (LOH) or deletions in melanoma. The importance of this locus is underscored by the identification of somatic as well as germline mutations in *CDKN2A*

(Hayward 2003; Jonsson et al. 2007). Patients with *CDKN2A* mutations carry an increased risk of melanoma of 70 % over their lifetimes (Hayward 2003). *CDKN2A* is unique in the way that it encodes for two different proteins, p16^{INK4A} and p14^{ARF}, each transcribed in a separate reading frame. The two genes share one exon but have two distinct first exons (exon 1α and exon 1β). The *CDKN2A* locus was discovered through linkage analysis of melanoma kindreds and molecular assays showing limited homozygous deletions in some melanoma cell lines (Kamb et al. 1994; Weaver-Feldhaus et al. 1994). It was subsequently shown that germline mutations in *CDKN2A* could explain a substantial fraction of melanoma kindreds (Hayward 2003; Lin et al. 2008). Mutations in *CDKN2A* are also detected in sporadic multiple primary melanoma patients with a frequency around 9 % (Puig et al. 2005). In cultured melanoma cells, *CDKN2A* is frequently inactivated by homozygous deletions whereas small deletions or mutations are more common as germline changes. For both germline and somatic mutations, many of the reported changes reside in exon 2, which impinges on both p16^{INK4A} and p14^{ARF} while mutations only affecting the p14^{ARF} transcript are rare. Within the retinoblastoma (RB) pathway, both the Rb protein and the p16^{INK4A} act as tumor suppressors by regulating the cell cycle. Whereas Rb has been shown to have a limited role in melanoma development, p16^{INK4A} appears to be the major tumor suppressor in this pathway for melanoma. The key function of p16^{INK4A} is as an inhibitor at the G1-to-S cell cycle restriction point where it binds to Cdk4 and thus unable Cdk4 to bind to cyclinD1, which subsequently would lead to phosphorylation and inactivation of Rb. This ultimately releases the E2f transcription factors thereby transcriptionally upregulating S-phase-related genes. The p16^{INK4A} also plays an important role in cellular senescence, a mechanism that restricts the emergence of immortalized cells; however, the explanation for this is elegantly reviewed elsewhere (Bennett 2003). By contrast, p14^{ARF} suppresses oncogenic transformation by binding and inhibiting Hdm2 whose function is to abrogate p53 function by targeting it for degradation via the ubiquitin pathway (Momand et al. 1992). The amplification of both *CDK4* and *MDM2* has been observed in a subset of melanomas and has also been associated with preserved expression of p16^{INK4A} and p14^{ARF} (Muthusamy et al. 2006). Additionally, melanomas with wild-type *BRAF* and *NRAS* frequently display increased gene copy number of *CDK4* and *CCND1*, which are direct downstream targets of the MAPK pathway (Curtin et al. 2005). These melanomas frequently represent chronically sun-damaged, acral- or mucosal-type lesions. The relevance of the *TP53* gene is uncertain since 22 % of melanoma cell lines are mutated at this locus while only 12 % of primary cutaneous melanomas are mutated (Hocker and Tsao 2007). In one study, a mutation frequency of 30 % of mucosal melanomas was found (Ragnarsson-Olding et al. 2002), suggesting subtype-specific mutation patterns. The low prevalence of p53 mutations in melanoma as compared to other cancers might be explained by the high frequency of *CDKN2A* inactivation. Evidence on the functional association between p14^{ARF} and p53 became clear when the interaction between Hdm2 and p14^{ARF} was elucidated (Zhang et al. 1998).

4.2 Somatic Changes in the Pigmentation Pathway of Melanoma

It was early demonstrated that the biology of pigmentation is an essential part of melanoma development. Several epidemiological studies demonstrate that dark skinned individuals have a decreased risk of developing melanoma and red-haired and light skinned people are at an increased risk of melanoma (Cho et al. 2005; Swerdlow et al. 1986). One of the key regulators of pigmentation is *MC1R* located on chromosome 16q and acts by inducing production of dark eumelanin over the red pheomelanin (Rouzaud et al. 2005). The dark eumelanin is thought to be more protective against UV radiation than the red pheomelanin. Genetic variants of the *MC1R* gene are, not unexpectedly, associated with an increased risk of melanoma as reviewed elsewhere (Nelson and Tsao 2009). Also, genetic variants in other genes or loci (*TYR*, *ASIP*, *OCA2*, *TYRP1*, *SLC24A4*, *TPCN2*, and 9p21) connected to the pigmentation pathway have been shown to confer melanoma risk through a recent set of with genome-wide association (GWAS) studies (Bishop et al. 2009; Brown et al. 2008; Gudbjartsson et al. 2008). Although these GWAS results clearly link *MC1R* to risk, no *MC1R* somatic mutations were identified in a cohort of 103 primary melanomas suggesting that *MC1R* is not a frequent target of somatic alteration (Kim et al. 2008). Furthermore, there are studies that demonstrate that the presence of *MC1R* variants increase the melanoma penetrance in *CDKN2A* carriers (Fargnoli et al. 2010). The r variant p.R163Q is associated with increased risk of melanoma in high sun exposed geographies (Córdoba-Lanús et al. 2014). In 2005, Garraway et al. used the NCI 60 panel of cell lines and an integrative genomics approach to map novel oncogenes. High-resolution genomic maps indicated lineage restricted copy number changes and integrated with gene expression profiling data-supervised methods identified *MITF* as amplified and highly expressed in a subset of melanomas (Garraway et al. 2005). It was at that time known that Mitf was a master regulator of the melanocyte lineage. When investigating *MITF* gene copy number status in a set of primary melanomas, an inverse relationship was observed between gene copy number and survival. Intriguingly, all *MITF*-amplified cell lines harbored *BRAF* mutation as well as inactivated p16^{INK4A}, suggesting a cooperative effect of these genetic defects. Consequently, the overexpression of *MITF* in combination with oncogenic *BRAF* in immortalized melanocytes transformed these thereby confirming the oncogenic potential of *MITF* in melanoma. Furthermore, two independent studies identified the rare functional variant in *MITFE318K* that increases melanoma risk and also predisposes to renal cell carcinoma. *MITFE318K* occurs at a conserved SUMOylation position, decreasing the number of SUMO-modified Mitf isoforms. This leads to increased Mitf transcriptional activity (Yokoyama et al. 2011; Bertolotto et al. 2011). Several major pathways such as normal pigment cell physiology, melanocyte survival, cell cycle regulation, and growth are known to be downstream activities of Mitf. An elegant study by McGill et al. showed that *BCL2* is a transcriptional target of Mitf confirming the growth regulatory function of Mitf since Bcl2 is an antiapoptotic factor (McGill et al. 2002). Moreover, additional targets of Mitf include Tbx2, Met, and Cdk2 representing mechanisms that provide

growth advantage (Carreira et al. 2000; Du et al. 2004; McGill et al. 2006). The regulation of Mitf has been investigated and regulatory pathways include Notch, Mc1r, Wnt, endothelin receptor, KIT, and MAPK pathways. These pathways are known to genetically interact with *MITF*, such as *PAX3* and *SOX10*, and are involved in neural crest development (Potterf et al. 2000; Verastegui et al. 2000) (see Chaps. 2 and 4). The critical function of Mitf in melanoma as well as the finding of *MITF*-amplified melanomas led to the discovery of somatic mutations of *MITF* in a subset of melanomas. In the study by Cronin et al., eight cases of 50 metastatic melanoma lines were either amplified or mutated for *MITF*. In line with these results, an additional three cases harbored mutation of the *SOX10* gene, an upstream regulator of *MITF*, in a mutually exclusive fashion with *MITF* alterations (Cronin et al. 2009). Correlation between mutations in the Mitf pathway and *BRAF*/*NRAS* mutation status supported the observation made by Garraway et al. that these alterations are cooperative genetic events (Garraway et al. 2005). Overall, this study observed that approximately 20 % of metastatic melanoma harbors genetic alterations of the Mitf pathway. Chromosome 7p gains/amplifications is commonly found in melanoma and with this in mind, Jane-Valbuena et al. set out to search for a potential novel oncogene in melanoma. Using high-density SNP arrays, *ETV1* was pinpointed as an attractive target (Jane-Valbuena et al. 2010). Functional analysis revealed that the oncogenic potential of *ETV1* was dependent on concomitant activation of the MAPK pathway and upregulated mRNA levels of *MITF*. Additional studies of other members of the Mitf pathway will most likely unravel additional gene alterations.

As an initiator of several melanocytic signaling pathways, *KIT* has a central role in the development of melanoma. It has been demonstrated that *KIT* mutations in melanoma are mutually exclusive with *BRAF* and *NRAS* mutations. c-Kit is a receptor tyrosine kinase and has been proven to be able to activate both the MAPK and PI3K pathways (Fig. 4.1). Particular high frequency of mutations was identified in melanomas arising on palms, soles, and subungual sites (acral melanomas) as well as on mucosal membranes (mucosal melanomas) (Curtin et al. 2006). Additionally, the gain or amplification of the *KIT* locus (chromosome 4q12) was observed preferentially in mucosal, acral, or chronically sun-damaged melanomas and a substantial number of mutated tumors also harbored amplification suggesting an additive effect of amplification and mutation. In melanomas that harbor c-kit mutations, mutations leading to substitutions at L576P and K642E have been shown to account for 55 % of c-Kit mutated melanomas (Beadling et al. 2008). These findings set the stage for clinical targeting of c-Kit in melanoma patients. Initial trials with imatinib, an ATP-competitive inhibitor of several tyrosine kinases including c-Kit, did not demonstrate statistically significant results, likely because these early trials did not select for patients with known c-Kit mutated tumors (Ugurel et al. 2005). More recent trials have selected for patients with known c-kit mutated metastatic melanoma. In one phase II, single-group, open-label trial exploring the effect of c-Kit inhibition with imatinib mesylate in patients with *KIT*-altered melanoma, clinically significant results were found. In this study, 23.4 % of the patients had *KIT* mutations and/or amplifications, with a median PFS of 12.0 weeks and an OS of 46.3 weeks. The K642E or L576P substitutions within c-Kit were present in all responses observed

(Carvajal et al. 2011). Further molecular studies are required to characterize specific genetic alterations in c-kit to allow for more effective therapeutic targeting.

Like *KIT*, β-catenin is also involved in melanocyte lineage development. Wnt ligand binding results in nuclear translocation of β-catenin, resulting in gene transduction. The overexpression of β-catenin in neural crest cells results in pigment cell formation, while conditional inactivation of β-catenin led to loss of melanoblasts (Dorsky et al. 1998; Hari et al. 2002). Many studies have shown that β-catenin is critical for the transformation of melanocytes. Increased level of nuclear β-catenin is a consequence of a cascade initially started by canonical Wnt signaling and has been found in 50 % of melanomas (Kielhorn et al. 2003). In mice, functional analysis has revealed that β-catenin cooperates with *NRAS* oncogenesis by repressing *p16INK4A* (Delmas et al. 2007). However, somatic mutations of β-catenin are detected in only a small fraction of melanomas suggesting that there are other unknown mechanisms responsible for the localization of β-catenin in the nucleus. Recent work has suggested that Wnt signaling in melanoma cells regulates their interaction with the tumor microenvironment. One study demonstrated that Wnt1 is anti-lymphangiogenic by suppressing melanoma derived VEGF-C expression (Niederleithner et al. 2012). Intriguingly, recent evidence has demonstrated that melanoma intrinsic β-catenin signaling results in the lack of T cell infiltration in melanoma animal models and patient-derived biopsies (Spranger et al. 2015). Further work will be required to define strategies to effectively target β-catenin signaling in melanoma cells as this has implications for not only targeted therapies in melanoma but also checkpoint blockade regimens.

4.3 Novel Pathways in Melanoma

To identify novel key regulators of melanoma oncogenesis and metastasis several approaches have been taken where an integrative approach combining gene expression profiling and SNP arrays identified *MITF* as lineage-specific oncogene in melanoma as discussed previously. An alternative approach employed by Kim et al. includes comparative oncogenomics using a Ras inducible nonmetastatic mouse model to identify clones that had accumulated genetic changes conferring metastatic capacity (Kim et al. 2006). With this approach, a focal amplification on mouse chromosome 13 containing eight genes was identified using high-resolution comparative genomic hybridization. When analyzing mRNA transcript levels, *NEDD9* was pinpointed as the target gene. To confirm the results found in the mouse model, a number of primary and metastatic melanomas were genome-wide screened for copy number changes. In all, 36 % of metastatic and 8 % of primary melanomas harbored the amplification of 6p25-p24 (syntenic to mouse chromosome 13) as well as increased gene and protein expression of *NEDD9*. Furthermore, the knockdown of *NEDD9* inhibited proliferation and invasion corroborating the metastatic capacity of Nedd9-activated melanoma cells.

There has also been a systematic genetic screen of the matrix metalloproteinase (MMP) superfamily of genes (Palavalli et al. 2009) in melanomas. Matrix

metalloproteinases (MMPs) are proteolytic enzymes that degrade the basement membrane and the extracellular matrix. This suggests an involvement of MMPs in cancer metastasis; however, clinical trials using MMP inhibitors are largely unsuccessful in clinical trials.

MMP8-deficient mice, however, exhibit an increased skin cancer risk suggesting a tumor suppressor role of *MMP8* in skin cancers including melanoma (Lopez-Otin et al. 2009). Notably, *MMP8* and *MMP27* were mutated in 6–8 %, respectively, of the melanoma cases investigated. Furthermore, mutations of *MMP8* were frequently accompanied by LOH, suggesting that *MMP8* acts as a tumor suppressor gene in melanoma. Functional analysis of *MMP8* mutant cells displayed decreased proliferation as compared to *MMP8* wild-type cells. By contrast, when investigating migratory capacity of *MMP8* mutant cells these displayed decreased migration ability. Taken together, MMPs seem to play some role in melanoma development.

Recent work has identified alterations in genes that play a role in telomere maintenance as promoting susceptibility to melanoma. Telomeres consist of tandem nucleotide repeats located at chromosomal ends and maintain genomic stability and chromosomal stability by protecting chromosome ends from degradation, end-to-end fusion, and atypical recombination. The enzyme telomerase replenishes telomeres as the telomere ends become shorter with each cell division. In one recent study, Horn et al. used multipoint linkage analyses and target-enriched high-throughput sequencing to identify a germline mutation in the promoter of telomerase reverse transcriptase (*TERT*) in a melanoma-prone family. Tert is part of a ribonucleoprotein complex that maintains telomere length. They also found recurrent ultraviolet signature somatic mutations within the *TERT* promoter in 74 % of human cell lines derived from metastatic melanomas that they examined (Horn et al. 2013). In another recent report, germline mutations in *ACD* and *TERF2IP*, two members of the shelterin complex that protects telomeres, were found in melanoma-prone families using next-generation sequencing. The study included 510 melanoma-prone families without mutations in known melanoma-susceptibility genes (Aoude et al. 2015). Furthermore, two independent studies identified germline variants in protection of telomere 1 (*POT1*) in *CDKN2A* wild-type melanoma-prone families. Pot1 plays a role in protecting telomere ends by preventing inappropriate processing of the exposed chromosome ends and regulating telomerase function (Robles-Espinoza et al. 2014; Shi et al. 2014). These findings suggest that telomere maintenance plays a role in melanoma susceptibility.

4.4 Genome-Wide Screening Approaches in Melanoma

Recent technical developments have allowed researchers to investigate gene copy number changes on a global basis. In melanoma, the availability of frozen tissue is a major drawback when it comes to genome-wide analyses; hence, many studies in melanoma have been performed with melanoma cell lines or short-term cultures. Several research groups have investigated the detailed landscape of gene copy number changes in melanoma lines (Gast et al. 2010; Jonsson et al. 2007; Stark and Hayward 2007). Interestingly, a fairly homogenous pattern of DNA copy number

changes was observed with several chromosomes altered in more than 50% of the cases. Frequent losses were observed on chromosomes 4, 6, 8, 9, 10, and 11, whereas frequent gains were found on chromosomes 1, 7, 8, 17, and 20. These regions are corroborated by high-density SNP array analysis with frequent LOH at these regions (Stark and Hayward 2007). Additionally, 11q13, 3p14, and 1p12 were recurrently amplified including candidate oncogenes such as *CCND1*, *MITF*, and *NOTCH2*. Homozygous deletions are frequently identified in melanoma lines with the *CDKN2A* locus being affected in 40–60% and *PTEN* in 10–15%. Moreover, less frequent deletions include genes such as *PTPRD*, *HDAC4*, and *PARD3* suggesting novel tumor suppressor genes in melanoma.

In 2005, a vanguard study described genome-wide assessment of DNA copy number changes in 126 primary melanomas (Curtin et al. 2005). Tumors were divided into four groups based on sun-induced damage and on whether the tumor was classified as acral or mucosal melanoma. Specifically, acral and mucosal melanomas were found to harbor an increased frequency of genomic alterations as well as higher frequency of gene amplifications. Furthermore, chromosome 10q deletions were more common in melanomas without chronic sun-damaged skin, whereas focal gains of the *CCND1* locus were more frequent in the group with chronic sun-damaged skin. This elegant analysis of genetic alterations displayed that subgroups can be identified in melanoma and a strong relationship between sun exposure and genome-wide DNA copy number patterns exist.

Primary and metastatic melanomas have been subjected to transcriptomic analysis. Using supervised and integrative methods a number of genes with a significant expression deregulation between primary and metastatic melanoma were identified. The proinvasive ability of these genes was subsequently validated with functional assays (Kabbarah et al. 2010).

New genome-wide sequencing approaches have been used to identify epigenetic targets in melanoma pathogenesis. More specifically, regulators of chromatin remodeling such as members of the SWI/SNF complex, a nucleosome remodeling complex, have recently been implicated. Inactivating mutations in SWI/SNF family member genes (*ARID1A*, *ARID1B*, *ARID2*, and *SMARCA4*) and in members of another chromatin remodeling complex, the poly comb complex, (*EZH2*, *BMI1*, and *JARID1B/KDM5B*) were altered in melanoma (Hodis et al. 2012; Kuźbicki et al. 2013; Chang et al. 2015; Tiffen et al. 2015). While the exact mechanism of how alterations in chromatin remodeling drive melanoma disease progression is unclear, cellular de-differentiation is one proposed mechanism (Sarris et al. 2014). Preliminary studies have demonstrated that EZH2, a member of the poly comb complex, is overexpressed in melanoma cells and suppresses tumor suppressor proteins (Tiffen et al. 2015). Other epigenetic alterations that have been suggested as mediating melanoma disease progression are noncoding RNAs. These molecules have been known to be aberrantly expressed in melanoma; however, the specific role in melanoma pathogenesis is yet unknown (Greenberg et al. 2014). As new technologies emerge to identify genetic alterations in melanoma, functional studies will be required to characterize the mechanism by which these alterations contribute to disease progression.

In summary, during the past few years there have been an explosion of genomic analyses in melanoma. The heterogeneous nature of melanoma is clearly reflected in genome-wide DNA copy number changes and the numerous genetic alterations found.

4.5 Progression of Melanoma: The Genomic Approach

The classical histopathological pathway of progression described by Clark is initiated in the benign nevus where proliferation of normal melanocytes begins. The next step includes the formation of dysplastic nevi and this may occur in pre-existing nevi or at a new location. The formation of radial growth phase melanomas occurs when melanocytes acquire the ability to proliferate intraepidermally. One of the most critical steps in the development of melanoma includes the progression from radially growing melanomas (i.e., radial growth phase, RGP) to vertically advancing melanomas (i.e., vertical growth phase, VGP) where cells have the ability to invade the dermis. This deep invasion allows melanoma cells to spread to distant organs, form metastases, and potentially create a lethal event. Dissemination can occur by either lymphatic or hematogenous routes. Since access to these vascular structures is a prerequisite for metastatic spread, it is not surprising that a simple measure of vertical invasion, i.e., Breslow thickness, has survived the test of time.

The genetic factors related to this histopathological progression are starting to emerge. The presence of somatic *BRAF* mutations in nevi (Pollock et al. 2003) suggests that this is an early event and that *BRAF* activation alone is not sufficient for melanocytes to transform into a malignant state. Moreover, there is no significant difference in the frequency *BRAF or NRAS* mutations within control nevi or melanoma-associated nevi (Tschandl et al. 2013). Subsequent genetic events are more uncertain, though $p16^{INK4A}$ loss is considered to be an important early step since germline mutations in *CDKN2A* have been found in a significant fraction of melanoma kindreds and homozygous deletions and deleterious mutations of *CDKN2A* occur in a large fraction of melanoma cell lines. Also, it is evident that mice with *Cdkn2a* loss cooperate with activating MAPK lesions to drive melanoma formation (Chin et al. 1997). In addition to *CDKN2A*, loss of *PTEN* should also be considered to be a critical event. This is supported by human studies where deleterious *PTEN* alterations have been described (Tsao et al. 2000, 2004) and also by mice studies where *Pten* loss has been shown to induce metastatic melanoma in combination with activating *Braf* alleles (Dankort et al. 2009). Moreover, *PTEN* depletion and subsequent PI3K pathway activation abrogates $Braf^{V600E}$-induced senescence in melanocytes (Vredeveld et al. 2012). Furthermore, simultaneous *CDKN2A* and *LKB1* inactivation in $Braf^{V600E}$ melanocytes results in activation of both mTorc1 and Akt, resulting in rapid melanoma formation in mice (Damsky et al. 2015).

Beyond discrete genetic events, markers that define the switch from RGP to VGP have also been examined. One of the hallmarks in the switch between RGP and VGP include the loss of E-cadherin expression and simultaneously increased expression of N-cadherin affecting cell adhesion mechanisms. In addition, these

expression differences stimulate β-catenin and thereby increase the survival of melanoma cells. In a more comprehensive screen, Haqq et al. investigated gene expression difference between RGP and VGP (Haqq et al. 2005). Interestingly, loss of expression for a set of genes was observed in the vertical growth phase of the melanoma; these include *CDH3* and *MMP10* among other genes. Furthermore, the same gene set effectively partitions metastatic melanomas into two groups, which might indicate that metastases could develop from either the radial growth or the vertical growth phase of melanoma. The authors also identified gene sets that differed between normal skin, nevi, primary melanoma, and two types of metastatic melanoma suggesting that molecular patterns of progression do exist. Likewise, Sabatino et al. characterized melanoma cell lines derived from metachronous metastases from a single melanoma patient (Sabatino et al. 2008). Genome-wide copy number analysis supported a cancer stem cell model where a suggestive progenitor cell harbors a core set of genomic aberrations and that metastatic progression is not a sequential event. In a similar study by Harbst et al. metastases obtained from five patients were investigated for genomic imbalances, gene expression profiles, and methylation profiles (Harbst et al. 2010). Here, evidence for two models of metastatic progression was provided. The first model was supported by genomic aberration patterns of a case with three metachronous metastases. Homozygous deletions at chromosomes 3p26 and 6q23 were found in two consecutive metastases originating from the same primary tumor in a mutually exclusive manner corroborating the findings of Sabatino et al. (2008). Investigation of the E-cadherin and N-cadherin mRNA levels indicates variability as to when the actual switch in expression occurs. For one case the switch had obviously occurred prior to the metastases investigated, whereas in two of the cases the switch became apparent at the development of the second metastasis (Fig. 4.2). Interestingly, methylation analysis provided additional evidence for *PTEN*'s role in melanoma progression. Silencing of the *PTEN* gene was evident in the last of three consecutive metastases originating from a single primary melanoma. An alternative model of sequential progression was also found. In another case, the primary tumor as well as two consecutive metastases displayed almost indistinguishable genomic profiles. Furthermore, gene expression and methylation profiles support a common origin for metastases originating from the same primary tumor. In all, this indicates that at least two types of mechanisms can contribute to melanoma metastases.

To further detail the genetic landscape of melanoma progression, high-throughput DNA sequencing has begun to provide a framework of melanoma evolution. One recent study investigated the succession of genetic events contributing to the malignant transformation of melanocytes. In 37 melanocytic neoplasms specifically chosen for melanomas with histologically distinct precursors, benign lesions had *BRAFV600* mutations exclusively. Intermediate lesions harbored *NRAS* and additional driver mutations. Of the lesions examined, 77 % of intermediate lesions and melanomas in situ harbored *TERT* promoter mutations, suggesting that these mutations a selected for early in the malignant transformation. *CDKN2A* inactivation was found exclusively in invasive melanomas. *PTEN* and *TP53* mutations were found only in advanced primary melanomas. Moreover, the mutational burden characteristic of ultraviolet radiation

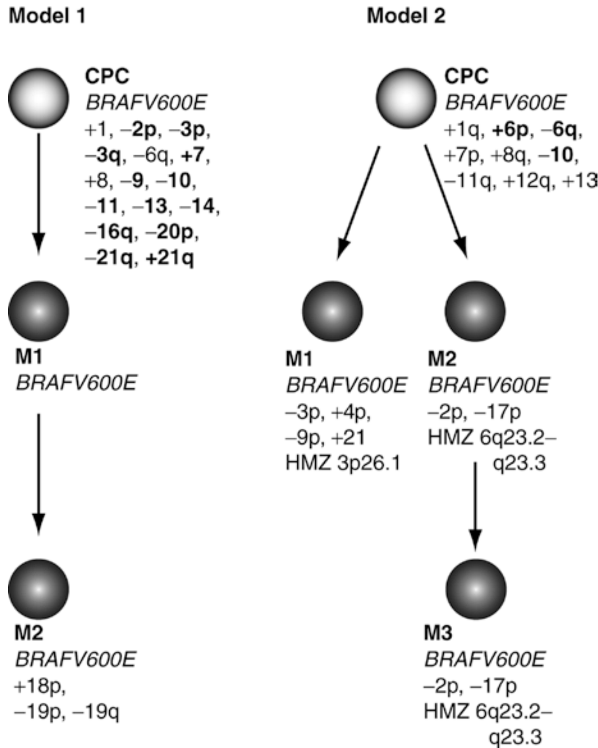

Fig. 4.2 Two models of tumor progression as suggested by Harbst et al. (2010). *Model 1*: The indistinguishable genomic profiles, as well as inheritance of the *BRAF* mutation, suggest sequential tumor progression. *Model 2*: Metastasis-specific gene copy number aberrations exclude the possibility of a sequential process and indicate to the evolvement of at least two distinct subclones with metastatic potential. However, both a core set of genomic aberrations and presence of the *BRAF* mutation in all lesions confirm common origin of the tumors. Major chromosomal changes are indicated. CPC indicates a common metastatic precursor cell/clone that harbors indicated chromosomal aberrations inherited by all the metastases from that tumor. In *bold*, copy number aberrations observed in the primary tumor. *HMZ* homozygous deletion

increased from benign through intermediate lesions to melanoma (Hunter Shain et al. 2015). In seeming contradiction to the traditional models of sequential genetic alterations and subsequent clonal selection leading the melanoma pathogenesis, recent studies have demonstrated that normal skin already contains a high burden of driver mutations. There are six genes (*Notch 1*, *2*, *3*, *P53*, *FAT1*, and *RBM10*) that were demonstrated to have an excess of nonsilent mutations, suggesting there is a selection pressure for these mutations, and as such, represent drivers. One-quarter of middle-aged skin contains a mutation in one of these genes (Martincorena et al. 2015). However, the sizes of clones containing these mutations are relatively limited and similar across individuals, indicating that the growth of clones slows early in their expansion. How this occurs is unknown at this time and warrants further investigation as it likely represents a checkpoint preventing malignant transformation.

These recent studies where a genomic approach was taken indicates the extensive complexity in progression of malignant melanoma and more in-depth genetic studies are eagerly awaited.

4.6 Molecular Classification of Melanoma

Several groups have used molecular parameters in an attempt to recover a subclassification of melanoma. Viros et al. used a combination of histomorphologic and genetic features to classify a comprehensive set of melanomas (Viros et al. 2008). Interestingly, *BRAF*-mutated melanomas displayed characteristic features such as nest formation of intraepidermal melanocytes and larger, rounder, and more pigmented tumor cells. By contrast, *NRAS*-mutated melanomas did not show any specific morphologic characteristics. A survival benefit was also identified in patients predicted to be *BRAF* mutated, most likely due to a different route of metastasis. As mentioned previously, Curtin et al. described a classification based on genetic alterations and primary tumor location and sun-damaged tumor surrounding skin (Curtin et al. 2005). A more recent study also used genome-wide DNA copy number changes as a way of subclassifying melanoma metastases. Here, three classes were found and showed different event-free survival (Kabbarah et al. 2010). An alternative way of molecularly classifying cancer is by using genome-wide assessment of mRNA transcript levels though the lack of frozen tissue from primary melanomas has led to only one study with sufficient clinical annotation (Kabbarah et al. 2010). In that study, 83 primary melanomas in the vertical growth phase with a Breslow thickness of at least 1 mm were subjected to global gene expression profiling. Applying supervised methods on melanomas from patients with or without 4-year distant metastasis-free survival a prognostic set of 254 genes was identified. The investigation of clinical parameters in the two prognostic groups revealed differences with regard to Breslow thickness, AJCC stage, melanoma type, ulceration, and mitotic rate—features all known to be strong prognostic factors in melanoma. Indeed, a comparison of the prognostic gene set and a gene set derived based on Breslow thickness revealed a considerable overlap. Genes related to DNA repair and cell cycle increased with thickness as well as with poor prognosis. A more careful analysis of the prognostic gene set showed that DNA replication and DNA repair are the most significant pathways (Kauffmann et al. 2008). These results have also been corroborated by a recent study applying an alternative microarray platform (Jewell et al. 2010).

Technical developments have enabled researchers to start exploring global gene expression profiles from formalin-fixed paraffin-embedded (FFPE) tissue (April et al. 2009). In melanoma, the first studies used a microarray platform consisting of a restricted set of cancer-related genes ($n = 502$) (Conway et al. 2009; Jewell et al. 2010; Mitra et al. 2010). However, these have been directed toward identifying single molecular biomarkers and not in classifying melanomas. Through such approaches, the expression level of osteopontin was identified as the gene with strongest association with reduced relapse-free survival (Conway et al. 2009).

Subsequent studies with sentinel lymph node status confirmed the prognostic significance of osteopontin and DNA repair genes (Jewell et al. 2010; Mitra et al. 2010). Studies using whole-genome approaches of gene expression analysis of archival FFPE RNA open up a wide range of opportunities for investigating the genomic landscape of primary malignant melanoma.

Global gene expression patterns have to a larger extent been applied to advanced stage or metastatic melanomas where availability of frozen tissue is not the main limitation. As alluded to earlier, one study used a gene set derived from a discriminatory analysis between radial growth and vertical growth phase of a single primary melanoma (Haqq et al. 2005). In this study, the investigators also detected two subtypes of metastatic melanoma. Type I metastases displayed increased expression of genes presumably downregulated in the vertical growth phase of primary melanoma, whereas type II metastases were characterized by increased expression of genes related to pigmentation such as *MITF*, *MLANA*, and *TYR*. More recently, Jönsson et al. set out to discern a biological subclassification with clinical impact in stage IV melanoma (Jonsson et al. 2010). Gene expression profiles were obtained for 57 lymph node or subcutaneous metastases. Unsupervised hierarchical clustering algorithms identified four molecular classes. These were subsequently characterized and named according to the gene expression pattern of the class. The first group, high-immune response, expressed high levels of immune response-related genes such as *LCK*, *CXCL12*, and *HLA* class I and II antigen. The second class, proliferative, expressed decreased levels of immune response-related genes as well as a trend toward higher Ki67 staining. High levels of genes such as *MITF*, *TYR*, *DCT*, and *MLANA* characterized the third class (pigmentation class). This is in line with the findings of Haqq et al. who identified a subclass in metastatic melanoma that had high expression of genes that characterize the radial growth phase of a single primary melanoma as well as high expression of pigmentation-related genes (Haqq et al. 2005). Finally, the normal-like subclass was characterized by melanomas expressing genes such as *TRIM29*, *KRT17*, and *KRT10*. Most importantly, this biological subclassification scheme displayed a significant association to clinical outcome with the proliferative subtype as a poor prognostic group, which was validated in an independent cohort of stage III melanomas (Fig. 4.3). In a study by Bugonvic et al., a prognostic gene expression signature supports the finding of improved survival in patients with tumors having increased expression of immune response-related genes (Bogunovic et al. 2009). Additionally, all patients included in the Jönsson et al. study were enrolled in a prospective trial of DTIC treatment. This allowed the investigators to correlate treatment response with molecular subclass. Indeed, a significant association between the pigmentation subclass and a stable disease at 3 months of treatment was identified suggesting that pigmentation might be a predictive target of DTIC response. Further molecular characterization of subclasses implied different frequencies of genomic imbalances and targeted gene deletions, e.g., *CDKN2A* homozygous deletion was significantly associated with the proliferative class. In all, this study indicates that a molecular classification is tenable and could be clinically meaningful. These results have

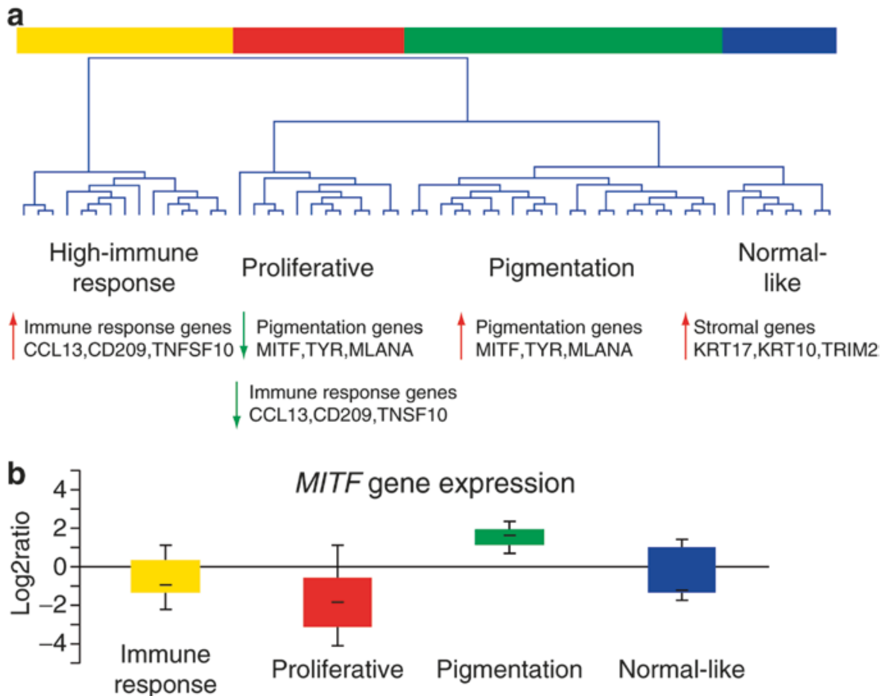

Fig. 4.3 Molecular classification of malignant melanoma. (**a**) Subclassification of stage IV melanomas based on gene expression patterns as suggested by Jönsson et al. (2010). Four classes were identified each broadly characterized by expression of set of genes involved in immune response and pigmentation. (**b**) Box plot of *MITF* gene expression levels in each class displaying an increased expression in the Pigmentation class

subsequently been validated in primary and metastatic melanomas (Harbst et al. 2012 and Cirenajwis et al. 2015). Finally, it has been demonstrated that tumors from CDKN2A germline mutation carriers are indistinguishable from sporadic melanomas and found in all four molecular subclasses (Staaf et al. 2014).

In one recent hallmark study, an integrative analysis of cutaneous melanomas establishes a genetic framework for molecular classification into four subtypes: mutant *BRAF*, mutant *RAS*, mutant *NF1*, and triple wild-type. Enrichment of *KIT* mutations, focal amplifications, and structural rearrangements were features of the triple-wild-type subgroup. There was no significant difference in clinical outcome between subtypes. However, a subset of each molecular subtype with a significant immune signature was associated with improved survival, which has significant implications for immunotherapy (Cancer Genome Atlas Network 2015).

In summary, the classification of melanoma based on molecular patterns will most likely be an important parameter in future medical oncology since the advent of targeted and immunotherapy showing promising results in melanoma will require molecular stratification prior to treatment.

4.7 Next-Generation Sequencing: Approaches in Melanoma

The availability of novel high-throughput sequencing methods such as human genome sequencing has revolutionized cancer research during the past few years. In a pioneering study from the Wellcome Trust Sanger Institute, a single melanoma cancer genome was sequenced with a tremendous gain in biologic insight (Pleasance et al. 2010). One of the main findings of the study was the observation that the melanoma genome was littered with C>T mutations 3′ to a pyrimidine site, a hallmark of mutations attributed by UV-induced DNA damage. This single finding puts to rest the controversy that melanomas are in fact not subject to UV mutagenesis (Hocker and Tsao 2007) (see also Chap. 3). It is also possible to sequence RNA transcripts using next-generation sequencing. An elegant study by Berger et al. used this approach to analyze short-term melanoma cultures (Berger et al. 2010). In the analysis, 11 novel melanoma gene fusions produced by underlying genomic rearrangements were found. However, none of these changes were recurrent suggesting that the gene fusions could be "passenger" events. Interestingly, in a screen for novel prostate cancer fusion genes, *BRAF* was identified and because of the high mutation rate of somatic mutations of *BRAF* in melanoma the screen was extended to include melanomas; however, only one case out of 131 harbored a rearranged *BRAF* gene corroborating the low prevalence of recurrent gene fusions in melanoma (Palanisamy et al. 2010).

A very important study demonstrating the significance and enormous potential of next-generation sequencing was published in 2009 (Emery et al. 2009). Here, a somatic *MEK* mutation was observed in a metastatic lesion showing resistance to treatment of an MEK inhibitor. In vitro analysis displaying further evidence of *MEK*-mutated clones demonstrated resistance to both a MEK inhibitor as well as to a Raf inhibitor. However, when combining Mek and Raf inhibitors resistance to targeted therapies of the MAPK pathway is circumvented.

As has been described above, next-generation sequencing has been leveraged to identify mechanisms of resistance to targeted therapy in metastatic melanoma. As an example, Van Allen et al. performed whole-exome sequencing on formalin-fixed, paraffin-embedded tumors and demonstrated that common pathways of selective Braf inhibitor resistance in melanoma cells include *BRAF* mutant amplification, STAT3 upregulation, or mutations in other members of the MAPK pathway (Van Allen et al. 2014). Mutations in downstream MAPK members results in constitutive activation of Mek, independent of the upstream Braf kinase and insensitive to Mek inhibitors.

With the recent success of immunotherapy in the treatment of metastatic melanoma (see Chap. 17), novel high-throughput technologies have been used to predict those patients that will likely benefit from immunotherapy targeting CTLA-4 or PD1/PD-L1. Whole exome sequencing was performed on tumor and blood samples from melanoma patients treated with the CTLA-4 inhibitors ipilimumab or tremelimumab. This revealed a neoantigen peptide landscape specifically present in tumors with a strong response to CTLA-4 blockade. More specifically, there was an association between high mutational load and clinical benefit with CTLA-4

immune checkpoint blockade (Snyder et al. 2014). In another recent study, whole exomes from CTLA-4 blockade pretreatment melanoma tumor biopsies and matched germline tissue samples were examined. Clinical benefit was associated with overall mutational load, neoantigen load, and expression of cytolytic markers in the immune microenvironment (Van Allen et al. 2015). Similar findings have been demonstrated with respect to PD-1 immune checkpoint inhibitors. Tumors that have a high somatic mutational frequency, above 10 somatic mutations per megabase of coding DNA, such as melanomas, were shown to respond best to PD-1 inhibitors (Lawrence et al. 2013).

4.8 Summary

In summary, the genetic and genomic landscape of melanoma has radically changed over the past decade. Advances in our understanding of cancer genetics and the advent of powerful technologies will undoubtedly transform our vision of the molecular underpinnings of melanoma within the next few years. In this chapter, we have been able to survey only some of the principles and technologies that underlie the ongoing genomic revolution. It is clear that only the tip of the iceberg has come into view.

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Transcriptional Regulation in Melanoma

5

Satoru Yokoyama and David E. Fisher

5.1 MITF (Microphthalmia-Associated Transcription Factor)

MITF is a basic helix–loop–helix leucine zipper protein, which binds to E-box sequences (5′-CA(C/T)GTG-3′) as a homo- or heterodimer with other MiT family members (itself, TFE3, TFEB, or TFEC) (Hemesath et al. 1994; Yasumoto et al. 1994). MITF has many isoforms, which are transcribed from different initial exons and exhibit tissue-specific expression patterns depending upon promoter usage (Hershey and Fisher 2005). Among isoforms, MITF-M, with its exon1M, is highly expressed in melanocyte-lineage cells (Fuse et al. 1996).

MITF has been shown to be required for melanocyte development, differentiation, and survival (see also Chap. 2). Mutations of MITF in humans cause Waardenburg syndrome (WS) 2A, which is an autosomal dominant auditory–pigmentary syndrome characterized by pigmentary abnormalities of the hair, skin, and eyes and congenital sensorineural hearing loss (Tassabehji et al. 1994). Appropriate regulation of MITF is required for cell growth/survival in melanocytes and MITF amplification has been reported in 10–20% of human melanomas (Garraway et al. 2005). In addition, human clear cell sarcoma is a frequently pigmented soft tissue sarcoma, which harbors a chromosomal translocation producing the EWS-ATF1 fusion protein (Davis et al. 2006). This chimeric oncoprotein is thought to mimic the normally cAMP-regulated ability of ATF1 to activate M-MITF expression, instead of constitutively activating the *M-MITF* promoter. The result is dysregulated M-MITF expression in clear cell sarcoma which likely accounts for the tumor's

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_5

melanin production as well as oncogenic behavior (Davis et al. 2006). In addition, ~20 % of metastatic melanomas were found to harbor somatic coding mutations, which may represent an additional oncogenic mechanism (Cronin et al. 2009) and recently a novel MITF mutation (E318K), which affects sumoylation of MITF, has been identified in familial and sporadic melanoma (Yokoyama et al. 2011).

MITF transactivates multiple genes related to cell cycle (CDK2, p16, p21, and Tbx2), pigmentation/differentiation (TYR, TYRP1, DCT, MART1, AIM-1, TRPM1, and PMEL17), apoptosis (HIF1A, BCL2, BCL2A1), and motility (c-MET, mir-211) (Fig. 5.1).

MITF transcription is regulated by other transcription factors, including PAX3, SOX10, CREB, FOXD3, LEF-1, and ONECUT-2. CREB/ATF1-mediated regulation of MITF is important for physiological expression in melanocytes, under the control of cAMP or other signals. MITF is also posttranslationally regulated by phosphorylation (via MAPK, GSK-3b, and RSK) (Mansky et al. 2002; Takeda et al. 2000; Weilbaecher et al. 2001; Wu et al. 2000), sumoylation (Miller et al. 2005; Murakami and Arnheiter 2005), caspase-dependent cleavage (Larribere et al. 2005), and ubiquitination. The MAPK-phosphorylated MITF is ubiquitinated and degraded via the proteosome pathway. MITF activity can be suppressed by the overexpression of PIAS3, which directly binds to MITF and inhibits DNA binding (Levy et al. 2002).

Recent work has indicated that nonspecific histone deacetylase inhibitors (HDACi) could potently repress MITF expression in melanoma cells. Suppressed MITF expression and cell growth were produced in melanoma cell lines and in mouse xenografts in response to treatment with a variety of HDACi (Yokoyama et al. 2008).

5.2 cAMP-Responsive Element (CRE)-Binding Protein/ Activating Transcription Factor-1 (CREB/ATF-1)

CREB/ATF-1 is one of the most important transcription factors in G-protein-coupled receptor (GPCR) signaling. In normal melanocytes, CREB/ATF-1 is required for pigmentation signaling from melanocortin receptor type 1 (MC1R) to MITF, which plays a central role in pigmentation as mentioned above (Price et al. 1998). α-Melanocyte-stimulating hormone (α-MSH) is produced after UV exposure in human skin (Lin and Fisher 2007; Yamaguchi and Hearing 2009) then bound to MC1R, which is a GPCR, in melanocytes. MC1R activates protein kinase A via cAMP production. Activated PKA phosphorylates CREB at Ser133, which induces transcription activity via a highly conserved CREB-binding site (cAMP-responsive element; CRE) (5′-TGANNTCA-3′) in the *M-MITF* promoter.

EWS-ATF1 fusion protein plays an oncogenic role in human clear cell sarcoma via MITF (Davis et al. 2006). It is reported that the transition of melanoma cells from radial to vertical growth phase is associated with the overexpression of CREB/ATF-1. Dominant-negative CREB (KCREB) can decrease melanoma tumorigenicity (possibly via MITF) and metastatic potential (via MMP-2 and MCAM/MUC18) in nude mice (Xie et al. 1997). Taken together, CREB/ATF1 contributes importantly to melanoma behavior.

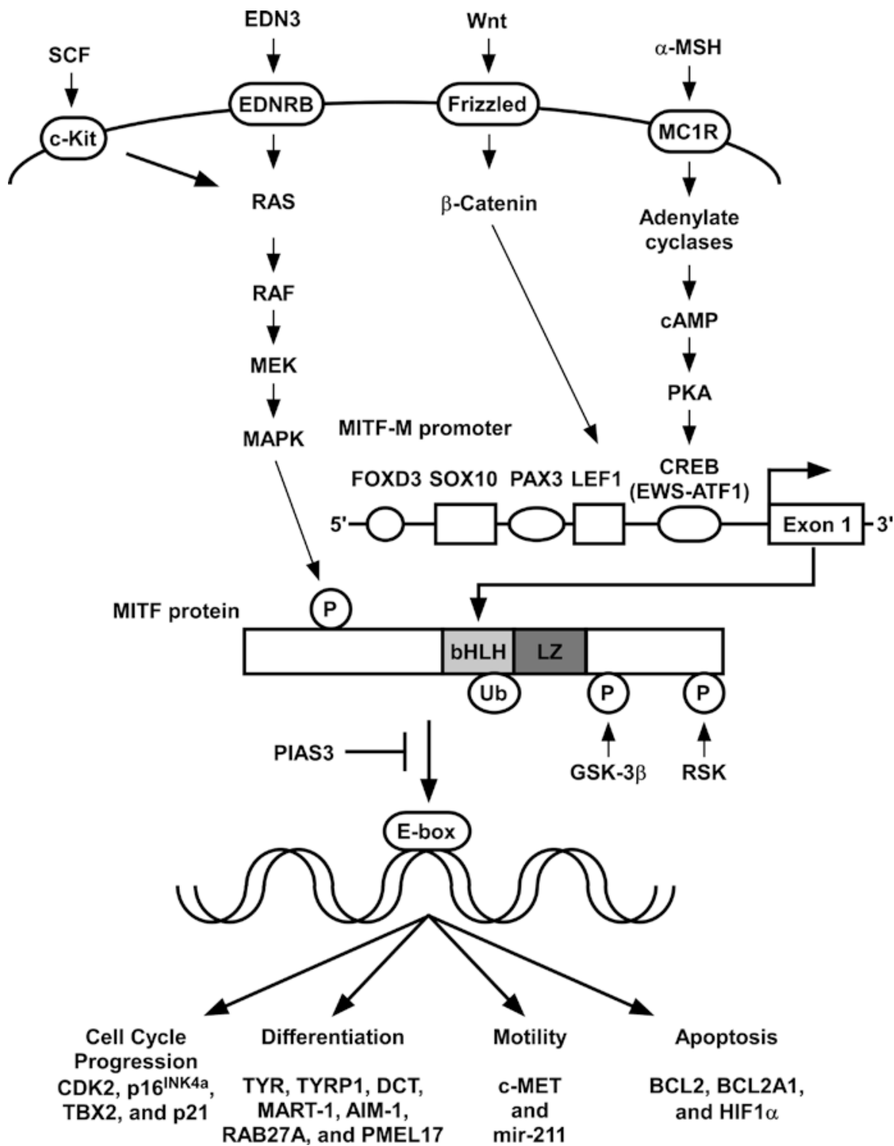

Fig. 5.1 MITF is involved in the induction of melanoma, melanocyte differentiation, cell-cycle progression, and survival. *Black lines* represent some of the signaling pathways connected to MITF, such as steel factor (*SCF*) and endothelin-3 (*EDN3*), and α-melanocyte-stimulating hormone (*α-MSH*). *White circles and squares* represent transcription factor-binding sites on the MITF-M promoter. MITF protein is posttranslationally regulated by phosphorylation via MAPK, GSK-3β, and RSK. PIAS3 suppresses the DNA-binding of MITF. MITF protein binds to consensus sequences, a subset of E-boxes, present in promoter or enhancer elements containing the consensus CATGTG or CACGTG. MITF regulates multiple targets in melanocytes and melanoma cells involved in various cellular processes such as cell-cycle control, survival, motility, invasion, differentiation, and/or pigmentation

5.3 SOX (Sry-Box) Family (SOX9 and SOX10)

The SOX family is comprised of approximately 20 transcription factors named after the original member, Sry (sex-determining region Y), because they all share a similar high-mobility group (HMG) domain (Soullier et al. 1999), which recognizes the consensus sequence (5′-(A/T)(A/T)CAA(A/T)G-3′). SOX proteins bind DNA as a monomer or a dimer and then regulate the transcription of target genes (Jiao et al. 2004; Ludwig et al. 2004; Peirano and Wegner 2000).

Among SOX family members, SOX10 is one of the major players in melanocyte development, and some recent data have suggested a significant role for SOX9 as well. In humans, SOX10 mutation causes WS type 4, which is characterized by sensorineural deafness/hypopigmentation associated with WS and enteric aganglionosis associated with Hirschprung's disease (Herbarth et al. 1998; Southard-Smith et al. 1998). SOX8 and SOX9 play roles in neural crest development, which is the origin of melanocytes (Cheung and Briscoe 2003; Maka et al. 2005).

UV exposure induces SOX9 via a cAMP/PKC-dependent pathway (Passeron et al. 2007) and SOX9 increases MITF and DCT expression in an SOX10-independent manner. In the case of SOX10, there is little information about its own transcriptional regulation. The tyrosine kinase TYRO3 can control SOX10 nuclear localization, resulting in subsequent SOX10-dependent increases in MITF, together with melanoma cell proliferation and survival (Zhu et al. 2009).

SOX10 regulates MITF, EDNRB, and c-ret, which are also related to WS and Hirschprung's disease. The function of SOX10 in melanomagenesis is still unknown, though SOX10 somatic mutations were reported in ~7 % of metastatic melanomas (Cronin et al. 2009) and SOX10 knockdown induced vemurafenib-resistance through activation of TGF-β signaling (Sun et al. 2014). These suggest that SOX10 could have a tumor-suppressive function in melanoma.

5.4 PAX3 (Paired Box 3)

PAX proteins represent a highly conserved family of transcription factors required for the development of multiple tissue types. Pax3 is a particularly interesting member of the Pax family, which coordinates the development of certain neural crest-derived lineages including skeletal muscle and melanocytes (Tassabehji et al. 1993). In humans, specific PAX3 mutations may cause either WS type 1 or type 3 characterized by abnormalities of the central nervous system, face, eye, nose, cochlea, and hair pigmentation.

PAX3 has two DNA binding domains: a paired domain and a homeodomain (Goulding et al. 1991). The paired domain binds the consensus sequence (5′-GT(T/C)(C/A)(C/T)(G/C)(G/C)-3′), whereas the homeodomain recognizes DNA containing a core sequence (5′-TAAT-3′). Pax3 can serve as a transcriptional activator or repressor by binding different partner proteins, such as SOX10 and TAZ (as activators) and TLE4 (Grg4 in mice), KAP1, and HP1γ (as repressors) (Bondurand et al. 2000; Hsieh et al. 2006; Lang et al. 2005; Murakami et al. 2006).

As predicted from WS types1/3, PAX3 regulates MITF, c-Ret, WNT1, and TGF-β2, which are required for the development of neural crest derivatives. PAX3 also regulates pigmentation genes (DCT and TRP1), which are related to melanocyte differentiation.

PAX3 overexpression is observed in many primary melanomas, melanoma cell lines, and melanoma sections (Barr et al. 1999; Medic and Ziman 2010; Plummer et al. 2008; Scholl et al. 2001). Even though Pax3's precise function in melanoma is not known, anti-apoptotic genes (PTEN, BCL-XL), differentiation-related genes (FGFR4, C-MET, MYF-5, MyoD, MSX2, HES1, and NGN2), and metastasis-related genes (CXCR4 and MET) (Kubic et al. 2015a, b) has been identified as PAX3 target genes, suggesting PAX3 could play an important role in melanoma.

5.5 Snail Superfamily (Snail and Slug)

Snail family members encode transcription factors of the zinc-finger type (Boulay et al. 1987). They share a similar organization, being composed of a highly conserved carboxy-terminal region, which contains 4–6 zinc fingers, and a more divergent amino-terminal region. The consensus binding site for Snail-related genes contains a core of six bases, 5′-CAGGTG-3′ (Mauhin et al. 1993), which also corresponds to a subset of the E-box motif (5′-CANNTG-3′). On binding to their E-boxes, Snail family members act as transcriptional repressors (Batlle et al. 2000; Cano et al. 2000). In humans, the mutation of SLUG causes WS type 2D, which is characterized by an auditory–pigmentary syndrome characterized by pigmentary abnormalities of the hair, skin, and eyes and congenital sensorineural hearing loss (Sanchez-Martin et al. 2002). SLUG was also identified as an MITF target gene.

SNAIL/SLUG is related to the epithelial-to-mesenchymal transition (EMT), which is thought to be a key step in development and cancer metastasis (Polyak and Weinberg 2009). The activation of Snail expression plays an important role in downregulation of E-cadherin, contributing to tumorigenesis of malignant melanomas (Poser et al. 2001) (see also Chap. 9). In vitro and in vivo experiments suggest that Slug is required for the metastasis of the transformed melanoma cells (Gupta et al. 2005) and for the survival through PUMA suppression.

5.6 FOXD3 (Forkhead Box D3)

FOX transcription factors represent a closely related family of proteins that mediate cell-cycle progression, survival, and differentiation (Myatt and Lam 2007). The FOX transcription factors contain a conserved FOX or winged helix domain, which is required for binding to a consensus DNA sequence and for activating target gene transcription.

Foxd3 acts as transcriptional repressor via a consensus binding site (5′-A(A/T) T(A/G) TTTGTTT-3′) and is expressed in migratory neural crest (NC) cells that give rise to multiple lineages. The downregulation of FOXD3 results in an increase

in the number of differentiating melanocytes in quail NC cultures, and in premature dorsolateral migration of chick NC cells (Kos et al. 2001). Conversely, the misexpression of FOXD3 in melanoblasts results in a failure of NC cells to enter the dorsolateral pathway. The ectopic expression of FOXD3 represses MITF in cultured NC cells and in B16-F10 melanoma cells via inhibition of PAX3 binding to MITF promoter (Thomas and Erickson 2009).

In melanoma, it was reported that B-RAF^{V600E}, which is one of the major mutations, suppresses FOXD3 levels in human melanoma cells and FOXD3 expression represses melanoma growth by inhibiting the G1-S transition (Abel and Aplin 2010). Recently, some of FOXD3 targets were identified such as ERBB3 and TWIST1, both of which are related to melanoma malignant phenotypes (Abel et al. 2013; Weiss et al. 2014).

5.7 ETS Family Members

ETS transcription factors have a conserved DNA-binding domain (the ETS domain) of about 85 amino acids. The ETS domain, which bears a winged helix–turn–helix protein fold, mediates binding to a core DNA sequence (5′-GGA(A/T)-3′), with adjacent sequences influencing binding affinities (Sharrocks 2001). ETS transcription factors are important in many biological settings such as cell growth, differentiation, and survival and in processes that include hematopoiesis, angiogenesis, wound healing, cancer, and inflammation. At least 27 ETS family members have been described in mammalian cells and nearly two-thirds are ubiquitously expressed in adult tissues.

Multiple members of the ETS family undergo oncogenic dysregulation in cancer, often through chromosomal translocation. In Ewing's sarcoma (EWS), EWS-ETV1 translocations result in highly transforming chimeric ETS fusion proteins (Bailly et al. 1994; Ouchida et al. 1995). Chromosomal translocations involving ETV1 and other ETS genes were found in more than 40 % of prostate cancers (Tomlins et al. 2005). Most commonly, these translocations interpose the promoter and 5′ coding exons of the TMPRSS2 gene upstream of an ETS factor gene (ERG, ETV1, ETV4, or ETV5), resulting in androgen-dependent regulation and elevated the expression of these genes.

In melanoma, translocations involving Ets family members have not yet been described. However, the amplification of the ETV1 locus (>40 %) and dependency on ETV1 expression for melanoma proliferation were reported (Jane-Valbuena et al. 2010). The oncogenic activity of ETV1 is thought to be mediated by another oncogene, MITF.

5.8 BRN2 (POU3F2/N-Oct-3)

POU domain transcription factors are present in many cell lineages where they perform varying functions, either as ubiquitous regulators of "house-keeping" genes or as developmental- and lineage-specific coordinators of cell fate decisions. The POU

domain is a highly conserved DNA binding structure, which was first found to recognize the canonical octameric sequence (5′-ATGCAAAT-3′) (Pruijn et al. 1986, 1987). Later, two subdomains, POUs and POUh, were identified to bind to DNA sequences 5′-ATGC and 3′-AAAT, respectively (Sturm and Herr 1988). POU proteins are capable of homodimerization on DNA target binding sites and interaction with a variety of other proteins, such as SOX family members (Wegner 2005). A major interaction between the POU family and SOX family involves OCT4 (POU5F1) and SOX2 in embryonic stem cells (Yuan et al. 1995). BRN2, SOX10, SOX9, and PAX3 are expressed in melanocytes and physical interactions among SOX10, PAX3, and BRN2 have been reported (Smit et al. 2000). BRN2 acts as a repressor of MITF expression in melanoma cells via direct binding to a region adjacent to the TATA box, resulting in suppressing the differentiated melanocytic phenotype and enhancing tumor metastasis (Goodall et al. 2008). BRN2 also regulates melanoma invasion and metastasis through suppressing PDE5A expression (Arozarena et al. 2011).

5.9 AP-1 (Activator Protein 1)/ATF2 (Activating Transcription Factor 2)

The AP-1 transcription factor is a dimeric complex that comprises members of the JUN, FOS, ATF (activating transcription factor), and MAF (musculoaponeurotic fibrosarcoma) protein families (Vogt 2002). AP-1 proteins are known as basic leucine-zipper (bZIP) proteins because they dimerize through a leucine-zipper motif and contain a basic domain for interaction with DNA. The AP-1 complex can form various different combinations of heterodimers or homodimers, and these combinations determine the genes that are regulated by AP-1. AP-1 upregulates transcription of genes containing the TPA response element (TRE; 5′-TGA(G/C)TCA-3′).

ATF2 is well characterized and thought to play a key role in melanoma (Bhoumik et al. 2007). ATF2 activity is regulated by phosphorylation on threonine (Thr) residues 69 and 71 via stress-activated kinases JNK, RalGDS-Src-P38 pathway, ATM, and Ras-MEK-ERK (Bhoumik et al. 2005; Gupta et al. 1995; Ouwens et al. 2002). Stimuli that activate these kinases, including exposure to proinflammatory cytokines, UV irradiation, DNA damage, or changes in ROS, are among the inducers of ATF2 transcriptional activity. Following phosphorylation, ATF2 activates transcription through heterodimerization with other transcription factors, among which c-Jun has been best characterized. ATF2/c-Jun heterodimers preferentially bind to the consensus sequence (5′-T(G/T)ACNTCA-3′). In addition to phosphorylation, ATF2 is also regulated by ubiquitin-dependent degradation by the proteasome, which is dependent on the association with JNK (Fuchs et al. 2000). ATF2 also functions in the DNA damage response by associating with the TIP60 HAT complex (Bhoumik et al. 2008). The TIP60 complex is required for ATM self-phosphorylation via acetylation. ATF2 regulates ATM activation by control of TIP60 stability and activity.

Through its dimerization with specific partners, ATF2 regulates the expression of its transcriptional targets. These are stress/DNA damage response genes (c-Jun,

c-fos, ATM, XPA, RAD23B, etc.), growth/tumorigenesis genes (cyclin A. cyclin D1, MMP2, TNF-α, etc.), and physiological homeostasis genes (tyrosine hydroxyrase, collagen, VCAM-1, PGC-1α, etc.).

There are several lines of striking evidence regarding ATF2 function in melanoma (Bhoumik et al. 2007). Nuclear ATF2 expression is a strong predictor of poor survival in melanoma patients (Berger et al. 2003) and interfering with ATF2 transcriptional activity can inhibit proliferation of melanoma cells in culture and formation of tumors and metastasis in mouse models. On the other hand, ATF2 located at mitochondria increases mitochondrial permeability and promotes apoptosis, suggesting that cytosolic ATF2 works as a tumor suppressor (Lau et al. 2012). Recent studies shows that AP-1 might be one of the key regulators of the invasive state in melanoma (Kappelmann et al. 2013; Verfaillie et al. 2015).

5.10 AP-2

The AP-2 family of transcription factors consists in humans and mice of five members, AP-2a, AP-2b, AP-2c, AP-2d, and AP-2e (Hilger-Eversheim et al. 2000). All AP-2 proteins share a highly conserved helix–span–helix dimerization motif at the carboxyl terminus, followed by a central basic region and a less conserved domain rich in proline and glutamine at the amino terminus. The proteins are able to form hetero- as well as homodimers. The helix–span–helix motif together with the basic region mediates DNA binding, and the proline- and glutamine-rich region is responsible for transactivation. AP-2 has been shown to bind to the palindromic consensus sequence (5′-GCCNNNGGC-3′) found in various cellular and viral enhancers. AP-2 activity is regulated through a number of signal transduction pathways. Phorbol esters and signals that enhance cAMP levels induce AP-2 activity independent of protein synthesis, whereas retinoic acid treatment of teratocarcinoma cell lines results in transient induction of AP-2 mRNA levels at a transcription level (Buettner et al. 1993; Luscher et al. 1989).

Many experiments have demonstrated the functions of AP-2 in melanoma (Bar-Eli 2001). For example, loss of AP-2 expression increases tumor growth and metastasis in melanoma cells (Jean et al. 1998) and dominant-negative AP-2 (AP-2B) augments melanoma tumor growth in vivo (Gershenwald et al. 2001). AP-2 function is mediated via its regulated expression of target genes, which appear to be involved in proliferation, cell-cycle regulation (HER-2, p21/WAF-1), apoptosis (c-KIT, Bcl-2, FAS/APO-1), adhesion (MCAM/MUC18, E-cadherin), and invasion:angiogenesis (MMP-2, plasminogen activator inhibitor type I, VEGF, and PAR-1).

5.11 LEF/TCF/β-Catenin (Canonical Wnt Signaling)

LEF1/β-catenins are downstream mediators of canonical Wnt signaling and are involved in a variety of processes in development and tumorigenesis. The Wnt family consists of over 19 members, all of which are hydrophobic cysteine-rich secreted

molecules that share a high level of homology. The ligand subtype determines which Wnt signaling pathway will be activated. For example, Wnt1, 3a, and 7 activate the canonical pathway, whereas Wnt5a, 5b, and 11 activate the noncanonical pathway (Weeraratna 2005). Receptors of Wnt ligands include the Frizzled (FZD) family of receptors (Zilberberg et al. 2004). The detailed WNT signaling are described in altered signal transdution pathway in melanoma (Chap. 7).

The transcription factors of the LEF/TCF (lymphoid enhancer factor/T-cell-specific factor) family are the most downstream components of the Wnt signaling cascade. Today, four family members are known in mammals: LEF-1, TCF-1, TCF-3, and TCF-4. All share a homologous “HMG box” DNA-binding domain and recognize the conserved consensus sequence (5′-AGATCAAAGGG-3′) in their target gene promoters, including cyclin D1, MITF, c-myc, MMP-7, and others.

Wnt1 and Wnt3A are the predominant family members involved in melanocyte development and both activate β-catenin. β-Catenin mutations were found in 6 out of 26 melanoma cell lines but appeared to be rare among primary melanoma tumor specimens. Truncating mutations of the adenomatous polyposis coli (APC) gene that regulates β-catenin levels are also rare in melanoma. β-Catenin is still required for melanoma survival even though mutations in Wnt signaling are rare (Takeda et al. 2000; Widlund et al. 2002). Recently, active b-catenin signaling in melanoma cells suppresses T-cell infiltration into tumor tissues, resulting in anti-PD-L1/anti-CTLA-4 monoclonal antibody therapy (Spranger et al. 2015).

5.12 Notch Signaling

The Notch signaling pathway is a key developmental cell–cell interaction mechanism, which regulates processes such as cell proliferation, cell fate, differentiation, or stem cell maintenance. All receptors and ligands are single-pass transmembrane proteins with large extracellular domains that consist primarily of epidermal growth factor (EGF)-like repeats. In mammals, four Notch receptors (Notch1-4) and five ligands (Jagged-1 and 2, and Delta-like [Dll] 1, 3, and 4) have been described (Greenwald 1998).

The detailed Notch signaling are described in “Altered Signal Transduction Pathway in Melanoma” (Chap. 7). After proteolytic cleavage of intracellular domain of Notch (NIC), it translocates to the nucleus where it binds to CSL transcription factors (CBF1 in humans) and thereby activates transcription of Notch target genes via consensus Notch-binding site (5′- TGGGAA -3′). In the absence of Notch signaling, CSL functions as a transcriptional repressor via interactions with several corepressors.

The Notch signaling pathway is involved in tumorigenesis, as aberrant Notch signaling is frequently observed in certain cancers. Depending on the cell type and context, Notch can promote cell proliferation and cancer growth, or act as a tumor suppressor (Radtke and Raj 2003; Wilson and Radtke 2006). There is increasing evidence that Notch acts as an oncogene in the development of melanomas, with several receptors (Notch1 and Notch2), ligands (Jagged-1, Jagged-2,

and Dll1), as well as target genes (HES1, HEY1, and MCAM) upregulated at early stages of melanocytes transformation and tumor progression (Hoek et al. 2004). Notch1 is thought to play an important role stage-specifically to promote the progression of primary melanoma (Balint et al. 2005). Recently, melanoma–keratinocytes contact could induce Notch signaling activation in melanoma, resulting in the induction of metastatic microRNA, mir-222/221, and finally melanoma metastasis (Golan et al. 2015).

5.13 NF-kB (Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells)

NF-kB proteins were initially identified as pivotal transcription factors in chronic inflammatory diseases (Sen and Baltimore 1986). Accumulating evidence indicates that NF-kB is activated by a wide variety of stimuli such as cytokines and chemokines via AKT-signaling, MAPK-signaling, or NIK (NF-kB interacting kinase) (Perkins 2012). NF-kB is a homo- or heterodimeric complex consisting of proteins of the Rel-family. Rel-proteins can be divided into 2 groups depending on their mode of synthesis and transactivation properties. One class combines p65 (also known as REL A), REL B, and c-REL, which are all directly synthesized in their mature forms. The second class consists of p105 (also known as NFkB1) and p100 (also known as NFkB2), which are synthesized as large precursors and matured via phosphorylation and proteolysis into p52 and p50, respectively. The most commonly detected dimers are p65/p50, p65/p65, and p50/p50. NF-kB recognizes the DNA consensus (5′-GGG(A/G) NN(C/T)(C/T)CC-3′) (Amiri and Richmond 2005).

In the absence of stimuli such as cytokines or chemokines, NF-kB binds with IkBα protein and localizes in the cytoplasm. In the presence of stimuli, IkB kinase (IKK) complex is activated via kinases including AKT, ERK, or NIK and then IkBα is phosphorylated, ubiquitinated, and degraded. IkBα degradation induces translocation of NF-kB from the cytoplasm to the nucleus, and target genes are transcriptionally activated.

Many NF-kB-regulated genes have been reported, which are related to carcinogenesis, including apoptosis (TRAF1, TRAF2, c-IAP1/2, ML-IAP, and survivin), proliferation (cyclin D1 and CDK2), metastasis (COX2, ICAM-1, VCAM-1, ELAM-1, and MMPs), and angiogenesis (CXCL-1/8, IL-1, and TNF).

Constitutive activation of NF-*k*B is an emerging feature in melanoma and various target genes, CXCL1 and CXCL8, are highly expressed in most melanoma cell lines (Yang and Richmond 2001). Recently, RIP1, which is one of the upstream genes of NF-kB, has shown to be upregulated in melanoma, resulting in NF-kB activation and melanoma proliferation (Liu et al. 2015). Furthermore, knockdown of IKKbeta reduces the growth of the melanoma lesions in mouse studies, and conditional deletion of Ikkbeta in melanocytes blocks HRAS-induced melanomagenesis. Taken together, NF-kB-signaling may play an important role in melanoma growth/survival and melanomagenesis (Yang et al. 2010).

5.14 SMAD/SKI

The transforming growth factor-β (TGF-β) family of growth factors comprises more than 40 members, including TGF-β, activins, bone morphogenetic proteins (BMP), and nodal. They are ubiquitous multifunctional cytokines that regulate cellular activities, such as proliferation, differentiation, migration, survival, embryonic development, angiogenesis, or immune surveillance (Massague 2000). Binding of the TGF family to their receptors causes the assembly of a receptor complex that phosphorylates Smad2/3, which in turn bind to Smad4 and accumulate in the nucleus where they act as transcription factors, usually via Smad-binding sites (5′-AGAC-3′) (Nakao et al. 1997).

In normal tissues and cells, including melanocytes, transforming growth factor β (TGF-β) contributes to homeostasis by blocking cell-cycle progression via upregulation of the cyclin-dependent kinase inhibitors p15 and p21Waf-1, and downregulation of c-MYC (Reynisdottir et al. 1995; Warner et al. 1999). There are other targets regulated by TGF-β-smad signaling, including VEGF, BMPR-II, ets2, and PAI-1. Epithelial-to mesenchymal transition is also induced by TGF-β, which is related to tissue development and cancer metastasis.

The ski oncogene was discovered in its viral form, v-ski, as the transforming gene of the defective Sloan–Kettering virus (Stavnezer et al. 1981). SKI has been shown to interact with Smad2/3/4 and to recruit a transcriptional repressor of the N-CoR family and histone deacetylases (HDACs), resulting in repression of Smad-driven transcription (Khan et al. 2001). As the inactivation of the TGF-β pathway has been observed in a variety of human cancers, including melanomas, primary invasive melanomas in vivo exhibit nuclear, and cytoplasmic localization of SKI, whereas in melanoma metastasis, SKI is mostly localized in the cytoplasm and expressed at very high levels (Reed et al. 2001). SKI might inhibit smad-driven transcription of p21 in melanoma, resulting in escaping TGF-β-induced anti-proliferative activity. SKI is also a potent stimulator of Wnt/β-catenin signaling in human melanoma cells by binding to FHL2, which in turn activates MITF and Nr-CAM promoters in a β-catenin-dependent manner (Chen et al. 2003). SKI may participate as a regulator of melanoma progression by activating β-catenin signaling and repressing the TGF-β pathway. Consistent with SKI oncogenic function, TGF-β plays a tumor suppressive role through MITF suppression in melanoma (Pierrat et al. 2012).

5.15 STAT3 (Signal Transducer and Activator of Transcription 3)

STAT proteins were originally discovered as mediators of cytokine receptor signaling and are both cytoplasmic signaling molecules and nuclear transcription factors that activate diverse genes (Schindler et al. 1992; Shuai et al. 1992). There are seven STAT proteins (STAT1–4, 5A, 5B, and 6) in mammals (Li 2008). In the canonical mode of JAK–STAT signaling, the activation of the pathway is initiated by binding

of a peptide ligand (e.g., a cytokine) to transmembrane receptors. This leads to receptor dimerization and cross-activation of receptor-associated JAK kinases (JAK1-3 or tyrosine kinase 2 (TYK2)), which in turn phosphorylate tyrosine residues in the cytoplasmic tail of the receptor. These phosphotyrosine residues function as docking sites for latent cytoplasmic STAT proteins, which are then phosphorylated by JAK on a crucial C-terminal tyrosine residue near the 700 amino acid position. Phosphorylated STAT proteins dimerize via Src-homology 2 (SH2)–domain–phosphotyrosine interactions and translocate to the nucleus, where they function as transcriptional activators, inducing the expression of target genes via their consensus binding site (5′-TTC(N)nGAA-3′).

STAT3 regulates genes related to survival/apoptosis (survivin, BCL2-XL, and MCL-1), proliferation (MYC and cyclin D1), metastasis (HIF-1, VEGF, and MMP-2), and immune checkpoint molecule (PD-L1) though some of its targets are not yet known to be directly regulated by STAT3 (i.e., may be indirectly regulated downstream of STAT3).

Protein inhibitor of activated STAT3 (PIAS3) has been identified as an inhibitor of STAT3 and MITF and may also regulate melanocyte growth. The overexpression of PIAS3 in melanoma cell lines inhibits the transcriptional activity of both MITF and STAT3 and induces apoptosis (Levy et al. 2002).

The overexpression of activated Stat3 containing two cysteine residue mutations can mediate cellular transformation (Bromberg et al. 1999). Among the human cancers that display constitutively activated STAT3 are a variety of hematologic malignancies, including myeloma, leukemias, and lymphomas, as well as solid tumors, including breast, lung, prostate, ovarian cancers, and melanoma (Buettner et al. 2002). In melanoma, several receptor tyrosine kinases including c-Met, EGFR, ERRB2, and ERRB4, which are known to activate src kinases, are overexpressed or activated. STAT3 inhibition in melanoma cell lines or melanoma tumor models has been shown to induce cell death/tumor regression (Niu et al. 1999; Niu et al. 2001), inhibit angiogenesis (Xu et al. 2005), prevent metastasis (Xie et al. 2004), and activate antitumor immune responses (Wang et al. 2004).

5.16 HIF1A (Hypoxia-Inducible Factor 1α)

HIF-1 is the key transcriptional regulator of the cellular response to a hypoxic environment. HIF1 plays a key role in many cellular processes that participate in responses to reduced oxygen and energy supply. HIF1 consists of a heterodimer with HIF1α and HIF1β and binds to the hypoxia responsive element (HRE, 5′-RCGTG-3′). Under normoxic conditions, HIF-1α is hydroxylated on proline residue 402 (Pro-402) and/or Pro-564 by prolyl hydroxylase domain protein 2 (PHD2). Prolyl-hydroxylated HIF-1α is bound by the von Hippel–Lindau tumor suppressor protein (VHL), which recruits an E3-ubiquitin ligase that targets HIF-1α for proteasomal degradation (Maxwell et al. 1999). Under hypoxic conditions, the prolyl and asparaginyl hydroxylation reactions are inhibited by substrate (O_2) deprivation and/or the mitochondrial generation of reactive oxygen species (ROS), which may oxidize Fe(II) present in the catalytic center of the hydroxylases.

HIF1 target genes have been identified in tumors, and are related to angiogenesis (VEGF), motility (CXCR4), proliferation (BNIP3), and the reprogramming of cancer metabolism (SLC2A1, SLC2A3, LDHA, MCT4, and PDK1).

HIF1α expression is regulated at the transcriptional and translational levels by the PI3K/AKT and MAPK/ERK pathways. Interestingly, PTEN loss or $BRAF^{V600E}$, which overactivate the PI3K/AKT or MAPK/ERK pathways, respectively, increase HIF1α expression and melanoma survival under hypoxic condition through HIF-1α (Kumar et al. 2007). HIF1α has been reported to be an MITF target gene (Busca et al. 2005) and also an regulator of MITF though DEC1 (Feige et al. 2011).

5.17 Tbx-2/3 (T-Box Binding Protein 2/3)

Members of the T-box family of transcription factors play important roles in the regulation of cell-fate decisions and morphogenesis during development. This family binds the 20 nucleotide partially palindromic sequence T[G/C] ACACCTAGGTGTGAAATT. Brachyury can bind as a monomer or a dimmer whereas Tbx-2 binds as a monomer to brachyury single half-sites, recognizing the consensus sequences GTGTGA, GGGTGA, or GTGTTA (Carreira et al. 2000). Several T-box genes are involved in the progression of cancer. The amplification of Tbx2/3 is reported in breast cancer (Fan et al. 2004; Sinclair et al. 2002). Both are also expressed in normal melanocytes and have been found to be strongly upregulated in a subset of melanoma cell lines (Carreira et al. 1998; Hoek et al. 2004; Vance et al. 2005).

Tbx2 and Tbx3 function as transcriptional repressors. Tbx2 represses TYRP-1, which is one of the pigmentation enzymes, p21, and p19. Through its targeting of p21 and p19, Tbx2 may be required to maintain proliferation and suppress senescence in melanomas (Vance et al. 2005). E-cadherin is also known to be a target gene of Tbx3 and potentially Tbx2. Tbx3 may be related to the transition from radial growth phase (RGP) to vertical growth phase (VGP) and could contribute to metastatic potential via suppression of E-cadherin expression.

TBX2 is regulated transcriptionally and posttranslationally by the p38 stress signaling pathway in response to UVC irradiation (Abrahams et al. 2008). TBX2 is also reported to be an MITF and PAX3 target gene (Carreira et al. 2000; Liu et al. 2013).

5.18 C-MYC

c-Myc is a basic helix–loop–helix leucine zipper protein, which binds to a subset of E-box (5′-CANNTG-3′) consensus sequences. This gene was discovered as a translocated oncogene in Burkitt's lymphoma, resulting in c-MYC overexpression under the control of the immunoglobulin enhancer or promoter (Taub et al. 1982). Dysregulated expression and/or amplification of C-MYC have been known to be an important event for many tumors. The c-myc locus (8q24) is amplified in 30–50 % melanoma (Kraehn et al. 2001). Moreover, the $BRAF^{V600E}$ and $NRAS^{Q61R}$-specific

senescence program is suppressed by c-myc overexpression in melanoma (Zhuang et al. 2008). c-MYC also regulates a melanoma oncogene, RAB7 (Alonso-Curbelo et al. 2014).

c-MYC-dependent transactivation requires heterodimerization with a partner protein Max. This dimerization with Max is also essential for Myc proliferative and oncogenic function (Amati et al. 1993; Ferre-D'Amare et al. 1993). The inhibition of the dimerization with Max might be a potential therapeutic target. Thus far, the most "drug-able" transcription factors are ligand-dependent ones (particularly nuclear hormone receptors), although efforts to successfully target other transcription factors will hopefully bear fruit in the future.

5.19 p53

p53 is one of the most important human tumor suppressors (Lozano 2007; Riley et al. 2008). It plays important roles in controlling the DNA damage response, cell-cycle progression (p21, p16, p14, etc.), and apoptosis (APAF-1, PUMA, etc.) by regulating its targets transcriptionally. Deletion or loss-of-function mutations are found in diverse human cancers (see also Chap. 4).

UV causes DNA damage in human skin and correlates with melanoma incidence. Pigmentation may prevent epidermal cells from UV-induced DNA damage. p53 plays a role in the pigmentation response via transcriptional activation of the POMC promoter in keratinocytes, which encodes α-melanocyte-stimulating hormone (Cui et al. 2007).

A number of groups have reported mutational analysis of the *p53* gene in melanoma. Low-frequency (0–10 %) *p53* mutation or loss of heterozygosity in melanoma has been observed (Volkenandt et al. 1991), suggesting that there are additional mechanisms to suppress the function of p53. One mechanism involves the MDM2 gene, which encodes an E3 ubiquitin ligase that binds directly to p53 and ubiquitinates it, targeting p53 for proteosomal degradation. In melanoma progression, MDM2 has been shown to be highly expressed in 6 % of dysplastic nevi, 27 % of melanoma in situ, and 56 % of invasive primary and metastatic melanomas (Polsky et al. 2001).

Conclusions

The importance of transcription factors in melanocyte development and melanomagenesis has been summarized in this chapter (Table 5.1). Multiple transcription factors have been shown to be activators or suppressors of melanoma proliferation, survival, metastasis, and apoptosis. The complexity of their activities remains to be fully elucidated and will undoubtedly employ newer technologies including analyses of epigenetic marks, chromatin remodeling, and identification of coactivator or corepressor multiprotein complexes. For most transcription factors, we do not currently have effective therapeutic strategies available to permit their pharmacologic modulation. Exceptions include nuclear hormone receptors, which require small molecule ligand-dependent activation or certain drug-able strategies affecting pathways that indirectly control

Table 5.1 Overview of relevant transcription factors for melanoma

Gene	Binding sequences	Cancer-related biological function	Cancer-related target genes
MITF	CA[C/T]GTG	Cell-cycle progression, survival	CDK2, p16, p21, TBX2, TRPM1, HIF1A, BCL2, c-MET
CREB	TGANNTCA	Tumorigenicity	CCND1, MITF (by EWS-ATF1)
SOX10	[A/T][A/T]CAA[A/T]G	Unknown	MITF
PAX3	GT[T/C] [C/A] [C/T] [G/C] [G/C], TAAT	Motility (by PAX3-FOXO1)	MITF, PTEN, BCL-XL, c-MET
Snail superfamily	CAGGTG	Epithelial–mesenchymal transition	E-cadherin (CDH1), claudin, occludin
FOXD3	A[A/T]T[A/G]TTTGTTT	Suppressor of growth	MITF
ETS family	GGA[A/T]	Tumorigenicity	MITF (not direct target)
BRN2	ATGC, AAAT	Metastasis	MITF
AP-1	TGA[G/C]TCA	Proliferation, metastasis	ATM, XPA, cyclin A, CCND1, MMP2, TNF-α
AP-2	GCCNNNGGC	Melanoma progression	p21, c-KIT, BCL2, MMP2, CDH1
LEF/TCF/β-catenin	AGATCAAAGGG	Survival	CCND1, MITF, c-MYC, MMP7
Notch (NIC)	TGGGAA	Melanoma progression	HES, HEY1, MCAM
NFkB	GGG[A/G]NN[C/T][C/T]CC	Growth, survival	c-IAP1/2, survivin, CCND1, CDK2, CXCL-1/8
SMAD/SKI	AGAC	Melanoma progression	p21, VEGF, ETS2, PAI-1
STAT3	TTCNnGAA	Melanoma survival, angiogenesis	Survivin, BCL-XL, MCL-1, HIF1A, c-MYC, CCND1, MMP2, VEGF
HIF1A	[A/G]CGTG	Angiogenesis, metastasis, proliferation	VEGF, CXCR4, BNIP
TBX2/3	G[G/T]GTTA, GTGTTA	Metastasis	p21, p19, CDH1
c-MYC	CANNTG	Suppress the senescence	BCL2
p53	[A/G] [A/G] [A/G]C[A/T] [T/A]-G[C/T] [C/T] [C/T]	Cell-cycle progression, DNA damage response	p21, p16, p14, PUMA

transcription factors. Hopefully increasing accumulation of information about transcription factor biochemistry and the pathways which modulate their activities will provide new clues to novel therapeutic targeting strategies aimed at transcription factors in melanoma.

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MiRNAs in Malignant Melanoma

6

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6.1 MiRNA Biogenesis and Function

6.1.1 Transcription of miRNA Genes and Processing in the Nucleus

MicroRNA genes are embedded in intergenic as well as intragenic regions of the human genome, encoding either a single miRNA species or a cluster of multiple miRNAs in a polycistronic manner (Lee et al. 2002). In the first step of miRNA expression, miRNA genes are transcribed into pri-miRNAs (primary miRNA transcripts; Fig. 6.1). In most cases, transcription is accomplished by RNA polymerase II, resulting in 5′-methyl-guanosine capped and polyadenylated pri-miRNAs, which contain local stem-loop structures and are up to several kilobases in length (Cai et al. 2004; Lee et al. 2004). Some specific miRNAs are initially transcribed by RNA polymerase III (Borchert et al. 2006). While still in the nucleus, the pri-miRNA is endonucleolytically cleaved by the so-called microprocessor complex composed of the RNAse III enzyme Drosha (RNASEN) and its co-factor DGCR8 [DiGeorge syndrome critical region on chromosome 8;

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_6

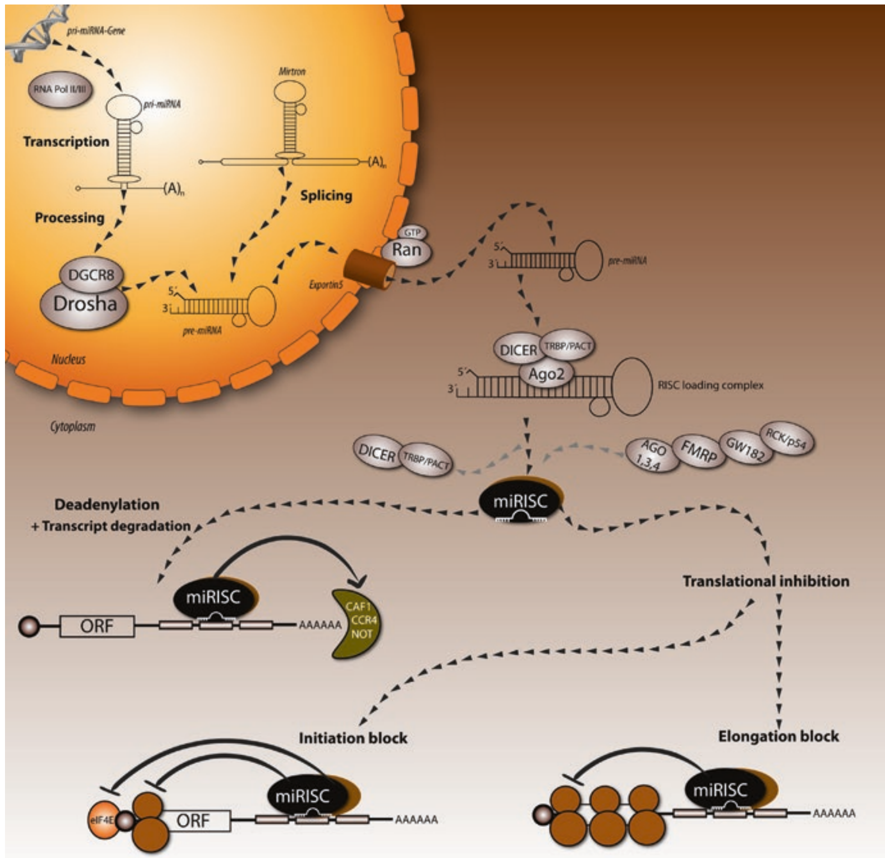

Fig. 6.1 Schematic overview of miRNA biogenesis and function. Please refer to Sect. 6.1 for detailed explanations on the molecular mechanisms involved in each step

also known as Pasha (Partner of Drosha) in *Drosophila melanogaster* and *C. elegans*] (Landthaler et al. 2004; Lee et al. 2003).

DGCR8 interacts with the ~33 bp stem-loop as well as with the adjacent unpaired flanking regions within the pri-miRNA, thus supporting Drosha-mediated cleavage in the stem region, about 11 bp away from the junction of ssRNA (single-stranded RNA) to dsRNA (double-stranded RNA) (Han et al. 2006; Zeng and Cullen 2005). The resulting pre-miRNA (~70 bp in length) is rapidly translocated to the cytoplasm via the Ran-GTP-dependent nuclear export factor exportin 5 (XPO5), a member of the nuclear transport receptor family (Kim 2004). Export of only correctly processed pre-miRNAs is ensured by recognition of the >14 bp dsRNA stem together with a short (1–8 nucleotides) 3′ overhang (Zeng and Cullen 2003). Exceptions to this rule have been reported, as in the case of cell adhesion complexes sequestering microprocessor components for the localized production of mature miRNAs from nuclear-exported pri-miRNAs (Kourtidis et al. 2015). Interestingly, a number of pre-miRNAs can be exported from the nucleus without undergoing processing by

Drosha. These so-called miRtrons are located in very short introns and are capable of forming a hairpin resembling pre-miRNA molecules after they were released from their host transcripts by splicing and debranching (Berezikov et al. 2007).

6.1.2 Cytoplasmic Processing and Modes of miRNA Mediated Gene Silencing

In the cytoplasm, maturation of pre-miRNAs occurs via a multi-enzyme complex called RISC (RNA induced silencing complex) loading complex (RLC). This RLC is composed of the RNase III enzyme Dicer, the double-stranded RNA-binding domain proteins TRBP (TAR RNA-binding protein) and PACT (protein activator of PKR), as well as AGO2 (Argonaute-2), which builds the core of the complex (Gregory et al. 2005; MacRae et al. 2008). TRBP and PACT facilitate Dicer-mediated cleavage of the pre-miRNA, which occurs near the terminal loop and results in an RNA duplex of ~22 nucleotides with two nucleotide overhangs on each 3′ terminus (Hutvagner et al. 2001; Knight and Bass 2001). In some cases, AGO2 – which exhibits robust RNaseH-like endonucleolytic activity – can support Dicer processing by cleaving the 3′ arm of specific pre-miRs (Diederichs and Haber 2007). Subsequently, Dicer and its interactors TRBP and PACT dissociate from the complex, and the miRNA duplex is separated into the guide strand (which is complementary to the target mRNAs and is thus functional in gene silencing) and the passenger strand (miRNA*), which usually gets degraded. It would appear that there is no universal helicase responsible for the unwinding of the miRNA duplex, but specific helicases may differentially regulate subgroups of miRNAs (Winter et al. 2009). In other cases, a helicase is not required at all for duplex unwinding (Pillai et al. 2005; Wu and Belasco 2005). The guide strand is characterized by the presence of a thermodynamically less stable base pair at the 5′ end of the duplex and is loaded onto the RISC complex after unwinding (Khvorova et al. 2003). Of note, miRNA* strands are not always simply by-products of miRNA biogenesis but are sometimes also acting as functional miRNAs on the miRISC (miRNA-containing RISC) (Chiang et al. 2010; Ghildiyal et al. 2010; Okamura et al. 2009). Nowadays, therefore the nomenclature changed to -5p or -3p miRNAs.

The assembly of the miRISC, also called miRNP (micro-ribonucleoprotein), is a dynamic process coupled with the preceding steps of pre-miRNA processing. Key components of the miRISC are proteins of the Argonaute (AGO) family, FMRP (fragile X mental retardation protein), and P-body components including GW182 and RCK/p54, which are essential for inducing miRNA-mediated gene repression (Filipowicz et al. 2008). Guided by the mature miRNA, the miRISC subsequently binds to target sequences in the 3′ untranslated regions (3′UTRs) of regulated transcripts, in order to inhibit their translation into functional proteins. To date, the general rules for the initial miRNA:mRNA interaction, which are fundamental for target recognition, are only incompletely determined experimentally and bioinformatically (Brennecke et al. 2005; Doench and Sharp 2004; Grimson et al. 2007; Lewis et al. 2005; Nielsen et al. 2007; Reyes Herrera and Ficarra 2012; Zheng et al. 2013; Peterson et al. 2014). In animals, miRNAs almost exclusively bind to their

target mRNAs with imperfect complementarity. Nevertheless, an indispensible prerequisite for efficient transcript targeting is continuous base-pairing of miRNA nucleotides 2–8, which are called the miRNAs "seed-sequence" (Bartel 2009). It is actually this miRNA seed which is the major determinant of a miRNA's target repertoire. Nevertheless, apart from the miRNA seed sequence targets, mRNAs usually have multiple cis-elements in their 3′UTRs that can be bound by other trans-factors, namely, RNA binding proteins (RBPs) (reviewed by Ho and Marsden 2014). Different mechanisms of interplay between RBPs and miRNAs/RISC can determine a synergistic or a competing relationship with an mRNA, destabilizing or stabilizing its translation. When both kinds of molecules target the same cis-element, the mechanism may involve the interaction between the RBP and the miRNA or components of the RISC. When they bind different cis-elements, the effect is more likely to depend on which molecule binds first (influenced by subcellular localization of the process), the number of cis-element in the 3′UTR of a given mRNA, and the ability of one molecule to displace the other from the target. The RBP involved in mRNA stability can be classified according to their target sequences into AU-rich elements (ARE)-binding proteins, and pyrimidine (C/CU)-rich element binding proteins. In any case RBP-mediated degradation or translation is dependent on miRNA-containing RISC (Ho and Marsden 2014). These additional regulatory layers of mRNA fate stress the necessity of experimentally validating any bioinformatically predicted miRNA target, i.e., based purely upon complementary nucleotide sequence.

The resulting miRNA-induced posttranscriptional silencing of target genes is mediated either by destabilization of the corresponding mRNA (Behm-Ansmant et al. 2006b; Giraldez et al. 2006; Wu et al. 2006; Wu and Belasco 2005; Guo et al. 2010) or by repression of protein translation (Pillai et al. 2005; Standart and Jackson 2007), both pathways acting cooperatively but yet independently of each other. Current knowledge suggests that destabilization of target mRNAs starts with recruitment of the P-body component GW182 (glycine-tryptophan protein of 182 kDa) by Argonaute proteins (Till et al. 2007). GW182 subsequently mediates binding of the CAF1:CCR4:NOT1 deadenylase complex to the target mRNA. Deadenylation is then followed by removal of the 5′-methyl-guanosine cap via the DCP1:DCP2 decapping complex, ultimately leading to 5′ → 3′ exonucleolytic degradation of mRNA by exonuclease XRN1 (Behm-Ansmant et al. 2006a; Eulalio et al. 2007, 2009; reviewed by Hausser and Zavolan 2014). By contrast, there still is a lack of consensus concerning the mechanism(s) by which miRNAs induce repression of translation. While many experiments refer to the initiation of translation as a target for repression, there is also evidence that various post-initiation steps could be affected (reviewed by Chekulaeva and Filipowicz 2009). Present research aims to unravel whether miRNAs are actually capable of controlling translation by multiple mechanisms or if these discrepancies were due to different experimental approaches utilized in the past (Cannell et al. 2008; Kong et al. 2008). Figure 6.1 summarizes the mechanisms involved in miRNA biogenesis and function.

6.1.3 Regulation of miRNA Biogenesis, Function, and Decay

The general pathway of miRNA biogenesis and function – as well as the clearance of miRNAs through decay – is complicated by a large number of regulatory mechanisms, in which a vast quantity of yet unidentified proteins is likely to be involved. There may be specific alterations to the pathway for every individual miRNA or at least distinct subgroups of miRNAs (reviewed by Krol et al. 2010).

The expression of miRNAs can be regulated at the level of transcription (reviewed by Davis and Hata 2009; Turner and Slack 2009) or at various steps during the processing of the pri-miR into mature miRNA (reviewed by Davis and Hata 2009). The latter mechanism commonly involves the regulation of constituents of the miRNA processing machinery, like Drosha/DGCR8 (Han et al. 2009; Triboulet et al. 2009) and Dicer/TRBP (Chendrimada et al. 2005; Melo et al. 2009; Paroo et al. 2009). Additionally, accessory proteins can directly interact with miRNA precursors either repressing or stimulating their further processing (reviewed by Winter et al. 2009). By contrast, modulation of miRNA function is mainly based on mechanisms affecting proteins of the miRISC, predominantly AGO2 and GW182 (reviewed by Krol et al. 2010). With regard to melanoma, it has been suggested that the transcriptional regulation of Dicer by MITF might be a major determinant in melanocyte cell survival and differentiation (Levy et al. 2010a, b; please refer to Sect. 6.2 for detailed information). In addition, recent literature described the strong reduction of AGO2 expression in melanoma, influencing miRNA functionality (Voeller et al. 2013; Sand et al. 2012a). Further studies analyzed changes in expression level of Dicer, Drosha and additional processing enzymes (Jafari et al. 2013; Jafarnejad et al. 2013; Ma et al. 2011), but leave the definition of the role of these changes mainly open.

To date, little is known about the regulation of miRNA decay. Nevertheless, recent findings implicate that sequences present at the 3′ end of specific miRNAs or, more generally, enzyme catalyzed modifications to the 3′ end of miRNAs may determine the rate of miRNA clearance (Hwang et al. 2007; Jones et al. 2009; Katoh et al. 2009). Interestingly, degradation of mature miRNAs seems to depend on their activity. In the absence of its complementary targets, a miRNA may be specifically released from the miRISC. Thereby, its 5′ end gets accessible to the 5′ → 3′ exonuclease XRN-2, which subsequently degrades the miRNA (Chatterjee and Grosshans 2009).

These findings clearly show that miRNAs are not only active regulators of gene expression but are themselves subject to sophisticated control. Adding even more complexity, miRNAs have recently been shown to not exclusively repress gene expression but to conversely enhance translation of specific transcripts under certain cellular conditions – although these cases are still clearly the exception rather than the rule (Vasudevan et al. 2007, 2008).

New studies recently further showed an important role of RNA editing via adenosine deaminase acting on RNA (ADAR) enzymes in miR functions (Shoshan et al. 2015; Dietrich and Bosshoff 2015).

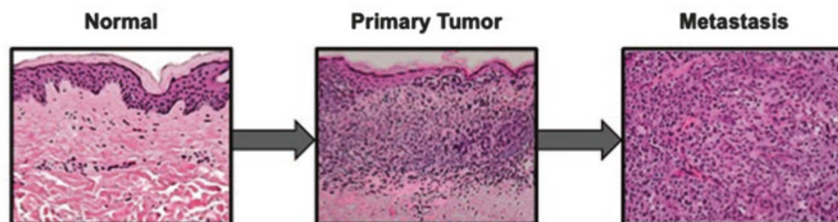

Fig. 6.2 Schematic overview of miRNAs deregulated during melanoma formation and progression (Reprinted from Mueller and Bosserhoff (2009), strongly modified. With kind permission from the *Br J Cancer*)

6.2 Impact of Specific miRNAs on Melanomagenesis

In contrast to other types of tumors, studies on the impact of specific miRNA species on melanomagenesis have not been conducted before the year 2008. Nevertheless, many miRNAs deregulated in melanoma cells compared to normal melanocytes have been characterized in regard to their target genes as well as their impact on melanoma cell function since then (Fig. 6.2 and Table 6.1). The following paragraph aims to integrate those findings in their meaningful context and to point out how miRNA-related research expanded our knowledge on the molecular mechanisms contributing to formation and progression of malignant melanoma. However, it cannot be comprehensive due to many new findings in the field. We try to integrate all miRs coming from arrays or miR sequencing approaches, which are confirmed by independent methods to be differentially expressed. However, based on multiple approaches published, we mainly focused on deregulated miRNAs, where functional consequences have been described.

6.2.1 Networking of miRNAs and MITF

It is not surprising that the very first miRNA identified to influence melanoma progression is involved in modulating the expression level of the key regulator of melanocyte cell fate—microphthalmia-associated transcription factor (MITF; see Chaps. 5 and 12). In their study, Bemis et al. (2008) set out to characterize an

Table 6.1 Summary of expression profiling as well as functional data available on miRNAs deregulated in melanoma cells

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-101	↑	Both	qRT-PCR, functional tests	Not yet determined	MITF, EZH2	(**80**)	miR-101 family
miR-106b	↑	Both	Microarray. qRT-PCR, functional tests	Not yet determined	CDKN1A	(**13**, **71**, **108**, **111**)	miR-17 family
mir-125a	↓	Both	Microarray, RT^2 Profiler Array, qRT-PCR	MAPK/ERK	LIN28B	(**13**, **17**, **108**, **144**)	miR-10 family
miR-125b	↓	Both	Microarray, qRT-PCR, functional tests	Hypermethylation, intracellular cAMP levels.	C-JUN, ITGA9, MAP3K11	(**38**, **49**, **56**, **58**, **104**, **108**, **140**, **141**)	miR-10 family
miR-126-3p & miR-126-5p	↓*	Both	Microarray, NGS, qRT-PCR, functional tests	TFAP2A level	ADAM9, MMP7	(**30**, **31**, **61**, **70**)	miR-126 family
miR-130a-3p	↓	Both	High-throughput RNA sequencing, qRT-PCR	Not yet determined	SMAD4°	(**22**)	miR-130 family
miR-130b-3p	↓(?)	Both	High-throughput RNA sequencing, qRT-PCR	Not yet determined	SMAD°	(**22**, **61**)	miR-130 family
miR-137	↓	Both	Microarray, qRT-PCR, functional tests	Amplification of a VNTR in the 5'UTR of the pri-miRNA	MITF, MET, YBX1, EZH2, CTBP1, PAK2	(**5**, **21**, **46**, **68**, **81**)	miR-137 family

(continued)

Table 6.1 (continued)

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-143	↓	Tissue	qRT-PCR, functional tests	Not yet determined	TCL1B	(**69**)	miR-143 family
miR-145	↓	Cell line	qRT-PCR, functional tests	Not yet determined	MYO5A, MYC°, FSCN1°	(**23**, **24**, **98**)	miR-145 family
miR-146a	↑	Both	Microarray, RT^2 Profiler Array, Small RNA profiling, qRT-PCR, functional tests	NRAS mutant (NRASQ61K), BRAF mutant (BRAFV600E), phosphorylation of myc by MAPK/ERK pathway, MITF level ?	NUMB	(**32**, **108**)	miR-146 family
	↓	Cell line	Microarray, qRT-PCR	MAPK/ERK, MITF level ?	Not yet determined	(**17**, **64**, **108**)	
miR-148a	↓*	Both	Microarray, RT^2 Profiler Array, qRT-PCR, functional tests	Hypermethylation	TGIF2, MITF	(**45**, **79**, **108**)	miR-148 family
miR-148b	↓*	Both	RT^2 Profiler Array, Microarray, qRT-PCR	MITF level, TFAP2A level	ALCAM, MITF	(**45**, **92**, **104**, **107**, **108**)	miR-148 family
miR-149*	↑	Both	qRT-PCR	p53 expression	GSK3A	(**55**)	miR-149 family
miR-15a	↓*	Tissue	qRT-PCR, functional tests	Not yet determined	IL-10RA	(**110**, **120**, **129**)	miR-15 family

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-155	↑	Tissue	RT^2 Profiler Array, qRT-PCR	IL1B	MITF, TYRP1	(**1**, **25**, **43**, **108**, **121**)	miR-155 family
	↓	Cell line	qRT-PCR, functional tests	Not yet determined	SKI	(**64**, **65**, **108**)	
miR-17	↑	Both	Microarray, qRT-PCR, functional tests	MAPK/ERK	ETV1, ADAR, LEF1, CCND1, CDKN1A	(**13**, **16**, **17**, **41**, **64**, **92**, **94**, **108**)	miR-17 family, miR-17-92 cluster
miR-18b	↓*	Both	Microarray, qRT-PCR, functional tests	Hypermethylation	MDM2	(**19**)	miR-17 family, miR-106-363 cluster
miR-182	↑	Both	Microarray, qRT-PCR, functional tests	Hypermethylation	MITF, FOXO3	(**74**, **122**)	miR-182 family
	↓	Both	Microarray	Not yet determined	Not yet determined	(**108**, **134**)	
miR-185	↑	Both	Microarray, qRT-PCR, functional tests	Not yet determined	IL10RA	(**92**, **108**, **129**)	miR-185 family
miR-19b	↑	Both	Microarray, qRT-PCR	MITF level	PITX1	(**92**, **103**, **104**, **108**)	miR-19 family, miR-17-92 cluster (19b-1), 106–363 cluster (19b-2)

(continued)

Table 6.1 (continued)

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-193b	↓	Both	Microarray, qRT-PCR, functional tests, Northern Blot	Not yet determined	CCND1, MCL1, STMN1	(**12**, **13**, **14**)	miR-193 family
miR-195	↑	Tissue	Taqman MicroRNA Assay, functional tests	Not yet determined	WEE1	(**6**)	miR-15 family
miR-196a	↓	Both	RT^2 Profiler Array, qRT-PCR, functional tests	Not yet determined	HOXB7, HOXC8	(**8**, **91**, **108**)	miR-196a family
miR-199a-3p	↑*	Both	Microarray, qRT-PCR, functional tests	Not yet determined	DNAJA4, MET	(**89**, **105**)	miR-199 family
miR-199a	↑	Both	Microarray, qRT-PCR, functional tests	Not yet determined	APOE, DNAJA4	(**105**)	miR-199 family
miR-1908	↑	Both	Microarray, qRT-PCR, functional tests	Not yet determined	APOE, DNAJA4	(**105**)	miR-1908 family
miR-20b	↑	Both	Microarray, qRT-PCR	MITF level ?	Not yet determined	(**92**, **104**, **134**)	miR-17 family, 106–363 Cluster
	↓	Cell line	Microarray	Not yet determined	F2R	(**114**)	
miR-200b	↓	Both	Microarray, qRT-PCR	Not yet determined	PTEN°, ZEB1°	(**57**, **115**, **134**)	miR-8 family

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-200c	↑	Cell line	qRT-PCR	Not yet determined	Not yet determined	(**26**, **92**)	miR-8 family
	↓	Both	Microarray, RT^2 Profiler Array, qRT-PCR, functional tests	Not yet determined	BMI1	(**76**, **108**, **128**, **134**)	
miR-203	↓	Both	Microarray, RT^2 Profiler Array, qRT-PCR, NGS, functional tests	Hypermethylation	E2F3, ZNF148, KIF5B, KIF2A, CREB1, VCAN, BMI1	(**9**, **11**, **61**, **96**, **97**, **99**, **100**, **108**, **134**)	miR-203 family
miR-205	↓	Both	Microarray, RT^2 Profiler Array, qRT-PCR, functional tests, NGS	Hypermethylation	E2F1, E2F5, MDM2	(**18**, **19**, **61**, **76**, **108**, **134**)	miR-205 family
miR-206	↓*	Tissue	Microarray, qRT-PCR, functional tests	Not yet determined	CDK4, CCND1, CCNC	(**36**, **127**)	miR-1 family
miR-21	↑	Both	Microarray, RT^2 Profiler Array, qRT-PCR, functional tests	MAPK/ERK, STAT3 activation	FBXO11, PTEN°, PDCD4°, BTG2°, TIMP3°	(**13**, **17**, **43**, **54**, **84**, **108**, **118**, **134**, **136**, **137**)	miR-21 family
miR-210	↑	Tissue	Microarray, RT^2 Profiler Array, qRT-PCR, functional tests	MAPK/ERK, HIF1A, HIF2A	MNT, PTPN1, HOXA1, TP53I11	(**17**, **101**, **108**, **117**, **143**)	miR-210 family

(continued)

Table 6.1 (continued)

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-211	↑	Both	Microarray, qRT-PCR	MITF level	IL-10RA	(**83**, **108**, **120**, **129**)	miR-204 family
	↓	Both	Microarray, NGS, qRT-PCR, functional tests	MITF level	RAB22A, AP1S2, SERINC3, M6PR, IGFBP5, PDE3A, SSRP1, SOX11, LIFR, SOX4, KCNMA1, TGFBR2, NGAT5, POU3F2, PRAME, NUAK1, IGF2R°, PDK4	(**4**, **7**, **10**, **13**, **61**, **66**, **83**, **86**, **88**, **113**, **134**)	
miR-214	↑	Both	Microarray, qRT-PCR, functional tests	Not yet determined	ITGA3, MET, TFAP2C, TFAP2A°, PAK2	(**106**, **107**, **121**)	miR-214 family
	↓	Tissue	RT^2 Profiler Array, qRT-PCR	Not yet determined	Not yet determined	(**13**, **77**, **108**)	
miR-218	↓	Both	RT^2 Profiler Array, qRT-PCR, functional tests	Not yet determined	MITF, KIAA1524, BMI1	(**44**, **108**, **133**, **142**)	miR-218 family
miR-22	↑	Tissue	Microarray, qRT-PCR	Not yet determined	Not yet determined	(**13**, **116**)	miR-22 family
	↓	Cell line	Microarray, qRT-PCR, functional tests	MAPK/ERK	LEF1, FOSL1, CDKN1A	(**17**)	

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-221	↑	Both	Microarray, qRT-PCR, functional tests, Northern Blot	HOXB7/PBX2, PLZF, hPNP^{old-35}, ADAR1	KIT, FOS, TFAP2A, CDKN1B	(**20**, **27**, **28**, **29**, **30**, **34**, **39**, **51**)	miR-221 family
	↓	Both	Microarray, qRT-PCR, functional tests	MITF/RBPJK, MAPK/ERK, hPNPase^{old-35}	TGFBR2, NFAT5	(**17**, **20**, **39**, **66**, **109**)	
miR-222	↑	Both	Microarray, qRT-PCR, functional tests, Northern Blot	HOXB7/PBX2, PLZF, hPNPold-35, ADAR1, Thr-38 phosphorylated Ets-1	ETS1, ICAM1, FOS, TFAP2A, CDKN1B	(**20**, **27**, **28**, **29**, **30**, **34**, **39**, **85**, **92**)	miR-221 family
	↓	Both	Microarray, qRT-PCR, Northern Blot	MITF/RBPJK, MAPK/ERK, ETS-1	ETS1	(**17**, **39**, **85**, **109**)	
miR-224	↑	Both	qRT-PCR, functional tests	E2F1, HIF1A	TXNIP	(**50**, **60**)	miR-224 family
	↓	Cell line	Microarray	Not yet determined	Not yet determined	(**108**, **134**)	
miR-25	↑	Both	Microarray, qRT-PCR	ERK dependent ?	PTEN°	(**15**, **57**, **108**)	miR-25 family
miR-26a	↓	Both	Microarray, NGS, qRT-PCR, functional tests	MAPK/ERK	BAG4, LIN28B, ZCCHC11	(**17**, **33**, **61**, **62**, **112**, **115**)	miR-26 family
miR-29a	↑	Tissue	RT^2 Profiler Array	STAT1	CDK6	(**108**, **119**)	miR-29 family

(continued)

Table 6.1 (continued)

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-29c	↓	Tissue	qRT-PCR	Hypermethylation	DNMT3A°, DNMT3B°, BCD276	(**95**, **132**)	miR-29 family
miR-30b	↑	Tissue	Microarray, qRT-PCR, functional tests	Not yet determined	GALNT7, GALNT1, SEMA3, CESLR3, TWF1	(**35**)	miR-30 family
	↓	Cell line	Microarray	MAPK/ERK	GALNT7	(**17**)	
miR-30d	↑	Tissue	Microarray, qRT-PCR, functional tests	Not yet determined	GALNT7, GALNT1, SEMA3, CESLR3, TWF1	(**35**)	miR-30 family
miR-31	↓	Both	Microarray, qRT-PCR, functional tests	Hypermethylation & EZH2-mediated histone H3K27 trimethylation	SRC, RAB27A, MAP3K14, MET, EZH2°	(**2**)	miR-31 family
miR-3151	↑	Tissue	qRT-PCR	BRAF	TP53	(**63**)	miR-3151 family
miR-33a	↓	Both	Microarray, qRT-PCR, functional tests	Not yet determined	HIF1A	(**146**)	miR-33 family
miR-338	↑	Tissue	Microarray, qRT-PCR	Aktivation of MAPK/ERK pathway	KIT°	(**51**, **53**)	miR-338 family
	↓	Tissue	qRT-PCR	Not yet determined	Not yet determined	(**10**, **109**)	

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-34a	↑	Tissue	RT^2 Profiler Array	CCL2	Not yet determined	(**108**, **130**)	miR-34 family
	↓	Both	Microarray, qRT-PCR, functional tests	Hypermethylation, B-Raf/ MKK/ER, p53	FLOT2, ULBP2	(**17**, **42**, **47**, **48**, **73**, **78**, **117**, **135**)	
miR-34b	↓	Both	Microarray, qRT-PCR, functional tests	Hypermethylation, p53	MYC, CDK6, MET	(**47**, **79**, **87**, **89**, **135**)	miR-34 family
miR-34c	↓	Both	Microarray, qRT-PCR, functional tests	Hypermethylation, p53	E2F3, ULBP2, MET	(**47**, **48**, **79**, **89**, **116**, **135**)	miR-34 family
miR-340	↑	Cell line	qRT-PCR, functional tests	Not yet determined	Modulator of MAPK/ERK signaling, RHOA, MITF	(**40**, **52**, **126**)	miR-340 family
miR-365	↓*	Both	qRT-PCR, functional tests	Not yet determined	NRP1	(**3**)	miR-365 family
miR-376a	↓	Both	Microarray, qRT-PCR, functional tests	Epigenetic modifications (?)	IGF1R	(**108**, **138**)	miR-368 family
miR-376c	↓	Both	Microarray, qRT-PCR, functional tests	Epigenetic modifications (?)	IGF1R	(**108**, **138**)	miR-368 family

(continued)

Table 6.1 (continued)

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-377	↓*	Both	Microarray, qRT-PCR, functional tests	Epigenetic modifications (?)	E2F3, MAP3K7	(**138**, **139**)	miR-154 family
miR-425	↓*	Tissue	Microarray, qRT-PCR	Not yet determined	IGF1	(**72**, **110**)	miR-425 family
miR-432	↑	Tissue	qRT-PCR	Hypomethylation	ADAR	(**94**)	miR-432 family
miR-452	↑	Both	qRT-PCR, functional tests	E2F1	TXNIP	(**60**)	miR-452 family
miR-455-5p	↓	Both	Microarray, functional tests	Not yet determined	CPEB1	(**114**, **123**, **134**)	miR-455 family
miR-514a	↑	Both	Microarray, qRT-PCR, functional tests	Not yet determined	NF1	(**124**, **125**)	miR-506 family
miR-524-5p	↓	Both	Microarray, qRT-PCR, functional tests	Activity of the MAPK/ERK pathway	BRAF, MAPK1	(**77**)	miR-515 family
miR-532-5p	↑	Both	qRT-PCR, functional tests	Not yet determined	RUNX3°	(**59**)	miR-188 family
miR-573	↓	Both	qRT-PCR, functional tests	Not yet determined	MCAM	(**131**)	miR-573 family
miR-638	↑	Both	Microarray, qRT-PCR, functional tests	AP2A expression, Hypermethylation	TP53INP2	(**6**, **108**)	miR-638 family
miR-7	↓	Cell line	Microarray, qRT-PCR, functional tests	Not yet determined	IRS2	(**37**, **108**)	miR-7 family

miRNA	Gain/loss in melanoma cells	Tissue/Cell line	Kind of evidence	Cause of loss/gain	Verified target(s) in MM	Reference(s)	Family/Cluster
miR-768-3p	↓	Tissue	Microarray, functional tests	MAPK/ERK	elF4E	(**13**, **17**, **53**)	~
miR-9	↑	Tissue	Microarray, RT^2 Profiler Array, qRT-PCR	Not yet determined	Not yet determined	(**13**, **108**)	miR-9 family
	↓	Both	qRT-PCR, functional tests	Hypermethylation, Transcription factor YY1	NFKB1, RYBP	(**75**, **79**, **145**)	
miR-92a	↑*	Both	Microarray, qRT-PCR	MAPK/ERK	PTEN	(**13**, **17**, **57**, **64**, **92**, **108**)	miR-25 family, miR-17-92 cluster (miR-92a-1), miR-106-363 cluster (miR-92a-2)
miR-98	↓	Tissue	qRT-PCR, functional tests	Lin28b, NF-kB (IL-6 signalling)	IL6	(**67**)	let-7 family
let-7a	↓	Both	Microarray, RT^2 Profiler Array, qRT-PCR, Northern Blot, functional tests	Not yet determined	NRAS°, ITGB3	(**13**, **82**, **90**, **93**, **108**, **120**)	let-7 family
let-7b	↓	Tissue	Microarray, qRT-PCR, functional tests	Not yet determined	CCND1, CCND3°, CDK4°, CCNA°	(**13**, **120**)	let-7 family

Question marks in brackets indicate lack of compelling evidence. Due to space limitations, only miRNAs with confirmed targets were included. Please refer to Sects. 6.2 and 6.3 for details
↓*/↑*: One available publication shows contradictory results
Gene°: Gene is regulated directly or indirectly by the mentioned microRNA

(continued)

Table 6.1 (continued)

~: mir-768 overlaps an annotated snoRNA, HBII-239. Phylogenetic analysis show poor conservation of the reported mature miRNA sequence (miRBase)

(**1**) Arts et al. (2015), (**2**) Asangani et al. (2012), (**3**) Bai et al. (2015), (**4**) Bell et al. (2014), (**5**) Bemis et al. (2008), (**6**) Bhattacharya et al. (2013), (**7**) Boyle et al. (2011), (**8**) Braig et al. (2010), (**9**) Bu and Yang (2014), (**10**) Caramuta et al. (2010), (**11**) Chang et al. (2015), (**12**) Chen et al. (2013), (**13**) Chen et al. (2010), (**14**) Chen et al. (2011), (**15**) Ciuffreda et al. (2012), (**16**) Cohen et al. (2015), (**17**) Couts et al. (2013), (**18**) Dar et al. (2011), (**19**) Dar et al. (2013), (**20**) Das et al. (2010), (**21**) Deng et al. (2011), (**22**) Ding et al. (2015), (**23**) Dynoodt et al. (2013a), (**24**) Dynoodt et al. (2013b), (**25**) El Hajj et al. (2015), (**26**) Elson-Schwab et al. (2010), (**27**) Errico et al. (2013), (**28**) Felicetti et al. (2008a), (**29**) Felicetti et al. (2008b), (**30**) Felli et al. (2016), (**31**) Felli et al. (2013), (**32**) Forloni et al. (2014), (**33**) Fu et al. (2014), (**34**) Galore-Haskel et al. (2015), (**35**) Gaziel-Sovran et al. (2011), (**36**) Georgantas et al. (2013), (**37**) Giles et al. (2013), (**38**) Glud et al. (2010), (**39**) Golan et al. (2015), (**40**) Goswami et al. (2015), (**41**) Greenberg et al. (2014), (**42**) Greenberg et al. (2011), (**43**) Grignol et al. (2011), (**44**) Guo et al. (2014), (**45**) Haflidadottir et al. (2010), (**46**) Hao et al. (2015), (**47**) He et al. (2007), (**48**) Heinemann et al. (2012), (**49**) Holst et al. (2011), (**50**) Hwang et al. (2014), (**51**) Igoucheva and Alexeev (2009), (**52**) Jian et al. (2014), (**53**) Jiang et al. (2014), (**54**) Jiang et al. (2012), (**55**) Jin et al. (2011), (**56**) Kappelmann et al. (2013), (**57**) Karreth et al. (2011), (**58**) Kim et al. (2014a, b), (**59**) Kitago et al. (2009), (**60**) Knoll et al. (2014), (**61**) Kozubek et al. (2013), (**62**) Kwon et al. (2015), (**63**) Lankenau et al. (2014), (**64**) Levati et al. (2009), (**65**) Levati et al. (2011), (**66**) Levy et al. (2010a, b), (**67**) Li et al. (2014a, b), (**68**) Li (2016), (**69**) Li et al. (2014a, b), (**70**) Lin et al. (2015a), (**71**) Lin et al. (2015b), (**72**) Liu et al. (2015a, b), (**73**) Liu et al. (2015a, b), (**74**) Liu et al. (2013), (**75**) Liu et al. (2012a), (**76**) Liu et al. (2012b), (**77**) Liu et al. (2014), (**78**) Lodygin et al. (2008), (**79**) Lujambio et al. (2008), (**80**) Luo et al. (2013a), (**81**) Luo et al. (2013b), (**82**) Ma et al. (2009), (**83**) Margue et al. (2013), (**84**) Martin del Campo et al. (2015), (**85**) Mattia et al. (2011), (**86**) Mazar et al. (2010), (**87**) Mazar et al. (2011), (**88**) Mazar et al. (2016), (**89**) Migliore et al. (2008), (**90**) Mueller and Bosserhoff (2009), (**91**) Mueller and Bosserhoff (2011), (**92**) Mueller et al. (2009), (**93**) Muller and Bosserhoff (2008), (**94**) Nemlich et al. (2013), (**95**) Nguyen et al. (2011), (**96**) Noguchi et al. (2014a), (**97**) Noguchi et al. (2014b), (**98**) Noguchi et al. (2012a), (**99**) Noguchi et al. (2015), (**100**) Noguchi et al. (2012b), (**101**) Noman et al. (2012), (**102**) Nyholm et al. (2014), (**103**) Ohira et al. (2015), (**104**) Ozsolak et al. (2008), (**105**) Pencheva et al. (2012), (**106**) Penna et al. (2011), (**107**) Penna et al. (2013), (**108**) Philippidou et al. (2010), (**109**) Pinto et al. (2015), (**110**) Poliseno et al. (2012), (**111**) Prasad and Katiyar (2014), (**112**) Reuland et al. (2013), (**113**) Sakurai et al. (2011), (**114**) Saleiban et al. (2014), (**115**) Sand et al. (2012a, b), (**116**) Sand et al. (2013), (**117**) Satzger et al. (2010), (**118**) Satzger et al. (2012), (**119**) Schmitt et al. (2012), (**120**) Schultz et al. (2008), (**121**) Segura et al. (2010), (**122**) Segura et al. (2009), (**123**) Shoshan et al. (2015), (**124**) Stark et al. (2015a, b), (**125**) Streicher et al. (2012), (**126**) Strong et al. (2014), (**127**) Tian et al. (2015), (**128**) van Kempen et al. (2012), (**129**) Venza et al. (2015), (**130**) Vergani et al. (2016), (**131**) Wang et al. (2013a), (**132**) Wang et al. (2013b), (**133**) Wei et al. (2014), (**134**) Xu et al. (2012), (**135**) Yamazaki et al. (2012), (**136**) Yang et al. (2015), (**137**) Yang et al. (2011), (**138**) Zehavi et al. (2012), (**139**) Zehavi et al. (2015), (**140**) Zhang et al. (2014), (**141**) Zhang et al. (2015b), (**142**) Zhang et al. (2006), (**143**) Zhang et al. (2009), (**144**) Zhang et al. (2015c), (**145**) Zhao et al. (2015), (**146**) Zhou et al. (2015)

allele for melanoma susceptibility, previously determined to be located on chromosome 1p22 (Gillanders et al. 2003; Walker et al. 2004). It turned out that this genomic locus encodes a miRNA – miR-137 – capable of negatively regulating MITF expression (Bemis et al. 2008). Interestingly, the miR-137 primary transcript harbors a significantly higher number of a 15 bp VNTR (variable number of tandem repeats) in a subset of melanoma cell lines displaying high MITF expression than it does in melanoma cell lines displaying low levels of MITF. Those extra copies of the 15 bp VNTR alter the pri-miR-137 secondary structure in a way precluding its further processing into mature miR-137 (Bemis et al. 2008). Strikingly, the re-expression of functionally active miR-137 in those cell lines resulted in a decline of MITF levels.

As already suggested in the report cited above, it meanwhile turned out that several other miRNAs are involved in regulating MITF as well. Also based on a melanoma associated genomic aberration – amplification of chromosomal locus 7q31-34, which harbors the *c-MET* and *BRAF* oncogenes – Segura et al. (2009) detected enhanced expression of miR-182 in melanoma cell lines and tissue samples. MiR-182 levels increased with tumor progression and were additionally inversely correlated with the expression of MITF and FOXO3 (a transcription factor of the forkhead family). Both transcription factors were confirmed to be direct targets of miR-182. Furthermore, it was shown that for the expression of this miRNA the migration of melanoma cells in vitro as well as their metastatic potential in vivo, whereas silencing of miR-182 impedes melanoma cell invasion and triggers apoptosis (Segura et al. 2009). The stimulatory effect of miR-182 over-expression on melanoma cell migration was abolished by the concomitant over-expression of MITF or FOXO3, making those proteins the key mediators of miR-182's effects.

Interestingly, it has been reported that cancer cells can circumvent the miRNA-mediated regulation of oncogenes to which they are addicted, by expressing transcripts with shortened 3′UTRs (Mayr and Bartel 2009). Thereby, selective pressure favors cells that generate alternative transcripts of oncogenes missing the miRNA target sequence(s) in their 3′UTR. Prompted by those findings, Goswami et al. (2015) unraveled that a specific MITF isoform, harboring a shortened 3′UTR, is preferentially expressed by their set of melanoma cell lines in contrast to normal melanocytes. This alternative MITF transcript lacks the miR-137 and miR-182 binding sites but yet contains two target sequences for another miRNA, miR-340 (Goswami et al. 2015). The authors were able to show that miR-340 actually inhibits the expression of the alternative MITF transcript in their melanoma cells. Even more remarkable, a protein commonly over-expressed in melanoma termed CRD-BP (coding region determinant binding protein; Elcheva et al. 2008) is able to bind to the alternative MITF 3′UTR, thereby masking the miR-340 target sites and restoring MITF expression (Goswami et al. 2015). On a functional level, either the inhibition of miR-340 or the over-expression of CRD-BP resulted in a significantly enhanced ability of melanoma cells to form colonies in soft agar.

In addition to these findings, Haflidadottir et al. (2010) attempted to perform a comprehensive analysis on miRNA binding sites located in the MITF full length 3′UTR. Although not paying attention to a potential alternative 3′UTR shortening, they identified miR-148 and miR-101 as additional regulators of MITF expression

in melanoma cell lines (Haflidadottir et al. 2010). In addition, miR-218, previously shown to be deregulated (Philippidou et al. 2010), miR-101, and miR-155 were revealed to directly modulate MITF expression (Guo et al. 2014; Luo et al. 2013a, b; Arts et al. 2015). Interestingly, miR-155, which is down-regulated in melanoma cells compared to melanocytes, is an example of a miRNA exerting a multilevel regulation over a signaling pathway, the WNT pathway, making it an attractive candidate as a therapeutic weapon. MiR-155 also interacts with the 3′UTR of the transcriptional regulator SKI, reducing its levels when exogenously expressed (Levati 2011). SKI is an important WNT/β-catenin inducer, leading to high MITF expression. Therefore, MITF should also be indirectly regulated by miRNAs that act upon WNT signaling, such as miR-155. Another indirect regulator of MITF is miR-203, which down-regulates MITF and its downstream effector Rab27a in melanoma cell lines by targeting its promoting transcription factor CREB1 (cAMP response element-binding protein 1) (Noguchi et al. 2014a, b).

MiRNAs can also mediate environmental effects via key transcription factors as MITF in melanocytes. Dynoodt et al. (2013a, b) observed a dramatic 15-fold decrease of miR-145 in UV-irradiated and forskolin treated mouse melanocytes. Over-expression or knockdown of this miRNA, respectively, decreased and increased the levels of MITF (among other proteins) and had a direct impact on the pigmentary process.

Taking all these findings into consideration, the question is how miRNA-mediated regulation impacts MITF expression in melanomagenesis. Reports on the role of MITF in formation and progression of malignant melanoma had initially been controversial. Nowadays, a model is proposed in which an intermediate level of MITF expression favors the proliferation and tumorigenic potential of melanoma cells, while a too high level results in cell cycle arrest and differentiation and a too low level results in cell cycle arrest and apoptosis (see Chaps. 4 and 5, and references therein). Although the expression of MITF has already been shown to be regulated through a plethora of mechanisms (including e.g., transcriptional control and post-translational modifications), miRNAs as well as 3′UTR binding proteins (acting as “target protectors”) seem to represent an additional layer of complexity in adjusting MITF levels. Most likely, in melanoma cells the favored outcome “intermediate MITF level” can be achieved by the deregulation of every single wire (or a combination of several wires) in the MITF network. Hereby, the deregulation of miRNA-based mechanisms is one way to adjust MITF at the preferred level or to fine-tune it, respectively. This may also provide additional insights into melanoma’s heterogeneity regarding MITF expression (reviewed by Bell and Levy 2011).

However, MITF is not only subject to regulation by miRNAs – vice versa, MITF regulates the expression of a subset of miRNAs in melanoma cell lines and may even influence global miRNA expression in cells of the melanocytic lineage. Ozsolak et al. (2008) identified the putative transcription start sites of as much as 175 miRNAs by combining nucleosome positioning patterns with chromatin immunoprecipitation (ChIP) screens for promoter signatures. This report significantly contributed to our current knowledge on the characteristics of miRNA promoters. Furthermore, the authors utilized the obtained data to identify a subset of 10

miRNAs and miRNA clusters, respectively, whose expression is MITF regulated by screening miRNA promoters for E-box elements (the binding sites for MITF in gene promoters; see Chap. 5). The cohort of miRNAs identified to be MITF transcriptional targets includes, e.g., miR-148b, miR-221/222, the miR-106a-363 cluster, miR-125, as well as several members of the let-7 family (Ozsolak et al. 2008). Interestingly, some of these miRNAs have already been proven to target genes playing a role in pigmentation and survival of melanocytes or to be involved in melanomagenesis. In melanocyte primary cultures, Wang et al. (2012) found 16 up-regulated and 6 down-regulated miRNAs through microarray profiling in MITF knocked-down (with siRNAs) melanocytes, compared to normal melanocytes. Using the TAM annotation method (Lu et al. 2010), they found that the miR-188 family was enriched, while the miR-221 family was depleted. Both groups of miRNAs were involved in cell-cycle, cell proliferation, and stem-cell regulation, and both groups were reported to be enriched in melanoma. Thus, the effects of MITF knockdown do not recapitulate the changes of microRNA expression reported in melanoma.

As miRNAs can be localized in introns of protein-coding genes, the expression of the protein and the miRNA are highly correlated. Noteworthy, intronic miR-211 was found responsible for the tumor suppressor effect attributed to its host gene, TRMP1 (Melastatin), upon MITF-promoted expression (Levy 2010; De Luca et al. 2015). MiR-211 contributes to melanoma cell adhesion by targeting NUAK1 (Bell et al. 2014), but as new targets for this miRNA emerge, its role in melanoma as a therapeutic target or as a biomarker has to be further characterized (Margue et al. 2013).

Additionally, Levy et al. (2010b) reported that MITF is able to transcriptionally regulate Dicer expression in melanocytes. Their study started with the observation that during melanocyte differentiation a population of miRNAs was up-regulated at the pre-miR level, whereas another population of miRNAs displayed stabile pre-miR but yet enhanced mature miRNA levels. Subsequently, it turned out that MITF is capable of inducing Dicer transcription by interacting with two E-box elements in the Dicer promoter, thus enforcing pre-miRNA processing (Levy et al. 2010a, b). Interestingly, melanocyte-specific knockout of Dicer in an in vivo mouse model resulted in a profound loss of both melanocyte stem cells and differentiated melanocytes. The authors assigned this impressive impact of Dicer on melanocyte survival to its ability to enhance the expression of miRNAs of the miR-17–92 cluster. Hereby, miR-17 targeting the pro-apoptotic Bim (Bcl-2 interacting mediator of cell death) protein may play a major role (Levy et al. 2010a, b) and further links the MITF-mediated regulation of Dicer to mechanisms involved in melanomagenesis.

Interestingly, further miRs regulating MITF were identified in the context of vitiligo and other pigmentation disorders. Here, miR-25 and miR-675 were proven to directly control MITF (Shi et al. 2016; Kim et al. 2014b).

These findings indicate that the melanocyte-specific transcription factor MITF might exert its powerful impact on pigmentation and lineage survival genes not only by direct transcriptional mechanisms but also that the effects of MITF are further enhanced by miRNAs as intermediates. Obviously, this will also enhance – or even potentiate – its oncogenic properties in melanoma formation and progression.

6.2.2 Oncogenic Role of miR-221/222 in Melanoma

Clustered on the X-chromosome, miR-221 and miR-222 are thought to be transcribed as a common precursor and to regulate overlapping or functionally related targets. Reports on an over-expression of these miRNAs in several types of tumors in which they regulate the expression of the c-KIT receptor tyrosine kinase (e.g., Felli et al. 2005; He et al. 2005; le Sage et al. 2007) led Felicetti et al. (2008b) to investigate a potential involvement of miR-221/222 in melanomagenesis (for further information on c-KIT in melanoma please refer to Chap. 7). They unraveled that loss of the tumor suppressive protein PLZF (promyelocytic leukemia zinc finger) leads to a stepwise up-regulation of miR-221/222 during melanoma progression (Felicetti et al. 2008a, b). These two miRNAs in turn are able to induce two distinct but functionally convergent oncogenic pathways by repressing c-KIT as well as $p27^{Kip1}$ (CDKN1B). In accordance, the over-expression of miR-221/222 in a moderately aggressive melanoma cell line resulted in an enhancement of proliferation rate, migratory and invasive potential, as well as anchorage-independent growth, paralleled by a significantly increased in vivo tumorigenicity (Felicetti et al. 2008a, b). Vice versa, the treatment of highly aggressive metastatic melanoma cells with inhibitory molecules (antagomiRs) against miR-221/222 not only caused obverse effects in vitro but – most strikingly – effectively inhibited in vivo tumor growth after bolus intratumor injection.

Expanding those findings, Igoucheva and Alexeev (2009) implicated that loss of c-KIT receptor expression in melanoma might be completely depending on miR-221/222 over-expression instead of being related to the down-regulation of the transcription factor AP-2 (see Chap. 5) as previously reported. This conclusion was drawn as there was no correlation detectable between AP-2 and c-KIT expression levels in their set of 27 melanoma cell lines but a strong inverse correlation of c-KIT and miR-221 expression levels was observed (Igoucheva and Alexeev 2009).

Including data generated in other tumor types, Howell et al. (2010) constructed an interesting network focusing on miR-221/222's regulatory effects in melanoma. It has been demonstrated that miR-221/222 regulate the expression of PTEN in aggressive non-small lung cancer and hepatocarcinoma cells (Garofalo et al. 2009). In the light of an increasingly important role of the AKT/PI3K pathway in melanomagenesis as well as a confusingly low rate of PTEN mutations detected in melanoma tissue samples (see Chap. 7), studies investigating a miRNA-mediated regulation of PTEN seem to be a promising option.

Additionally, Das et al. (2010) reported the existence of a $3' \rightarrow 5'$ exonuclease, human polynucleotide phosphorylase ($hPNPase^{old\text{-}35}$), specifically degrading mature miR-221 in melanoma cells. In response to the over-expression of $hPNPase^{old\text{-}35}$, the cellular miR-221 level decreased and its repressive impact on $p27^{Kip1}$ expression was abolished (Das et al. 2010). As $hPNPase^{old\text{-}35}$ is a type I interferon (IFN) inducible protein, the authors were able to unravel a pathway in which IFN-β-mediated growth arrest is based on induction of $hPNPase^{old\text{-}35}$, which subsequently degrades miR-221 and therefore triggers the up-regulation of $p27^{Kip1}$. Of note, $hPNPase^{old\text{-}35}$ had previously been shown to degrade c-Myc mRNA, thereby causing G_1 cell cycle arrest by $p27^{Kip1}$ activation involving another pathway (Sarkar et al. 2003, 2006).

Nevertheless, the over-expression of miR-221 in melanoma cells was sufficient to render them resistant against IFN-β-mediated growth arrest (Das et al. 2010).

In conclusion, miR-221/222 have already been established to be potent oncogenic miRNAs in melanoma. This is further supported by newer findings even suggesting miR-221/222 as potent tumor markers for melanoma (Inada et al. 2015; Felli et al. 2016). Several mechanisms are involved in regulating their expression, including transcriptional control by PLZF (Felicetti et al. 2008a, b) and MITF (Ozsolak et al. 2008), as well as positive and negative feedback loops involving their target genes (reviewed by Howell et al. 2010) and specific exonucleolytic degradation by $hPNPase^{old-35}$ (Das et al. 2010). Although the up-regulation of miR-221/222 may not be the sole cause for loss of c-KIT in melanoma cells, miRNA-mediated c-KIT repression could serve as an additional mechanism to escape c-KIT dependent apoptosis in a subset of melanomas harboring activating mutations in BRAF (Igoucheva and Alexeev 2009). Further, miR-221/222 over-expression may be responsible for resistance against IFN-β treatment (Becker et al. 2002) in at least some melanomas (Das et al. 2010).

6.2.3 Tumor-Suppressive Role of let-7 miRNA Family Members in Melanoma

One of the major breakthroughs regarding miRNAs in biomedical research took place in 2005 when Frank Slacks' group reported a causal link between the down-regulation of miRNA let-7 and the formation of lung cancer (Johnson et al. 2005). Thereby, the tumor-suppressive effect of let-7 miRNA family members in lung carcinomas is based on their potential to regulate the expression of the RAS oncogene. Strikingly, it meanwhile turned out that loss of the expression of at least some let-7 family members seems to be a common event in the progression of several types of human cancers (reviewed by Boyerinas et al. 2010 – and that this is true for melanoma as well).

Performing an expression pattern analysis on 157 different miRNAs in laser-microdissected tissues comprising ten benign melanocytic nevi and ten primary melanomas, Schultz et al. (2008) found five members of the let-7 family (let-7a, let-7b, let-7d, let-7e, and let-7g) to be strongly down-regulated in the melanoma samples. Focusing only on let-7b in their subsequent experiments, they showed that introducing artificial let-7b molecules into melanoma cells decreased levels of a set of cell cycle regulators, in detail cyclins D1, D3, and A as well as cyclin-dependent kinase (CDK) 4 (Schultz et al. 2008). While the authors were able to give some evidence for a direct interaction of let-7b with the cyclin D1 3′UTR, they also point out that at least a few of the remaining let-7b effects described might be rather indirect. Nevertheless, the transfection of melanoma cell lines with artificial let-7b resulted in a reduced number of proliferating cells (less cells were detected to be in S-phase, accompanied with an increased number of cells in G_1) as well as a strong reduction of the cells' potential to form colonies in soft agar (Schultz et al. 2008).

Almost synchronously, Mueller and Bosserhoff (2008) reported loss of expression of a second member of the let-7 miRNA family – let-7a – to have a potent

impact on the invasive potential of melanoma cells. Analyzing the regulation of integrin beta3 expression in detail, they found that (in contrast to other cell types) promoter-dependent mechanisms are only subsidiary in melanoma cells whereas the cellular let-7a level is the main determinant of integrin beta3 protein production (Muller and Bosserhoff 2008). Strikingly, the introduction of artificial let-7a molecules into a highly aggressive melanoma cell line lacking endogenous let-7a expression resulted in an about 75 % reduced invasive potential in vitro. Vice versa, blocking endogenous let-7a in otherwise normal melanocytes using anti-miRs was sufficient to induce migratory behavior in this cell-type (Muller and Bosserhoff 2008). The authors attributed these effects mainly to let-7a's impact on integrin beta3, as the up-regulation of the classical vitronectin receptor alphaVbeta3 has repeatedly been shown to be correlated with malignant potential and aggressive tumor growth in melanoma (Albelda et al. 1990; Danen et al. 1994, 1995; Van Belle et al. 1999). However, like presumably every miRNA known, let-7a targets a cohort of additional transcripts and this fact might strengthen or even potentiate its role in melanoma progression. Mueller and Bosserhoff (2008) highlight the suppression of N-Ras by let-7a, which they used to monitor the efficiency of let-7a transfection in their experiments. Although the significance of additional Ras knockdown is uncertain against the background of activating BRAF mutations, it might prove particularly effective in a subset of melanomas harboring activating mutations in N-Ras (see Chaps. 7 and 12).

Importantly, new sequencing studies helped to clearly differentiate the subforms of let-7 and revealed the regulation of all of these in melanoma (Kozubek et al. 2013; Qi et al. 2014).

Corroborating findings in other tumor types, members of the let-7 family of miRNAs take on a role as potent tumor suppressors in malignant melanoma as well. The complete set of let-7 target genes still remains to be established to identify the most important mediators of let-7's effects. Nevertheless, already at this point, the most intriguing fact about let-7 is its ability to interfere with constituents of several distinct pathways involved in malignant transformation of cells in general and in melanomagenesis in special.

6.2.4 Tumor-Suppressive Role of miR-196a in Melanoma

Another miRNA confirmed to act as a tumor suppressor in malignant melanoma cells is miR-196a. Thereby, it currently seems that this miRNA exerts much of its function by regulating the expression of at least two class I homeodomain containing transcription factors, HOX-B7 and HOX-C8 (Braig et al. 2010; Mueller and Bosserhoff 2010a).

The expression of miR-196a has been shown to be strongly reduced in melanoma cell lines as well as in a small set of tissue samples derived from primary cutaneous melanomas and melanoma metastases, respectively (Braig et al. 2010; Mueller and Bosserhoff 2010a). In line with general reports characterizing miR-196a as a potent regulator of HOX gene expression (e.g., Yekta et al. 2004), reduced

miR-196a levels inversely correlate with strongly enhanced the expression of the HOX transcription factors HOX-B7 and HOX-C8 in melanoma cells (Braig et al. 2010; Mueller and Bosserhoff 2010a). 3′UTR reporter assays confirmed that miR-196a is actually capable to directly interact with the HOX-B7 (Braig et al. 2010) and HOX-C8 (Mueller and Bosserhoff 2010a) transcripts, thereby preventing their translation into protein.

Due to these efforts, Braig et al. (2010) were able to link loss of miR-196a to the over-expression of BMP4 (bone morphogenetic protein 4), an event strongly enhancing the migratory potential of melanoma cells (Hsu et al. 2005; Rothhammer et al. 2005, 2008). In their model proposed, diminished levels of miR-196a cause induction of the transcription factor HOX-B7, which in turn activates bFGF (basic fibroblast growth factor) production. In the end, this results in an enforced expression of BMP4, most likely mediated by transcription factors of the Ets family (Braig et al. 2010). Additionally, Mueller and Bosserhoff (2010a) demonstrated that miR-196a indirectly modulates the expression of several genes potentially involved in melanoma progression by regulating HOX-C8 levels. Many of the HOX-C8 target genes described in literature are related to oncogenic processes like cell adhesion, cytoskeleton remodeling, tumor formation, and invasive behavior of tumor cells (Lei et al. 2005, 2006). The authors demonstrated that at least three of them – *cadherin-11*, *calponin-1*, and *osteopontin* – appear to be deregulated in melanoma cells partially due to the over-expression of HOX-C8 (Mueller and Bosserhoff 2010a).

In the course of the studies cited, melanoma cell clones were generated, which stably re-express miR-196a at a level almost equal to that detected in normal melanocytes (Braig et al. 2010; Mueller and Bosserhoff 2010a). Remarkably, those cells were characterized by an about 50 % and 40 % reduction in their migratory (Braig et al. 2010) and invasive potential, respectively, as well as by diminished tumorigenicity after subcutaneous injection into nude mice as shown in a preliminary experimental setup (Mueller and Bosserhoff 2010a). Vice versa, the treatment of otherwise normal melanocytes with anti-miRs against miR-196a was sufficient to induce migratory behavior in this cell type (Braig et al. 2010).

As true for miR-221/222 and let-7, there is a need to extend the set of known, melanoma-relevant miR-196a target genes to eventually draw a complete picture of the molecules mediating this miRNAs' effects on melanomagenesis. This could be of importance as newer studies suggest a link of an SNP in miR196a to levels of intracellular reactive oxygen species and sensitivity to apoptotic signals (Huang et al. 2013). It also seems like the deregulation of HOX protein levels might well be worth a detailed examination as several studies clearly defined a link between aberrant *HOX* gene expression and cancer development (reviewed by Piérard and Piérard-Franchimont 2012; Shah and Sukumar 2010). Interestingly, a report highlighting miR-196 family members as metastasis suppressors in breast cancer (Li et al. 2010) additionally strengthens the tumor-suppressive role of miR-196a in malignant melanoma. By contrast, detection of increased miR-196a levels in other types of tumors when compared to their healthy biological correlates (e.g., Bloomston et al. 2007; Luthra et al. 2008; Maru et al. 2009; Schimanski et al. 2009) clearly point toward a tissue-specific role of this miRNA. Considering its targets,

the *HOX* genes, as “tumor modulators” harboring the potential to act as both oncogenes and tumor suppressor genes depending on the respective tissue context (Abate-Shen 2002), seem to be rather plausible but add a further level of complexity to this area of research.

6.2.5 Tumor-Suppressive Role of the miR-34 Family in Melanoma

Recently, several reports conclusively demonstrated that the p53 tumor suppressor protein directly induces the expression of miR-34 miRNA family members and probably exerts a significant portion of its expansive impact on cell-cycle control and regulation of apoptosis through these mediators (reviewed by Hermeking 2010). Accordingly, the ectopic expression of miR-34 genes has profound effects on cell proliferation and survival causing cell-cycle arrest in G1 phase, inhibiting colony formation in soft agar, as well as inducing cellular senescence and apoptosis, respectively (Bommer et al. 2007; Chang et al. 2007; Corney et al. 2007; He et al. 2007; Raver-Shapira et al. 2007; Tarasov et al. 2007; Welch et al. 2007). Therefore, genes involved in cell-cycle regulation and apoptosis control are significantly enriched in the large set of confirmed miR-34 targets comprising *Bcl-2*, *CCND1*, *CCNE2*, *CDK2*, *CDK4*, *CREB*, *MET*, *MYC*, *SIRT1*, and many more (for a comprehensive list, see Hermeking 2010). Some of the studies defining this potent onco-suppressive role of miR-34 members in various tumor entities also include data regarding melanoma (Lodygin et al. 2008; Lujambio et al. 2008; Migliore et al. 2008). Newer data revealed miR-34a/c to regulate the NK2D ligand ULBP2 and thereby modulate tumor cell recognition by NK cells (Heinemann et al. 2012). The following paragraph aims to summarize these findings and recombine them with results generated in other cancer types to present a perspective on the possibly far-reaching but yet underestimated influence of miR-34 on melanomagenesis.

In humans, the miRNAs of the miR-34 family are encoded by two different loci with one locus encoding miR-34a and the other locus encoding a common precursor for miR-34b/c. The high similarity shared by all three mature miR-34 forms generates points to an overlapping although not completely identical repertoire of target transcripts. Lodygin et al. (2008) demonstrated that the expression of miR-34a is commonly silenced in a variety of tumors as a consequence of promoter hypermethylation. This is also true for melanoma cells as 19 of 44 (43.2 %) investigated melanoma cell lines as well as 20 of 32 (62.5 %) primary melanomas investigated displayed hypermethylation of the miR-34a promoter resulting in loss of miR-34a pri-miRNA expression (Lodygin et al. 2008). CpG methylation presumably occurs at a region 100–500 bp upstream of the miR-34a transcription start site which also includes the p53 binding site. By contrast, normal melanocytes derived from two different donors did not display miR-34a promoter methylation, implicating that this mechanism represents a tumor-specific event. Interestingly, miR-34a is encoded at chromosome 1p36 – a chromosomal locus repeatedly described to harbor a gene for melanoma susceptibility, which surprisingly has not been identified to date (e.g.,

Ng et al. 2008; Poetsch et al. 2003). LOH for 1p36 had been detected in 77 % of nodular melanomas, 86 % of metastatic melanomas, but only 20 % of superficially spreading melanomas (Poetsch et al. 2003). Additionally, in neuroblastoma, a correlation between 1p36 loss and miR-34a down-regulation had been confirmed (Wei et al. 2008). It is therefore tempting to speculate, that in melanoma cells either loss of 1p36, hypermethylation of the miR-34a promoter or a combination of both mechanisms may result in loss of miR-34a and its tumor-suppressive function.

CpG island methylation had also been found to be the cause for silencing of miR-34b/c in melanoma and several other types of cancer (Kozaki et al. 2008; Lujambio et al. 2008; Toyota et al. 2008). Thereby, the study of Lujambio et al. (2008) strikingly unraveled a highly significant correlation between miR-34b/c promoter hypermethylation and the presence of lymph node metastases in a panel of melanoma tissue samples. A report published by Migliore et al. (2008) implicates that this might be due to the release of c-MET expression from miR-34b/c-mediated repression. Performing a bioinformatical search for miRNAs targeting c-MET they found miR-34b/c as well as miR-199* to be able to suppress c-MET production and therefore the ability of cancer cells to respond to HGF (hepatocyte growth factor) stimulation – even if they harbored c-MET gene amplification or over-expression resulting in high levels of constitutively active receptor (Migliore et al. 2008). In their study, melanoma-derived primary cells expressed only limited amounts of miR-34c (and therefore possibly also of miR-34b). The transfection of synthetic miR-34b/c molecules into these cells led to the down-regulation of c-MET expression as well as to impairment of c-MET-mediated motility in vitro (Migliore et al. 2008). The MET oncogene encodes a tyrosine kinase receptor which upon activation by binding its ligand HGF (also called scatter factor) activates a complex biological program which in the end results in invasive growth and metastatic dissemination of tumor cells (Birchmeier et al. 2003). In melanoma, the over-expression of c-MET has repeatedly been reported and its transcriptional activation had been linked to increased levels of SOX10, PAX3, and MITF whereas loss of Plexin B1 prevents suppression of c-MET activation by absent receptor–receptor interactions (e.g., Cruz et al. 2003; Kiriakidou et al. 2004; Mascarenhas et al. 2010; Puri et al. 2007). It is known from other tumor types that c-MET expression can also be induced during hypoxia (through binding of HIF-1α to the MET promoter; Pennacchietti et al. 2003) or activation of several oncogenes including *Ets* family members (Gambarotta et al. 1996; Ivan et al. 1997; Webb et al. 1998). While HGF is a paracrine mitogen for normal melanocytes, most melanoma cells gain the potential to produce HGF themselves thereby establishing sustained autocrine stimulation of the receptor (Li et al. 2001). Notably, correlations between c-MET expression and the metastatic potential of melanoma cells as well as patient survival have been described (Barnhill and Mihm 1993; Natali et al. 1993; Slominski et al. 2001).

These findings implicate that the loss of expression of miR-34 family members – potentially caused by multiple mechanisms (including aberrant methylation and chromosomal aberrations) – might be a central event in the progression of primary melanomas on to invasive and metastatic disease. They may additionally shed new light on the controversially disputed role of p53 in melanomagenesis. Whereas

silencing of p53 is acknowledged to be one of the most influential events in the formation of virtually all tumor types, its role in melanoma has been neglected due to a low frequency of missense mutations detected (0–10 %; reviewed by Box and Terzian 2008). However, as indicated by several studies performed in animal models, the impact of p53 on melanomagenesis should be revisited; e.g., Terzian et al. (2010) recently demonstrated that high levels of p53 prevent the conversion of nevi into malignant and metastatic melanomas. Some of the conflicting effects observed might be explained by alterations in the expression of miR-34 family members impairing p53's ability to maintain its tumor suppressive function in advanced melanomas. That is, their rapidly growing *TP-ras0/+* tumors (Terzian et al. 2010) might overcome the tumor-suppressive effect of high-level stabilized wild-type p53 by losing miR-34 expression. In summary, these observations might also harbor some exciting insights into a key aspect contributing to the pronounced chemotherapy resistance of melanoma (Soengas and Lowe 2003) as loss of miR-34 expression had been linked to resistance against apoptosis induced by p53 activating agents (Zenz et al. 2009; Yamasaki et al. 2012).

6.2.6 Other miRNAs with a Functional Impact on Melanomagenesis

6.2.6.1 MiR-193b

Profiling the expression of 470 miRNAs in a set of formalin-fixed paraffin-embedded (FFPE) specimens derived from eight benign nevi and eight metastatic melanomas, Chen et al. (2010) detected 31 miRNAs to be differentially expressed in the melanoma samples (13 up-regulated, 18 down-regulated miRNAs). The authors selected the miRNA most strongly down-regulated, miR-193b, for a more detailed functional analysis. The introduction of artificial miR-193b molecules into three different melanoma cell lines resulted in a reduction of their proliferative capacity of between 30 and 60 % marked by an increased number of cells in G1 phase whereas the fractions of cells detected to be in S phase or G2 phase decreased (Chen et al. 2010). Combining whole genome cDNA microarray profiling and bioinformatic miRNA target prediction the authors identified cyclin D1 (CCND1) as a potential target for miR-193b. Subsequent 3′UTR reporter assays confirmed miR-193b to be a further miRNA targeting CCND1 in melanoma – in addition to let-7b (see above). The same group followed up the finding on the deregulation of miR-193b and revealed Stathmin1, a regulator of microtubule dynamics, and MCL1 (myeloid cell leukemia sequence 1) to be a direct target (Chen et al. 2013).

6.2.6.2 miR-532-5p

Members of the Runt-related (RUNX) family of transcription factors function as scaffolds interacting with co-regulatory factors often involved in tissue differentiation (Javed et al. 2005). Driven by findings on a tumor-suppressive role of RUNX3 in several types of tumors, Kitago et al. (2009) detected a significant down-regulation of this transcription factor on mRNA level in their set of 82 primary melanomas and 41 melanoma metastases as compared to 12 normal skin samples. Thereby, RUNX3

down-regulation displayed a nonlinear association with AJCC stage of the tumors with AJCC stage IV classified metastatic melanomas showing an even stronger reduction of RUNX3 expression compared to primary tumors. Interestingly, multivariate analysis revealed that the down-regulation of RUNX3 mRNA was related to disease outcome in those patients (Kitago et al. 2009). In contrast to tumors derived from other tissues (Ito 2004), loss of RUNX3 expression was not due to promoter hypermethylation in the melanoma samples but seems to be associated with increased expression of miR-532-5p. Kitago et al. (2009) detected this miRNA to be significantly over-expressed in their metastatic cell lines compared to melanocytes as well as in the metastatic melanoma samples compared to primary tumors pointing toward an enforced expression of miR-532-5p as progression to metastatic disease occurs. While the transfection of anti-miRs against miR-532-5p into a metastatic melanoma cell line resulted in the increased expression of RUNX3 on mRNA and protein level, a direct interaction of the miRNA with a target site in the RUNX3 3′UTR has not yet been confirmed. Another interesting fact is that miR-532-5p is actually encoded at Xp11.23 which is located next to Xp11.3 – the genomic locus encoding miR-221/222 (see above).

6.2.6.3 miR-155

Levati et al. (2009) analyzed several miRNAs known to be involved in oncogenic processes for their expression in a panel of melanoma cell lines. In the majority of cell lines used, they detected an enhanced expression of miRNAs belonging to the miR-17-92 cluster – miR-17-5p, miR-18a, miR-20a, and miR-92a – as well as a diminished expression of miR-146a, miR-146b, and miR-155 compared to normal melanocytes (Levati et al. 2009). As miR-155 down-regulation was most prominent, the authors decided to examine this miRNA's impact on melanoma cell function. The transfection of artificial miR-155 molecules into melanoma cells inhibited proliferation by between 30 and 98 % in 12 out of 13 cell lines tested. Subsequent experiments performed in four of the cell lines implicated that this impairment of melanoma cell proliferation was at least partly based on induction of apoptosis in miR-155 transfected cells, further they revealed the regulation of the transcriptional repressor SKI (Levati et al. 2009; Levati et al. 2011). Reports on other types of tumors point towards a tissue-specific function of miR-155 ascribing the latter oncogenic as well as a tumor-suppressive potential depending on cellular context (e.g., Gartel and Kandel 2008; Volinia et al. 2006). The functional impact of miR-155 down-regulation on melanomagenesis remains to be defined as it appears to be only weakly expressed already in normal melanocytes. MiR-155 was recently shown to regulate MITF (Arts et al. 2015; see Sect. 6.2.1) and TYRP1 (El Hajj et al. 2015) shedding some light on its potential function. In addition, miR-155 was associated with patient outcome and might therefore be of prognostic potential (Jayawardana et al. 2015).

6.2.6.4 miR-210

Zhang and co-workers reported a correlation between elevated pri-miR-210 levels in melanoma tissues and shorter metastasis-free survival of the patients in Kaplan–Meier analysis (Zhang et al. 2009). MiR-210 is the miRNA species most prominently

induced during hypoxia (reviewed by Huang et al. 2010). In their study, Zhang et al. (2009) demonstrated that the over-expression of this miRNA overrides hypoxia-induced cell-cycle arrest by indirectly activating the cell cycle and metabolic regulator MYC via repression of its antagonist MNT in several tumor cell lines. It was therefore hypothesized that miR-210 is involved in the regulation of mitochondrial biogenesis and central carbon metabolism to adapt tumor cells to hypoxic episodes and to further provide them a competitive advantage in developing tumors once normal oxygen levels are restored (Morrish 2009). Hypoxia and HIF signaling have also been shown to be implicated in melanomagenesis and melanoma metastasis (reviewed by Bedogni and Powell 2009), especially as a potential constitutive HIF-1 activity has been described for melanoma (Kuphal et al. 2010). Unfortunately, besides the Kaplan–Meier analysis cited above, no data regarding melanoma cells is provided by Zhang and colleagues which performed all their experiments in cell lines derived from other tumor types. However, Satzger et al. (2010) detected significantly enhanced the expression of miR-210 in 16 primary melanoma samples as compared to 11 melanocytic nevi investigated. miR-210 expression did not correlate with recurrence-free survival or overall survival in an extended set of 112 primary tumors (Satzger et al. 2010). Additionally, considering a currently disputed bipolar effect of hypoxia and miR-210 on tumorigenesis and a modulation of tumor cell susceptibility to lysis by antigen-specific cytotoxic T cells (Huang et al. 2009; Noman et al. 2012), a lot more research will be necessary to define the specific impact of miR-210 on the progression of this disease. Recently, miR-210 was clearly associated with early recurrence and may be useful as a diagnostic marker (Ono et al. 2015).

6.2.6.5 miR-125b

Several studies revealed a strong downregulation of miR-125b expression in melanoma (Glud et al. 2010). Based on these studies, multiple effects on melanoma cells have been shown, for example, Kappelmann et al. (2013) described a regulation of c-Jun by miR-125b. Here, the regulative effect can only be found on protein level hinting at a post-transcriptional regulation by modulation translation of c-Jun. Further, Mixed linked kinase 3 (MLK3) was proven to be regulated by miR-125b and thereby induced in melanoma, supporting cell growth and invasion (Zhang et al. 2014). Nyholm et al. (2014) could implicate miR-125b in the induction of senescence, thereby further confirming that the lack of miR-125b in melanoma is implicated in tumor development.

6.2.6.6 miR-21

A recent review by Melnik (2015) summarized the importance of miR-21 in melanoma. In contrast to most described and previously analyzed miRs, miR-21 is upregulated in melanoma and associated with progression (Grignol et al. 2011; Satzger et al. 2012). A functionally regulation of melanoma cell apoptosis but also a probably even more important regulating of invasive processes were demonstrated (Satzger et al. 2012; Yang et al. 2015; Martin del Campo et al. 2015). Here, FBXO11 and TIMP3 were shown to be direct targets of miR-21. Interestingly, NRAS and BRAF mutations are associated with miR-21 induction (Melnik 2015).

6.2.6.7 Additional Deregulated miRNAs

In Table 6.1, all miRNAs are listed, where deregulation was confirmed by more than one group and target genes have been identified. Additionally, miR-101, miR-106a, miR-106b, miR-107, miR-130a, miR-135b, miR-142, miR-149*, miR-150, miR-15b, miR-17, miR-18a, miR-1826, miR-185, miR-19a, miR-19b, miR-195, miR-199a, miR-1908, miR-20a, miR-21, miR-210, miR-212, miR-224, miR-25, miR-29a, miR-301a, miR-3151, miR-335, miR-340, miR-373, miR-378, miR-432, miR-509-3p, miR-514a, miR-532-5p, miR-638, miR-93 were described as upregulated and miR-10b, miR-125a, miR-125b, miR-126*, miR-127-3p, miR-130a-3p, miR-130b-3p, miR-134, miR-137, miR-141, miR-143, miR-144-3p, miR-145, miR-149, miR-183, miR-184, miR-191, miR-192, miR-193a, miR-193b, miR-194, miR-196a, miR-200b, miR-203, miR-204, miR-205, miR-218, miR-23b, miR-23b-3p, miR-26a, miR-27b, miR-29c, miR-30b, miR-30a-3p, miR-31, miR-33a, miR-34b, miR-34c, miR-376a/c, miR-429, miR-451a, miR-455-3p, miR-455, miR-524, miR-573, miR-663, miR-7, miR-768-3p, miR-98, miR-99a, let-7a, let-7b, and let-7e were downregulated in melanoma (see also Fig. 6.2).

6.3 Lessons to Learn from miRNA Expression Profiling in Malignant Melanoma

In addition to analyses focusing upon the functional impact of single miRNA species on melanoma progression, several studies performing global miRNA expression profiling or determining expression patterns of miRNA subsets in melanoma cell lines and tissue samples have been conducted. In the beginning, this topic was only addressed as a part of large scale profiling studies in which few melanoma samples had been included (Blower et al. 2007; Gaur et al. 2007; Lu et al. 2005; Zhang et al. 2006; reviewed by Mueller and Bosserhoff 2009). Therefore, it had been difficult to deduce information on alterations in the miRNA profile of melanoma cells clearly distinguishing them from normal melanocytes with only these data published.

From 2008 on, research specifically aimed to identify differences in the miRNA expression patterns of normal melanocytes, nevi, and melanoma cells. The objective was to either identify single deregulated miRNAs as targets for in-depth functional characterization (Chen et al. 2010; Molnar et al. 2008; Mueller et al. 2009; Philippidou et al. 2010; Schultz et al. 2008) or to identify potential melanoma biomarkers (Caramuta et al. 2010; Glud et al. 2009; Jukic et al. 2010; Leidinger et al. 2010; Liu et al. 2009; Ma et al. 2009; Segura et al. 2010). Further, some groups analyzed limited sets of miRNAs already known to be involved in tumorigenic mechanisms to confirm their deregulated expression in melanoma as well (Levati et al. 2009; Satzger et al. 2010). Another experimental approach utilized next generation sequencing to potentially identify the complete miRNAome of the melanocytic lineage (Stark et al. 2010; discussed in detail in Mueller and Bosserhoff 2010b). The data obtained during these studies can be exploited in different ways. On the one hand, the deregulated expression of miRNAs that had already been

functionally characterized in melanoma before can be confirmed in larger sets of cell lines or tissue samples, as well as new candidates for functional analysis can be identified depending on the degree of deregulation detected. On the other hand, miRNA classifiers suited for diagnostic and/or prognostic purposes could be created – based less presumably on a single miRNA but rather on a larger set of miRNAs. Ding et al. (2015) focused on 21 miRNAs that were up-regulated and 42 that were down-regulated in a metastatic as well as in a nonmetastatic melanoma cell line compared to a melanocyte cell line. These included miR-148-3p, miR-129-5p, miR-34-5p, miR-363-3p, and miR-374b-5p, and the networks constructed for their putative target genes were related with cell morphology, cell development, and DNA replication. They also discovered a novel miRNA to be annotated (Ding 2015). Nevertheless, Kozubek et al. (2013) observed a consistent lower expression of miR-203, miR-204-5p, miR-205-5p, miR-211-5p, miR-23b-3p, miR-26a-5p, and miR-26b-5p in human melanoma biopsies compared to nevi. Furthermore, miR-211-5p expression levels could accurately distinguish invasive melanoma, in situ melanoma, and dysplastic nevi from common nevi in a discovery cohort using Next Generation Sequencing, (NGS) and in an independent validation cohort using qRT-PCR. Importantly, this signature differed significantly from the one discriminating between melanoma cell lines and melanocytes (Kozubek 2013), stressing the importance and feasibility of this approach with archived FFPE specimens matched to invaluable clinical information. The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov/) has recently released data on microRNA expression in melanoma, which, together with the genomic alterations through DNA, RNA, and protein-based analysis of 333 primary and/or metastatic melanomas, has allowed classification of cutaneous melanomas into four categories (TCGA network 2015).

6.3.1 Confirmation of Deregulated Expression of Single miRNAs and Identification of New miRNA Candidates for Functional Characterization

The mis-expression of many of the miRNAs discussed in Sect. 6.2 was confirmed in independent sample sets during profiling studies highlighting their importance in melanoma progression (data included in Table 6.1). For example, down-modulation of let-7a in melanoma tissues has repeatedly been detected. In summary, those findings point to a model in which normal skin melanocytes as well as benign nevi show the high expression of let-7a which then successively gets diminished during melanoma progression with melanoma metastases displaying the lowest let-7a levels (Chen et al. 2010; Glud et al. 2009; Ma et al. 2009; Philippidou et al. 2010). Further, a reduced expression of let-7b (Chen et al. 2010) and miR-196a (Caramuta et al. 2010; Philippidou et al. 2010) as well as an enhanced expression of miR-221/222 (Mueller et al. 2009; Philippidou et al. 2010) has concurrently been observed in different sets of melanoma cell lines and tissue samples. These are examples of positive correlations, indicating that miRNA profiling studies can clearly be utilized to support and strengthen findings obtained during functional studies, which are

usually performed in vitro in rather small sets of cell lines. However, microarray data should be taken into account with care unless validated by qRT-PCR or Northern blotting.

Although consistent results have been obtained in regard to specific and prominent aberrations in miRNA expression, there are also some discrepancies, and the overall correlation between independent miRNA profiling studies in melanoma is rather poor. For instance, Caramuta et al. (2010) reported let-7b to be up-regulated in their melanoma specimens and Satzger et al. (2010) did not find miR-222 significantly up-regulated in their set of tissue samples. Further, Caramuta et al. (2010) detected reduced levels of miR-15b in melanoma cells – in sharp contrast to Satzger and co-workers (2010) who detected miR-15b to be strongly up-regulated (see below). Adding to the confusion, a highly pronounced variation was observed in the expression levels of single miRNAs between melanoma cell lines and tissue samples, respectively (Caramuta et al. 2010; Philippidou et al. 2010; Satzger et al. 2010). As an example, Segura et al. (2010) found the expression of miR-155 rather up-regulated in melanoma lesions compared to benign nevi, which is in stark contrast to the functional report published by Levati et al. (2009) (see above). This caveat might be resolved by the findings from Philippidou et al. (2010) who detected miR-155 to be down-regulated in melanoma cell lines compared to normal melanocytes, but up-regulated when comparing melanoma tissue samples to benign nevi.

This, among other things, indicates that the interpretation of data obtained during miRNA profiling studies is heavily depending on the type of sample used as baseline control. As isolated melanocytes show, a high degree of variation in their miRNA expression profiles dependent not only on donor source but also on passage number (as already known from their cDNA and protein profiles), each study should include an extended set of melanocytes derived from different donors and intervals of propagation in cell culture should be adequately limited. Interestingly, the miRNA profiles generated from tissue samples seem between independent studies than do expression profiles generated from cell lines (e.g., Philippidou et al. 2010, compared to Glud et al. 2009). However, when analyzing tissue specimens most commonly benign nevi are used as baseline control and therefore serve as biological correlate for melanoma cells. Undoubtedly, nevi cells cannot be considered equivalent to normal skin melanocytes as they already harbor potentially malignant genetic alterations rendering them abnormal by definition (for a detailed discussion, see Mueller and Bosserhoff 2010b). Additionally, tissue samples are prone to contamination by nonmelanocytic cells derived from the surrounding tumor stroma. Therefore, microdissection should be established a standard in sample preparation in order to obtain reliable results.

Of note, not only the baseline control will influence interpretation of miRNA profiling data but so will data processing. Although challenging, a consensus must be aspired on normalization of miRNA microarray data as well as on a common stable reference gene for qRT-PCR based miRNA profiling methods (reviewed by Meyer et al. 2010). Moreover, as NGS setups find their way into everyday laboratory use (see also Stark et al. 2010, and related discussion by Mueller and Bosserhoff 2010b) the general debate on the best method for miRNA expression profiling is fueled further (reviewed by Git et al. 2010).

The high variability observed in the profiling studies cited above indicates that miRNAs consistently detected to be deregulated in independent sample sets might be best candidates to actually playing a central role in melanomagenesis. Indeed, some miRNAs meet this claim. The most prominent example is miR-17-5p, which together with other members of the well-characterized oncogenic miR-17-92 cluster (reviewed by Olive et al. 2010) has been reported to be up-regulated in melanoma cells (Chen et al. 2010; Levati et al. 2009; Mueller et al. 2009; Philippidou et al. 2010). Interestingly, Levy et al. (2010a, b) recently hypothesized that miR-17-mediated suppression of the proapoptotic BIM protein may play a significant role in survival of melanocytic cells in the absence of Bcl-2. Further, reduced expression of miR-200 family members which play a major role in epithelial-to-mesenchymal transition (reviewed by Bracken et al. 2009) had concordantly been demonstrated by several groups indicating a role of these miRNAs in the progression to advanced melanoma as well (Chen et al. 2010; Philippidou et al. 2010; Schultz et al. 2008). The down-regulation of miR-194 (Caramuta et al. 2010; Mueller et al. 2009) and miR-211 (Caramuta et al. 2010; Chen et al. 2010; Jukic et al. 2010) as well as the up-regulation of miR-210 (Caramuta et al. 2010; Philippidou et al. 2010) and miR-373 (Mueller et al. 2009; Philippidou et al. 2010) are further examples for deregulated expression of miRNAs not yet analyzed for their specific functions in malignant melanoma. Thereby, the role of miR-373 in melanoma might be more sophisticated than in other types of tumors (Mueller et al. 2009; Satzger et al. 2010).

This implicates that a lot more miRNAs might be functionally involved in the formation and progression of malignant melanoma than the few we started to analyze in detail so far. Fortunately, a reasonable amount of miRNA profiling data are now available to support the identification of candidate miRNAs for future functional validation. Nevertheless, for every single miRNA, it remains to be elucidated if its deregulated expression really is a defect driving melanoma progression or if it just has to be considered a passenger defect not reflective of the fundamental molecular mechanisms underlying tumorigenesis.

6.3.2 Identification of miRNA Biomarkers Suitable for Diagnostics and Prognostics in Melanoma

In the beginnings of cancer-related miRNA research, groundbreaking publications demonstrated that (1) many miRNAs are encoded at genomic loci matching to fragile sites or regions associated with cancers (Calin et al. 2004) as well as that (2) miRNA expression profiles reflect the developmental lineage and the differentiation state of solid tumors (Lu et al. 2005; Volinia et al. 2006). Strikingly, poorly differentiated tumors can be successfully classified by their miRNA expression profile, in contrast to their mRNA profile – leading Rosenfeld and colleagues to construct a 48 miRNA classifier capable to determine the origin of metastatic tumors of unknown primary origin with high accuracy (Gaur

et al. 2007; Lu et al. 2005; Rosenfeld et al. 2008). With regard to melanoma, these studies unrevealed that 85.9 % of genomic loci harboring one or more of 283 examined miRNA genes exhibited DNA copy number alterations and that some of these changes were specific to this tumor type (Zhang et al. 2006). In addition, a set of 15 miRNAs expressed at significantly different levels separated the eight melanoma cell lines included in the NCI-60 panel from the other cancer cell lines investigated (Gaur et al. 2007). Taken together, these findings paved the way to explore the potential of miRNA expression profiling to identify miRNA biomarkers with a diagnostic and/or prognostic value in malignant melanoma.

Fortunately, in accordance to other tumor tissues, FFPE specimens derived from melanocytic lesions are suitable starting material for miRNA expression profiling (Glud et al. 2009; Liu et al. 2009; Ma et al. 2009). In the light of a very limited availability of fresh-frozen melanoma samples (due to therapeutic guidelines requiring microscopic analysis of the whole primary tumor to determine histopathological prognostic parameters, including Breslow thickness), as well as of the large quantity of archival material stored in pathological institutes worldwide this really opens avenues for miRNA biomarker discovery. First interesting results already point out that miRNA profiles actually do harbor the potential to be utilized in diagnostics and prognostics of melanoma in the future. In this way, Satzger and colleagues (2010) found significant association of high miR-15b expression with poor recurrence-free survival and overall survival in Kaplan–Meier analysis of a total of 128 melanoma patients (median follow-up 43.1 months). They also showed that the up-regulation of miR-15b was a statistically significant independent parameter of disease-free survival and overall survival in multivariate Cox analysis in addition to Breslow thickness and ulceration of the primary tumor (Satzger et al. 2010). In addition, Caramuta et al. (2010) described low levels of miR-191 and high levels of miR-193b in melanoma lymph node metastases to be associated with shorter survival after diagnosis of metastatic dissemination into the regional lymph nodes analyzing 16 melanoma patients.

In contrast to these studies focusing on single miRNA markers associated with melanoma survival, Segura et al. (2010) constructed a miRNA classifier consisting of six miRNAs (miR-150, miR-342-3p, miR-455-3p, miR-145, miR-155, and miR-497) indicative for melanoma prognosis. Based on this classifier, they were able to predict post-recurrence survival in their set of 59 FFPE melanoma metastases (derived from patients with detailed clinical follow-up) with an estimated accuracy of about 80 %. Notably, the miRNA classifier was also able to significantly risk-stratify stage III melanoma patients into “better” and “worse” prognostic categories based on survival probability in contrast to the AJCC standard classification system (stages IIIB and IIIC; Segura et al. 2010, detailed discussion in Mueller and Bosserhoff 2010b). Pointing towards clinical application by using three retrospective cohorts (training, validation cohorts profiled with miRNA arrays and an independent cohort assessed through qPCR), Hanniford

et al. (2015) identified a miRNA classifier in primary melanoma tumors. The classifier consists of a four miRNA signature including miR-150-5p, miR-15b-5p, miR-16-5p, and miR-374-3p, which, combined with the current clinico-pathological staging system, could increase the prognostic value for the risk of developing brain metastasis of the clinicopathological staging alone. Interestingly, miR-150-5p, which was less abundant in primary melanomas that metastasized to the brain, correlated with tumor infiltrating lymphocytes, reflecting tumor-tissue-specific miRNA signature, as opposed to just a cancer-cell-specific signature, putting forward a new advantage of approaches profiling miRNAs from human FFPE samples (Hanniford 2015).

In the era of personalized medicine, miRNA classifiers may represent good prognosis markers and can help in decision making. Pinto et al. compared BRAF mutated versus BRAF nonmutated melanoma patients, finding that miR-192 exhibited a higher expression in BRAF-mutated patients compared to a panel of 15 other miRNAs. This high expression, together with miR-193*, and low expression of miR-132 was associated with worse clinical outcome (Pinto et al. 2015).

6.3.3 Circulating miRNA in Melanoma

While the reports cited above rely on miRNA expression profiling in tissue samples, Leidinger et al. (2010) analyzed blood cell miRNA profiles in 35 melanoma patients and 20 healthy control individuals. Utilizing a subset of 16 significantly regulated miRNAs, they were able to separate melanoma patients from healthy individuals with an accuracy of 97.4 %, a specificity of 95 % and a sensitivity of 98.9 % (Leidinger et al. 2010). This study provides first evidence that miRNA expression profiles generated from peripheral blood cells could be engaged to distinguish patients suffering melanoma from healthy individuals in a noninvasive routine diagnostic approach. In 2008, Mitchell et al. made an important breakthrough when they made a comparative profiling of circulating miRNAs extracted from plasma and serum samples. Furthermore, they were able to detect specific miRNAs in circulation from a murine prostate tumor xenograft model and to find circulating miRNAs which can be used to distinguish between healthy and prostate cancer patients (Mitchell et al. 2008). Having demonstrated that miRNAs were degraded when exposed to RNases present in blood plasma and serum, one of the most intriguing questions regarded the mechanism through which miRNAs could be stably detected in samples after at least 24 h at room temperature or after repeated freezing and thawing cycles.

To date, there are three stabilizing mechanisms for circulating miRNAs. These are (1) exosome-transported miRNAs (Valadi et al. 2007; Hunter et al. 2008), (2) HDL-transported miRNAs (Vickers et al. 2011), and (3) miRNA–protein complexes, where AGO2 appears to play a central role, raising the hypothesis that cells release functional miRNA-induced silencing complexes (miRISC) into the circulation (Arroyo et al. 2011). Although most of the efforts have been directed towards the structural and functional characterization of exosomes

(which are also loaded with functional mRNA and proteins), the group headed by Muneesh Tewari has more recently generated strong evidence showing that the exosomal fraction of miRNA represents around 15 % of the total circulating miRNA, being the great majority of these molecules in the vesicle-free (i.e., miRNA-protein complexes) compartment (Arroyo et al. 2011; Chevillet et al. 2014). Moreover, these mechanisms are not mutually exclusive, and, as their roles and mechanisms are progressively understood, they open new avenues for diagnostic and therapeutic clinical use.

In brief, miR-320 and miR-150 were found particularly abundant in exosomes from cells of different types (normal and cancer cells) (Zhang et al. 2015). MiR-451 is abundant in exosomes derived from non-cancer cells, while miR-214 and miR-155 are enriched in exosomes derived from tumor cell lines or peripheral blood of cancer patients (Zhang et al., 2015). Further, miR-17, miR-19a, miR-21, miR-126, and miR-149, were found at significantly higher levels in plasma exosomes derived from a cohort of metastatic melanoma patients compared to a cohort of healthy controls, a cohort of nonrecurrent treated familial melanoma patients carriers of mutated CDKN2A/p16, and a cohort of carriers of the same mutation that had no evidence of disease (Pfeffer et al. 2015).

Stark et al. (2015b) tested a panel of seven miRNAs in blood samples of stage III and IV melanoma patients compared to controls that detected the presence of melanoma with a sensitivity of 93 % and a specificity of at least 82 %. This panel was found to be a better predictor for progression recurrence and survival than current serological clinical markers (Stark et al. 2015a, b). In a broader approach, Margue et al. (2015) used qRT-PCR arrays to compare the serum miRNomes of 52 melanoma patients with 30 healthy volunteers, and the serum profiles with tissue miRNA profiles. They found miR-30b-5p, miR-3754a-5p, among others, to be exclusively expressed in tissue samples, while miR-3201 and miR-122-5p had a higher expression in serum samples. Importantly, the serum miRNome could distinguish only later stage melanoma patients from healthy volunteers, raising doubts about circulating miRNAs as early diagnostic biomarkers, while establishing an important database platform as a reference for further studies (Margue 2015).

As true as for other cancers where tumor markers for early neoplastic transformation remain to be discovered, melanoma biomarkers distinguishing between nevi and early melanoma are urgently needed – especially considering the ability to metastasize a very early event in the progression of malignant melanoma. MicroRNA biomarkers hopefully not only suite this purpose but may also be utilized for separating primary melanomas which will lead (or have already led) to metastasis from nonmetastasizing tumors. Regarding prognostic applications of miRNAs, a central goal has to be the identification of miRNA classifiers (which are representative of molecular biomarkers in general) that can subsequently be incorporated into established staging systems relying solely on morphological criteria. In the end, this will hopefully allow better separation of patients into treatment groups, based on their risk and prognosis. Thereby, patients responsive to one kind of adjuvant treatment could be identified while

nonresponders could be spared negative effects of treatment. However, for prognostic as well as diagnostic applications, large-scale studies in comprehensive sets of tissue or blood samples (for which detailed clinical data have to be available) are needed ultimately to prove the usefulness of single miRNAs or miRNA classifiers which already have been proposed as melanoma biomarkers to date. Taken together, although miRNA analysis is unlikely to replace the existing tools for tumor diagnosis and management (like immunohistological staining or established serum marker-proteins), miRNA biomarkers promise huge benefits if used to complement established methodologies.

6.3.4 MiRs as Therapeutical Targets or Therapeutic Options

The unique features of miRNAs and the increasing evidence of their role in disease have stimulated the biotechnology industry to develop miRNA targeting strategies or use synthetic miRNAs as therapeutic agents. MiRNAs are often conserved among species in sequence and function, which makes them particularly attractive for the development and testing of anti-miR drugs. The success of antisense technologies in research has paved the way to the development of anti-miRs, using specific chemistries to increase stability and pharmacokinetics (reviewed by van Rooij et al. 2012). Anti-miRs are modified antisense oligonucleotides targeting a mature miRNA through complementary sequence. An anti-miR should be cell-permeable, stable in the circulation and have high specificity to the target microRNA. The most successful anti-miRs are based on LNA (Locked Nucleic Acid) chemistry, one of them, the antagomiR Miravirsen, targeting miR-122, has reached phase II clinical trial in patients infected with Hepatitis C virus (HCV) (https://www.clinicaltrials.gov/ct2/results?term=Miravirsen&Search=Search). In addition, synthetic microRNAs, called mimics or mimetics, are being designed to use them as weapons against specific gene products. These are usually RNA duplexes carrying specific modifications that will increase their stability and cell permeability. These modifications include linkers, such as cholesterol or nanoparticles (reviewed by Esau and Monia 2007). To increase tissue or cell specificity and prevent side-effects due to general uptake, delivery in viral vectors, such as the AAV has been used. Specificity is achieved via different virus serotypes with tissue tropism, or tissue-specific promoters.

Only a few of these microRNA therapeutic agents have reached clinical trials, among them MRX34, mimicking the tumor suppressor miR-34, is being tested in a phase I trial in different cancers including metastatic melanoma (https://www.clinicaltrials.gov/ct2/show/NCT01829971?term=mirna+therapeutics&rank=115). In summary, there is great hope that the success of these clinical trials will boost further development from the pharmacological industry.

Conclusion

Undoubtedly, a sense of euphoria encases the scientific community when considering future therapeutic and biomarker applications based on miRNAs. This attitude is strengthened as first diagnostic kits (Asuragen Inc.; Rosetta

Genomics) made their way into commercial application and a first miRNA-based drug entered phase IIa clinical trials in the USA and Europe (Santaris Pharma A/S) – with some equally promising miRNA therapeutics subjected to tests in preclinical studies (Regulus Therapeutics Inc.; Mirna Therapeutics Inc.). While we successfully demonstrated that miRNAs are new players in melanomagenesis, we are yet far away from a complete understanding of the impact those tiny molecules exert on formation and progression of this malignancy. Expanding our knowledge on melanoma-relevant miRNAs will be an indispensable prerequisite to develop therapeutic approaches based on miRNA to eventually allow the effective treatment of melanoma patients in the future. Nevertheless, already at this point it seems like miRNAs could at least fill some of the gaps in our yet limited knowledge of the molecular mechanisms underlying this deadly disease. A beginning has been made – a long but promising way is still ahead.

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7 Altered Signal Transduction Pathways in Melanoma

Yann Cheli, Eric Lau, and Ze'ev A. Ronai

7.1 Introduction

Signal transduction pathways are central to all cellular biological processes, as they provide the link between extracellular or intracellular stimuli and an array of regulatory proteins, including protein kinases, ubiquitin ligases, and transcription factors. Given this, it is not surprising that signal transduction pathways are often deregulated in cancer. Indeed, melanoma is a paradigm for rewired signaling because most critical mutations discovered in this tumor type are centered around relatively few major signaling cues, the most significant of which are the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways. Both pathways contain regulatory components with catalytic activities, making them the preferred targets for therapy. Here, we summarize our current understanding of the major deregulated signaling pathways in melanoma and the implications of such deregulation for tumor biology.

7.1.1 Extracellular Receptors

Among the receptors reported to be deregulated in melanoma are numerous membrane-bound G protein-coupled receptors and receptor tyrosine kinases (RTKs), including MC1R (melanocortin 1 receptor), c-Kit (mast/stem cell growth factor receptor), c-Met (hepatocyte growth factor receptor), IGFR (insulin-like

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_7

growth factor receptor), and Frizzled (WNT receptor). Deregulation of other RTKs, including AXL, epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), and vascular endothelial growth factor receptor (VEGFR), has also been implicated in the resistance of melanomas to certain treatments such as BRAF inhibitors (BRAFi) (Fargnoli et al. 2010; Landi et al. 2006; Mattei et al. 1994; Topcu-Yilmaz et al. 2010; van Ginkel et al. 2004).

7.1.1.1 MC1R

MC1R is a melanocyte-specific G protein-coupled receptor that binds to α-melanocyte-stimulating hormone (α-MSH, Fig. 7.1). MC1R–α-MSH interactions play a central role in the regulation of both pigmentation, by inducing generation of eumelanin and cAMP, and melanocyte proliferation (Hunt et al. 1995; Mountjoy et al. 1992; Robinson and Healy 2002; Suzuki et al. 1996).

MC1R exhibits genetic variance, with mutations at the hot spot residues R151C, R160W, and D294H being the most significant. The mutations reduce receptor function and result in a phenotype of fair, freckled skin and red hair (Kadekaro et al. 2003). Stimulation of MC1R by α-MSH normally potentiates p16INK4A tumor suppressor activity after UV exposure (Pavey et al. 2002); these specific mutations are associated with reduced UV-induced DNA damage repair efficiency and increased melanoma risk (Scott et al. 2002; Song et al. 2009). In contrast, overexpression of MC1R variants has been shown to render cells insensitive to α-MSH-mediated suppression of cell proliferation (Robinson and Healy 2002), suggesting that polymorphic variants of MC1R may enhance melanoma susceptibility or progression by attenuating p16INK4A function, at least in part.

Although MC1R is neither genetically nor epigenetically silenced (Kim et al. 2008a), expression of the functionally impaired variants compromises receptor activity and correlates with increased melanoma risk (Landi et al. 2006). Carriers of MC1R variants who have mutations in CDK2NA also have a higher melanoma risk (Fargnoli et al. 2010). Notably, germline mutations of MC1R are associated with an increased incidence of BRAF mutations in melanoma (Landi et al. 2006). Likewise, inactivation of MC1R in the $Braf^{V600E}$:$Pten^{-/-}$ mouse melanoma model increases the incidence of melanoma independently of UV radiation (Mitra et al. 2012).

Mutation of G proteins themselves, in particular the α-subunit of G(q) (GNAQ), may induce alterations in early melanoma lesions (Kusters-Vandevelde et al. 2010; Lamba et al. 2010; Van Raamsdonk et al. 2009). GNAQ is mutated within a RAS-like domain at position Q209L, which renders the protein constitutively active and amplifies PKC and MAPK signaling. Accordingly, overexpression of the GNAQ Q209L mutant is sufficient to confer anchorage independence and increase the tumorigenicity of immortalized melanocytes.

7.1.1.2 Receptor Tyrosine Kinases

Many cell surface receptors for growth factors, hormones, and cytokines are RTKs. Ligand binding activates the intrinsic RTK enzymatic activity, often by autophosphorylation, with subsequent phosphorylation of tyrosine residues on many substrates, including PLCγ, PI3K, and MAPK, which drive cell proliferation

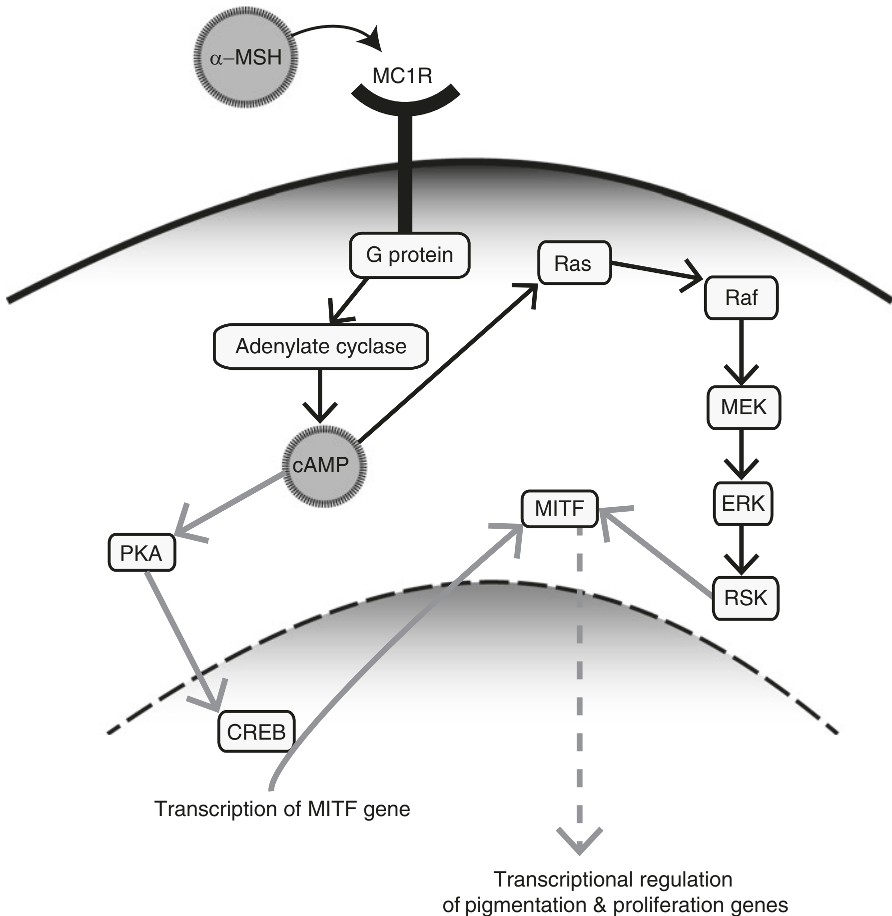

Fig. 7.1 α-MSH and MC1R receptor signaling. Binding of α-melanocyte stimulating hormone (*α-MSH*) to its cognate receptor melanocortin-1 receptor (*MC1R*) activates G protein-coupled adenylate cyclase and increases cytoplasmic cAMP levels. cAMP initiates a cascade that sequentially activates protein kinase A (*PKA*), CREB, and transcription of MITF. In parallel, cAMP activates the RAS–RAF–MAPK–RSK cascade, which results in MITF activation. MITF then modulates the transcription of downstream pigmentation and proliferation genes. Branches of this signaling pathway that are upregulated in melanoma are indicated in *black*. The *dashed arrow* indicates modulation of transcriptional programming by MITF to favor tumorigenesis

differentiation, survival, and cell migration. Among the growth factor RTKs deregulated in melanoma are AXL, EGFR, c-Kit, c-Met, and IGFR, which bind to growth arrest-specific 6 (Gas6), epidermal growth factor (EGF), stem cell factor (SCF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF), respectively (Fig. 7.2). Ligand binding by these receptors activates the downstream RAS–RAF–MEK–MAPK and PI3K–AKT signaling pathways independently of any existing MAPK pathway mutations and promotes cell survival and proliferation. Changes in

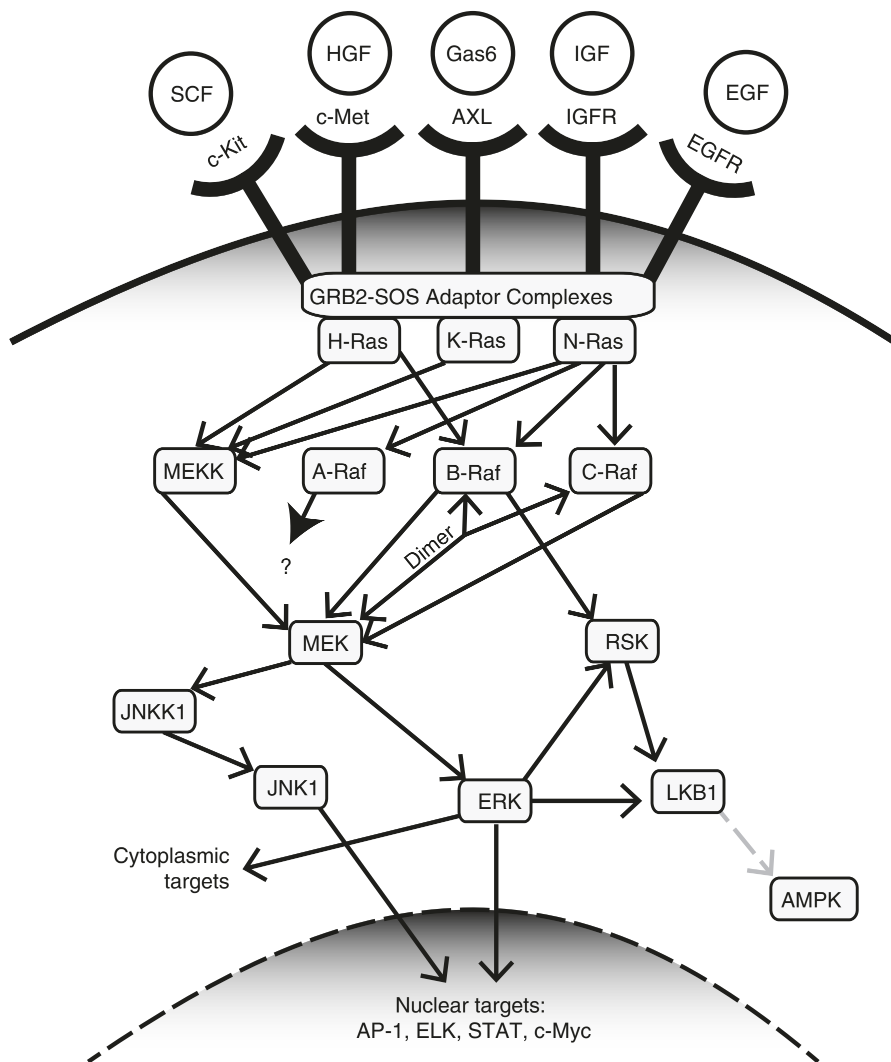

Fig. 7.2 Growth factor receptors and MAPK signaling. Membrane-bound growth factor receptors (*c-Kit, c-Met, IGFR, AXL, EGFR*) generally signal inwards through adaptor complexes containing proteins, such as GRB-SOS, which recruit the RAS family members HRAS, KRAS, or NRAS for activation. In melanoma, NRAS is the most commonly mutated protein and plays a predominant role in activation of the downstream effector kinases, RAF and MEKK. The RAF family of effector kinases includes A-, B-, and CRAF. Although signaling through ARAF has been less studied, in melanoma, it appears that BRAF- and CRAF-mediated signaling predominate. BRAF and CRAF activate downstream MEKK, MEK, and RSK. MEK activation leads to further downstream activation of JNKK1–JNK1 and ERK, as well as their cytoplasmic or nuclear transcriptional targets. Together, BRAF and ERK activate RSK to suppress LKB1, which would otherwise activate AMPK. In melanoma, this arm of AMPK activation, which normally regulates cell growth and survival, is downregulated (*gray dashed arrow*). Branches of the MAPK pathway that are upregulated in melanoma appear in *black*

the expression of these receptors have been implicated in several tumor types; in melanoma, altered expression has not been observed uniformly but may be associated with specific phases of tumor development.

7.1.1.3 AXL

AXL RTK is ubiquitously expressed in epithelial, mesenchymal, and hematopoietic tumors, and in the corresponding non-transformed cells. AXL was discovered in patients with chronic myeloproliferative disorder, and has since been implicated in the proliferation and drug resistance of numerous cancers, including melanoma (Paccez et al. 2014).

AXL is upregulated in ocular melanomas and melanoma cell lines, where it promotes cell proliferation and confers a survival advantage under serum starvation conditions (van Ginkel et al. 2004). Increased AXL expression is also found in both NRAS- or BRAF-mutated tumors, although the incidence is higher in NRAS mutant melanomas. Of interest, AXL expression inversely correlates with melanoma differentiation and microphthalmia-associated transcription factor (MITF) expression (Muller et al. 2014), and the combination of low MITF and high AXL expression is associated with a highly invasive phenotype. Pharmacological AXL inhibitors attenuate but do not abolish the invasive phenotype (Sensi et al. 2011), indicating a role for other RTKs and/or signaling pathways in the invasive phenotype. In response to chemotherapy, a subset of tumor cells that exhibit a senescence phenotype show elevated production and secretion of cytokines (Ohanna et al. 2011), with concomitant activation of STAT3 and AXL and increased migration and invasion properties in surrounding cells (Ohanna et al. 2013).

Lastly, a major clinical obstacle in melanoma therapy is the acquisition of resistance to either BRAFi or MEKi. The mechanisms underlying such resistance include upregulation of AXL, among many RTKs, which coincides with low levels of MITF and activation of NF-kB and JAK1 (Konieczkowski et al. 2014). Accordingly, treatment of resistant melanomas with a pharmacological inhibitor of AXL augments the effect of the BRAFi or MEKi and enhances cell death (Muller et al. 2014).

7.1.1.4 EGFR

EGFR is a transmembrane receptor for members of the EGF family of growth factors. Increased expression or mutation of the EGFR gene is commonly seen in a number of tumor types, including colorectal, breast, and non-small cell lung cancers, where it is thought to serve as an oncogenic driver.

EGFR upregulation has been implicated as a mechanism of therapy resistance for several tumor types, including melanoma. Consistent with this, co-administration of BRAFi and EGFRi appears to attenuate ERK activity and sensitizes resistant melanomas to BRAFi or MEKi inhibition (Girotti et al. 2013). The resistance of $BRAF^{V600E}$ mutant colorectal cancer to BRAFi therapy has also been associated with high EGFR expression, which enables reactivation of ERK via RAS and CRAF to bypass BRAF inhibition (Corcoran et al. 2012).

Elevated EGFR expression, as observed in resistant melanoma, has been linked to SOX10 and MITF expression (Ji et al. 2015). Furthermore, EGFR upregulation is often

accompanied by upregulation of other RTKs that are associated with SOX10 and TGF-β signaling, including platelet-derived growth factor receptor β (Sun et al. 2014).

Interestingly, SOX10 was also found to regulate the expression of the ubiquitin ligase RNF125, which controls JAK1 stability through ubiquitin–proteasome-dependent degradation. BRAFi-resistant melanomas exhibit downregulation of SOX10 and concomitant inhibition of RNF125 expression. Consequently, JAK1 stability and availability increase, and the expression of several RTKs, including EGFR and AXL, is stimulated. As might be expected, inhibition of JAK1 effectively reduces the expression of RTKs and overcomes the resistance of melanomas to BRAFi (Kim et al. 2015).

7.1.1.5 c-Kit

c-Kit expression is apparent in early or radial growth phase melanomas. Although the penetrance appears to be low, a c-Kit-activating mutation, L576P, has been reported in melanoma (Antonescu et al. 2007; Rivera et al. 2008; Willmore-Payne et al. 2006). Interestingly, however, downregulation of c-Kit expression is associated with melanoma progression (Giehl et al. 2007; Janku et al. 2005; Montone et al. 1997; Natali et al. 1992). These observations suggest that upregulation of c-Kit and its ligand SCF may be required to establish the primary lesions, but that continued expression of c-Kit is not needed for invasion and metastasis. The disparity of mutations in or altered expression of c-Kit among different melanomas was initially overlooked in clinical trials of c-Kit-specific inhibitors. In more recent trials, clinical efficacy has been observed when patient cohorts harboring c-Kit mutations were treated with the highly selective pharmacological inhibitor, Gleevec (Terheyden et al. 2010). How inhibition of c-Kit contributes to melanoma progression remains an important topic for investigation. The c-Kit ligand SCF is a keratinocyte-secreted factor, and it has been proposed that downregulation of c-Kit in melanoma cells may allow them to escape SCF-induced cell death. Indeed, *in vitro* studies have shown that re-expression of c-Kit in metastatic melanoma sensitizes the cells to SCF-mediated apoptosis and reduces their tumorigenic and metastatic potential in vivo (Bar-Eli 1997; Huang et al. 1996; Willmore-Payne et al. 2005).

Although the mechanism by which c-Kit is downregulated during melanoma progression remains unclear, a recent study found that downregulation may be epigenetically linked to expression of microRNAs (miRNAs) (see also Chap. 6), specifically miR-221 and miR-222, which were shown to suppress expression of both c-Kit and p27Kip (Felicetti et al. 2008).

7.1.1.6 c-Met

c-Met-dependent signaling is amplified in melanoma, although genetic mutations or modifications that result in aberrant activation of c-Met do not appear to be common. Two c-Met mutations, N948S and R988C, have been identified in melanoma cell lines and tumor tissues and shown to activate c-Met signaling through several downstream effectors, including MITF, tyrosinase, and AKT and its effectors (Chin et al. 2006; Puri et al. 2007). However, c-Met upregulation has also been observed in melanoma, particularly in the later stages of disease (Natali et al. 1993). This has been suggested to play a role in metastasis, especially in the liver (Rusciano et al. 1995). Genetic amplification and activation of c-Met concomitant with Src

activation has been reported in BRAFi-resistant melanoma cells; accordingly, genetic or pharmacological inhibition of c-Met attenuated the proliferation and invasion of the BRAFi-resistant cells (Vergani et al. 2011).

c-Met upregulation can be induced by a number of mechanisms. One is via MITF, which is induced by MC1R–α-MSH signaling, as mentioned above (Rouzaud et al. 2006; Rusciano et al. 1999). Indeed, impaired MC1R function, which is frequently observed in melanoma, is indicative of deregulated c-Met at both the genetic and protein levels.

The ubiquitin ligase skeletotrophin is another protein implicated in the regulation of c-Met. In melanoma, expression of skeletotrophin is lost due to increased SNAIL-mediated transcriptional repression. Re-expression of skeletotrophin impairs the invasive capacity of melanoma cells *in vitro*, and this correlates with a reduction in c-Met mRNA transcripts (Takeuchi et al. 2006). MicroRNAs have been shown to contribute to increased c-Met levels in melanoma. miR-34a is normally expressed in melanocytes but is downregulated in melanoma. Here too, re-expression of miR-34a *in vitro* reduces c-Met expression and suppresses the growth, migration, and invasive capacities of melanoma cells (Yan et al. 2009).

7.1.1.7 IGFR1

IGF1R is another growth factor receptor that is upregulated in progressively malignant melanoma (Mallikarjuna et al. 2006). In early melanoma lesions, IGF1R appears to enhance cellular growth and survival by promoting MAPK- and ß-catenin-dependent signaling pathways; however, IGF1R-dependent stimulation of these two pathways may be dispensable in later stage melanomas where other oncogenes are constitutively activated (Satyamoorthy et al. 2001).

Upregulation of IGF1R is associated with both malignant progression and resistance to apoptotic stimuli. Antisense-mediated inhibition of IGF1R is sufficient to inhibit the growth of mouse melanoma cells in nude mice (Resnicoff et al. 1994), and monoclonal antibody-targeted inhibition of IGF1R in human melanoma cells similarly inhibits their growth and invasion in xenograft mouse models (Maloney et al. 2003). Furthermore, disruption of IGF1R can sensitize melanoma cells to TRAIL-induced apoptosis (Karasic et al. 2010) and increase radiosensitivity of melanoma by impairing the ATM-mediated DNA damage response (Macaulay et al. 2001). Moreover, inhibition of IGF1R is sufficient to suppress growth of human melanomas harboring the BRAF^{V600E} mutation (discussed further below), indicating that IGF1R inhibition can override signaling events that circumvent the known IGF1R effector, the RAS-MAPK signaling axis (Yeh et al. 2006). High levels of IGF1R have been reported in BRAFi-resistant cells, and have been implicated in upregulation of the PI3K pathway (Villanueva et al. 2010).

7.2 Cellular Kinases and Transcription Factors

7.2.1 WNT–ß-catenin

The WNT–ß-catenin signaling pathway plays an important regulatory role in melanocyte development and is deregulated in melanoma (see also Chap. 5). WNT is a

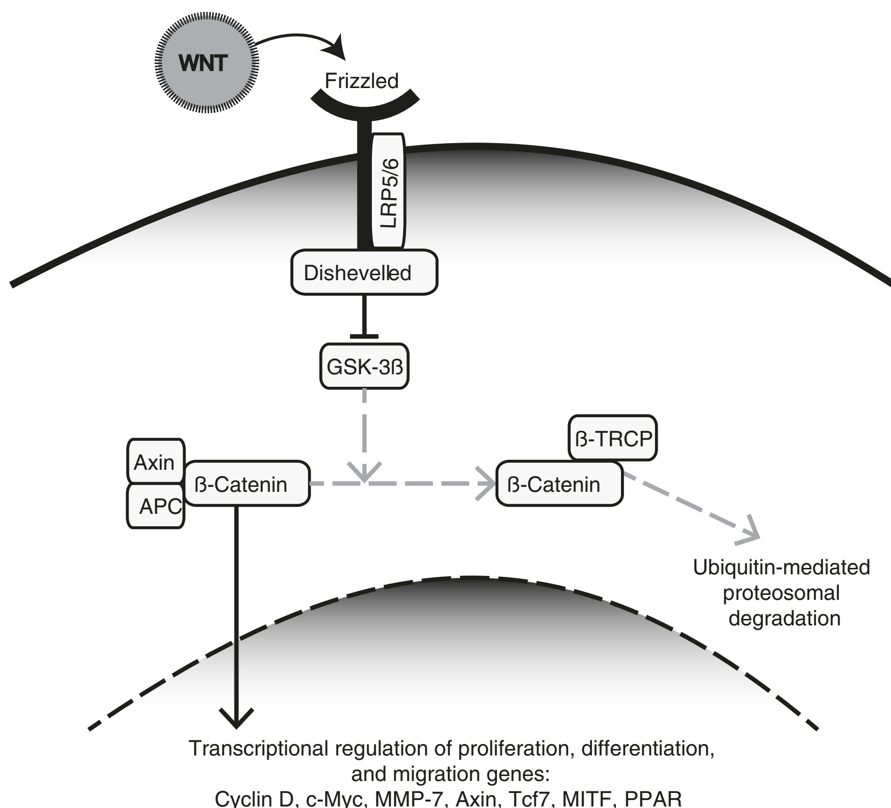

Fig. 7.3 WNT signaling pathway. Binding of WNT to its cognate receptor Frizzled and co-receptor LRP5/6 results in inactivation of GSK3ß via Dishevelled. Inactivation of Dishevelled stabilizes ß-catenin by releasing it from its degradation complex, which includes Axin and APC. Under non-stimulated conditions, ß-catenin is bound by ß-TRCP, which facilitates its ubiquitination and subsequent proteasomal degradation. Stabilized ß-catenin is then imported into the nucleus to facilitate transcriptional regulation of proliferation, differentiation, and migration genes. Branches of this signaling pathway that are upregulated in melanoma are indicated in *black*; downregulated branches are indicated by *dashed gray arrows*

secreted ligand for the membrane receptor, Frizzled (Fig. 7.3), and WNT binding activates the cytoplasmic Frizzled effector, Dishevelled. Consequently, Dishevelled inhibits GSK3ß–Axin–APC-mediated degradation of ß-catenin, stabilizing its levels and allowing nuclear import to execute its transcriptional functions. Activation of the WNT–ß-catenin pathway facilitates ß-catenin-mediated upregulation of MITF, which promotes melanocyte differentiation and development (Dorsky et al. 2000; Takeda et al. 2000). MITF can itself bind to ß-catenin, thereby tilting the transcriptional activity towards MITF targets and generating a positive feedback loop (Schepsky et al. 2006).

As expected, melanomas harboring activating ß-catenin mutations also have increased MITF levels (Doglioni et al. 2003). In turn, MITF upregulation has been

shown to increase multivesicular body synthesis and consequently WNT signaling, allowing the cells to enter a proliferative stage (Ploper et al. 2015). Interestingly, ß-catenin also upregulates the transcription factor Brn-2, which transcriptionally represses Mitf and simultaneously enhances invasive melanoma behavior. Brn-2 expression also characterizes distinct subsets of MITF-negative melanoma cells (Goodall et al. 2008). The implications of MITF heterogeneity within the same and different tumors is the subject of intense investigation.

WNT–ß-catenin signaling is upregulated in melanoma, and although only ~3 % of melanoma biopsies harbor ß-catenin mutations, ~30 % of human melanomas exhibit increased nuclear localization of ß-catenin (Larue and Delmas 2006). Increased WNT signaling directly upregulates Mitf and Brn-2 expression, with concomitant suppression of p16INK4A transcription; these changes act in concert to drive melanoma growth and proliferation (Delmas et al. 2007; Goodall et al. 2004a; Widlund et al. 2002). Nuclear localization of ß-catenin is observed in melanomas carrying mutant phosphatase and tensin homolog (PTEN) phosphatase and has been implicated in melanomagenesis by cooperating with $NRAS^{Q61K}$ to increase caveolin-dependent transcriptional activity. This effectively bypasses the senescence phenotype elicited by mutant NRAS alone and promotes metastasis, in part by internalization of E-cadherin (Conde-Perez et al. 2015).

Interestingly, the functional role of WNT signaling in melanoma development varies with the specific WNT isoform. WNT3, an activator of the canonical WNT signaling axis, is anti-tumorigenic and its expression correlates with primary/nevi lesions and decreased proliferation of tumor models *in vitro* and *in vivo*. Furthermore, WNT3 expression correlates with upregulation of genes controlling melanocyte development and differentiation, including *Axin*, *Tcf7*, and *Mitf* (Chien et al. 2009). In contrast, WNT5A, which activates the non-canonical WNT signaling axis, appears to antagonize the transcriptional effects of WNT3A. Indeed, WNT5A is pro-tumorigenic, cooperating with other signaling pathways (e.g., PKC) to enhance the metastatic and invasive behavior of melanoma cells, which most likely occurs through its known function in the re-distribution of adhesion receptors (Weeraratna et al. 2002; Witze et al. 2008). Consistent with this, WNT5A-positive melanoma tumors appear to exhibit increased invasiveness and decreased proliferation compared with WNT5A-negative but MITF- and Melan-A-positive tumors, supporting a model of proliferative vs invasive phenotype switching during tumor progression (Eichhoff et al. 2010). Of note, increased WNT5A expression was also seen in tumors with acquired BRAFi resistance, and its inhibition re-sensitized tumors to BRAFi and reduced their proliferation, in part via attenuation of p-AKT activity (Anastas et al. 2014).

Melanomas with activated WNT–β-catenin signaling have a strong immunosuppressive effect on dendritic cells and cytotoxic T lymphocytes, mediated by increased IL-10 secretion and reduced IFN-γ secretion by the T cells. In this regard, it is interesting to note that activated WNT–β-catenin signaling has been linked with resistance to immunotherapy (Spranger et al. 2015; Yaguchi et al. 2012).

7.2.2 MAPK Signaling Axis

Alterations in the expression or activity of AXL, EGFR, MC1R, c-Kit, c-Met, IGFR, and WNT are examples of the most external layers of perturbed signaling cues that promote melanoma formation and progression. Several of the signaling pathways downstream of these receptors are themselves deregulated in melanoma.

The majority of melanomas display deregulated MAPK signaling due to mutations in the NRAS or BRAF genes. As a consequence, the downstream kinases and transcription factors are rendered constitutively active, regardless of aberrations upstream of NRAS or BRAF. In this section, we review our current understanding of alterations in the MAPK signaling axis and the implications for melanoma development or progression.

The MAPK pathway is coupled to upstream membrane receptors by the RAS family of small G proteins; HRAS, KRAS, and NRAS (Bos 1989; Dhillon et al. 2007). In non-transformed cells, the RAS proteins are responsive to activation by c-Kit, c-Met, IGFR, and WNT, for example, and transduce activating signals through interplay with the RAF family of effector serine/threonine kinases; ARAF, BRAF, and CRAF. Signals are then transduced by sequential activation of a cascade of MAP kinases: MEK, MEKK, and finally, ERK (Fig. 7.2). Of these proteins, NRAS and BRAF are the most commonly mutated in melanoma, with ~15 % and more than 50 % of melanomas harboring NRAS and BRAF mutations, respectively (Davies et al. 2002; Fecher et al. 2007). Further downstream, MEK mutations have also been reported, particularly in the context of acquired resistance to chemotherapy. For instance, MEK1 mutations occur at low incidence overall, but they are most frequently reported following BRAFi therapy and confer resistance to MEKi and BRAFi (Emery et al. 2009; Murugan et al. 2009).

While most melanoma-associated mutations in NRAS occur at amino acid 61, BRAF deregulation is attributed to mutations at several hotspots, most prominently V600E, resulting in a constitutively active kinase (Wan et al. 2004). Indeed, the catalytic activity of $BRAF^{V600E}$ was calculated to be ~10-fold higher than that of wild-type BRAF (Brummer et al. 2006). While mutant BRAF and NRAS share some downstream effectors, most notably ERK, they each also activate unique downstream components. As illustrated in Fig. 7.2, BRAF activity also affects MEK–ERK kinases and RSK. These kinases in turn suppress the activity of the LKB1–AMPK signaling pathway, thereby promoting melanoma proliferation (Esteve-Puig et al. 2009; Zheng et al. 2009). As a result of their unique contributions to signaling, BRAF and NRAS mutants have distinct characteristics with respect to melanoma development and progression.

As noted above, ERK is the downstream kinase most commonly affected by NRAS and BRAF mutations in melanoma, and constitutive or super-activation of ERK perturbs critical regulators of cellular behavior. For example, $BRAF^{V600E}$ antagonizes apoptosis via ERK-dependent inhibition of the apoptotic proteins Bad, Bim, and PUMA, and upregulation of anti-apoptotic proteins such as Mcl-1 (Jiang et al. 2008; Sheridan et al. 2008; Wang et al. 2007b). Enhanced ERK activation also alters cell cycle control and proliferation by suppressing the negative cell cycle

regulator p27/Kip1 (Kortylewski et al. 2001) and, importantly, by modulating the expression of melanocyte MITF isoforms and inducing the M-MITF 6a isoform (Primot et al. 2010; Wellbrock et al. 2008). MAPK/ERK activation can further enhance the proliferative capacity of melanoma cells by promoting upregulation of other transcription factors such as c-Jun and Brn-2, either by increasing their stability (c-Jun) or increasing their expression (Brn-2) (Goodall et al. 2004a, b; Lopez-Bergami et al. 2007). Mutant BRAF-mediated ERK signaling also impinges on invasive cellular behaviors resulting from changes in growth or invasion regulatory proteins such as Plexin B or matrix metalloproteinase-1 (Argast et al. 2009; Huntington et al. 2004). Indeed, activation of the RAS–RAF–MAPK–ERK pathway has been implicated in immune evasion by modulating the production of immunosuppressive cytokines such as IL-6, IL-10, and VEGF by melanoma cells (Sumimoto et al. 2006).

Constitutive upregulation of ERK signaling results in rewiring of signaling pathways, a common occurrence in many tumors, including melanoma. For example, rewired ERK signaling causes constitutive activation of c-Jun via two complementary pathways. ERK-mediated upregulation of RSK–CREB increases c-Jun transcription, whereas ERK-mediated phosphorylation of GSK3ß inhibits its ability to phosphorylate c-Jun on residue 243, which is required for targeting of c-Jun for ubiquitination and degradation by FBW7 (Nateri et al. 2004; Wei et al. 2005), thereby resulting in increased c-Jun stability (Lopez-Bergami et al. 2007). In turn, c-Jun induces transcription of a large set of targets genes that include cell cycle regulators such as Cyclin D as well as components of other signaling pathways. One is the PKC adaptor protein RACK1, which potentiates both PKC and JNK signaling. As a result, RACK1–PKC signaling increases JNK activity and further activates its substrates, including c-Jun, thus enforcing a feed-forward signaling pathway. Another c-Jun transcriptional target implicated in melanoma development is the kinase PDK1, which activates AKT. The c-Jun-mediated increase in PDK1 transcription, and thus activation of both the AKT and PKC pathways. Inhibition of c-Jun effectively attenuates melanoma development in a xenograft mouse model, and this can be rescued by re-expression of PDK1 (Lopez-Bergami et al. 2010). Thus, ERK signaling causes activation of the JNK, PKC, PDK1, and AKT pathways, representing a paradigm for rewired signaling.

Constitutive activation of MAPK itself is sufficient for transformation of immortalized melanocytes through elevation of angiogenic and invasive behavior secondary to upregulation of VEGF and MMP-2 (Govindarajan et al. 2003). However, synergistic crosstalk between upregulated MAPK signaling and other major signaling axes (e.g., PI3K–AKT–MTOR), can further promote additional tumorigenic behaviors such as the enhanced proliferation observed in uveal melanoma (Babchia et al. 2010).

Upregulated or constitutive activation of the MAPK signaling cascade correlates with poor clinical outcome (Houben et al. 2004), which is commonly attributed to activating mutations at different branch points along the signaling pathway. Thus, HRAS and KRAS mutations appear to correlate with benign Spitz nevi and primary lesions, whereas NRAS is most frequently mutated in primary and metastatic

melanoma and is characteristic of chronically sun-exposed lesions (Ball et al. 1994; Jafari et al. 1995; Jiveskog et al. 1998; Shukla et al. 1989; van Dijk et al. 2005; van Elsas et al. 1995). Immediately downstream of the RAS proteins are the RAF kinases, of which BRAF is the most frequently mutated (specifically $BRAF^{V600E}$) in melanoma. The more dominant oncogenic role of BRAF compared with ARAF and CRAF is most likely due to its higher kinase activity (Emuss et al. 2005; Lee et al. 2005). Although BRAF germline mutations have been reported, they are not common in familial melanoma (Lang et al. 2003), suggesting that BRAF mutations occur during melanoma development. Interestingly, $BRAF^{V600E}$ and NRAS mutations appear to be mutually exclusive in melanoma; a phenomenon that may be influenced by type and site of origin of the melanoma tumor. For example, BRAF mutation does not appear to correlate with the degree of sun exposure, as is the case for NRAS mutations (Davies et al. 2002). Interestingly, NRAS mutations induce a switch in the dominant usage of RAF isoforms from BRAF to CRAF (Dumaz et al. 2006).

Mutant BRAF is also found in congenital nevi and other non-malignant lesions, where it is associated with upregulation of senescence markers such as senescence-associated ß-galactosidase (SA-ß-gal) and mosaic p16INK4A induction (Michaloglou et al. 2005). Indeed, mutations of NRAS and BRAF (V600E) alone can promote cellular senescence *in vitro*, which not only illustrates oncogene-induced senescence but also highlights the need for other oncogenic events to drive tumor progression (see also Chaps. 4, 5, and 11). Nevertheless, ERK activity does not always correlate with BRAF activation, most likely due to variations in the functional status of ERK phosphatases. It has been proposed that some ERK phosphatases might be less active in malignant than non-malignant cells, although it is not yet clear whether and how ERK phosphatases might be deregulated in melanoma. In this regard, BRAF mutation alone is insufficient to transform melanocytes, and secondary mutations that confer uncontrolled cell cycle progression are required. The notion that BRAF acts cooperatively to induce melanoma development is supported by observations in zebrafish, where mutant BRAF promotes nevi development but additional oncogenic changes, such as loss of p53, can promote progression to invasive lesions (Patton et al. 2005).

Other secondary mutations that can support uncontrolled proliferation include p16INK4A and p19INK4D (see cell cycle section). Although it is unclear what role p16INK4A may play in BRAF-driven senescence, loss of p16INK4A can facilitate melanoma tumor formation driven by mutant RAS (Ackermann et al. 2005; Chin et al. 1997). Interestingly, cooperative stabilization of ß-catenin results in silencing of p16INK4A, and in combination with mutant NRAS, this is sufficient to promote melanoma progression (Delmas et al. 2007). Additional signaling mechanisms that contribute to oncogene-induced senescence and earlier barriers to melanoma progression continue to be identified. For example, early oncogene-induced activation of the ER stress-activated unfolded protein response was found to halt tumorigenesis independently of conventional senescence mechanisms (Denoyelle et al. 2006).

Although epigenetic perturbations that promote activation of these pathways remain largely obscure, the mutational status of melanoma tumors is known to correlate with some characteristic epigenetic profiles. For example, melanomas with BRAF mutations exhibit changes in several miRNAs, such as downregulation of

miR-193a, miR-338, and miR-565, and upregulation of miR-191 (Caramuta et al. 2010). A further level of complexity is suggested by the finding that pseudogene transcripts can act as false miRNA “decoy” targets (a.k.a., competitive endogenous RNAs), thereby absorbing and nullifying the function of miRNAs targeting specific endogenous transcripts (Chen 2010; Poliseno et al. 2010; Karreth et al. 2015). Such regulation has been demonstrated for PTEN and BRAF and may effectively alter the expression of other genes that are central to the control of melanoma development. As increasing effort is devoted to sequencing the melanoma genome, a wealth of pseudogenes contributing to different stages of melanoma biology are expected to be identified (Pleasance et al. 2010).

7.2.3 PTEN–PI3K–AKT

The phosphatidylinositol-3-kinase–AKT (PI3K–AKT) pathway is also frequently deregulated in melanoma (Inoue-Narita et al. 2008; Robertson 2005), although the mechanisms underlying the deregulation of many components remain largely elusive. Like the MAPK pathway, the PI3K pathway is an effector signaling cascade positioned downstream of the membrane receptors described earlier, including c-Met and IGFR1. PI3K converts phosphatidylinositol-4,5 bisphosphate (PIP2), located on the cytoplasmic face of the plasma membrane, into the secondary lipid signaling molecule, phosphatidylinositol-3,4,5 trisphosphate (PIP3). In turn, PIP3 activates the downstream effector AKT/protein kinase B. The three members of the AKT family of serine/threonine kinases (AKT1, AKT2, and AKT3) have well-characterized pro-survival functions (Datta et al. 1999; Madhunapantula and Robertson 2009) (Fig. 7.4), with AKT3 appearing to be the isoform most affected in melanoma.

One mechanism by which AKT signaling is enhanced is via deregulation of PIP2 processing by perturbation of inositol polyphosphate 4-phosphatase type II (Gewinner et al. 2009). However, upregulation of AKT activity in melanoma can largely be attributed to deregulation of its negative regulator, PTEN (Parmiter et al. 1988). Although loss of PTEN protein is prevalent in melanoma (Chudnovsky et al. 2005), deregulation by mutation accounts for only a small fraction of melanomas with deregulated PTEN. Accordingly, while PTEN is commonly mutated in melanoma cell lines, such genetic mutations are rare in actual tumor samples, particularly those of metastatic grade (Goel et al. 2006; Pollock et al. 2002; Wu et al. 2003). These observations indicate that downregulation or loss of PTEN in patient’s tumors results from additional transcriptional and post-translational modifications, with the latter being the most common. Although not shown yet in melanoma, the ubiquitin ligase NEDD-4, which targets PTEN for destruction, is upregulated in numerous cancer types, including gastric and colorectal cancers (Kim et al. 2008b; Trotman et al. 2007; Wang et al. 2007a). Oncogenic deregulation of PTEN also occurs via Fyn-related kinase (FRK, previously known as RAK), which is overexpressed in melanoma and numerous other cancers. FRK phosphorylates PTEN, thereby abrogating its interaction with NEDD-4 and increasing its availability (Brauer and Tyner 2009; Yim et al. 2009).

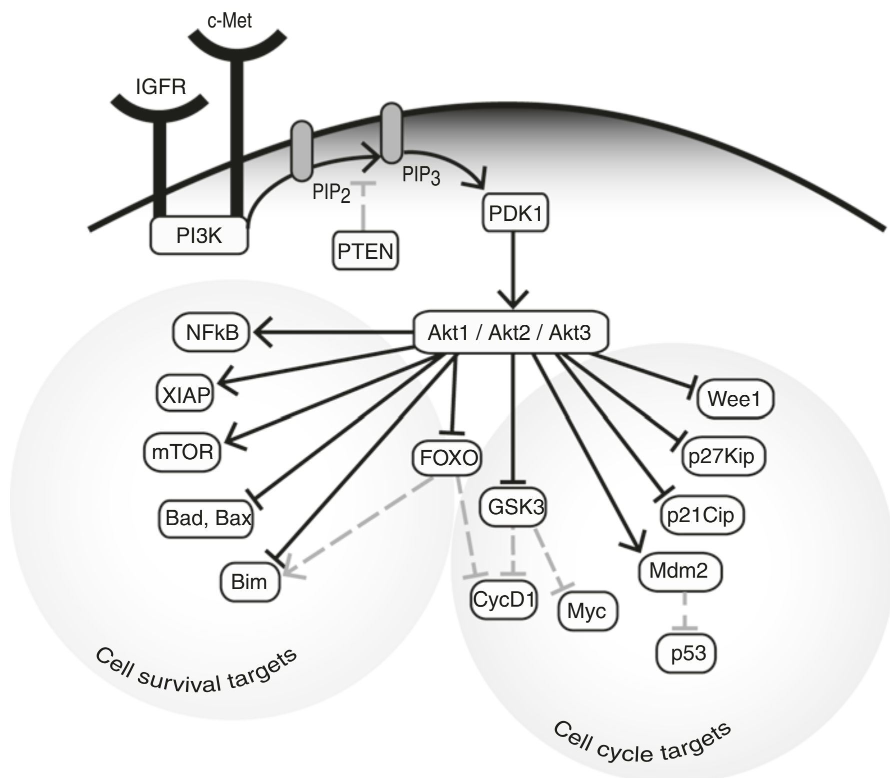

Fig. 7.4 AKT signaling. Various membrane-bound growth factor receptors (c-Met and IGFR) signal inwards via PI3K, which phosphorylates PIP_2 to produce the secondary messenger molecule PIP_3. PIP_3 then functions to activate PDK1, which in turn activates AKT family kinases (*AKT1, AKT2*, and *AKT3*). AKT is a widely recognized pro-survival effector kinase that acts by upregulating or activating numerous cell survival-related proteins, such as NF-kB, XIAP, and mTOR, and inhibiting cell death-related proteins such as Bad, Bax, and Bim (see also Chap. 10). AKT also inhibits transcription factors, such as FOXO, which contribute to cell death or cell cycle arrest. AKT promotes cell cycle progression by inhibiting cell cycle inhibitors, including Wee1, p21Kip, p21Cip, and p53 (via activation of *Mdm2*), and relieving Cyclin D1 and Myc from suppression by GSK3. Branches of the AKT pathway that are upregulated in melanoma appear in *black*; downregulated branches appear in *dashed gray*

Other forms of epigenetic silencing of PTEN include promoter methylation, which is observed in up to 62% of patients with metastatic melanoma (Mirmohammadsadegh et al. 2006). In effect, loss of PTEN promotes an excess of PIP3 and activation of AKT and its downstream targets, resulting in increased growth and survival of melanoma. Notably, activated AKT which is affected by deregulated PTEN is inversely correlated with a positive clinical outcome for melanoma patients (Dai et al. 2005).

In addition to loss of PTEN, direct changes to AKT family members can modulate the PI3K pathway in melanoma. Of the three AKT isoforms, AKT3 is specifically and significantly upregulated in sporadic melanoma tumors, particularly those

of metastatic grade (Robertson 2005; Stahl et al. 2004). Although AKT3 upregulation has mainly been attributed directly to an increase in genomic copy number, a recent report has identified a novel activating mutation of AKT3 (E17K) in some melanoma cases (Davies et al. 2008). Targeted siRNA-mediated silencing of AKT3 is sufficient to suppress melanoma progression and induce cell death, emphasizing the oncogenic potential of deregulated AKT3 activation.

AKT signaling affects numerous cellular process: it influences cell cycle dynamics through regulation of the G1/S phase regulator Cyclin D3 (Spofford et al. 2006); affects cell growth, metabolism, and proliferation via control of VEGF expression and interplay with mTOR and the TORC1 and TORC2 complexes (Bhaskar and Hay 2007; Govindarajan et al. 2007; Levine et al. 2006); contributes to invasive behavior by NF-kB-mediated regulation of matrix metalloproteinase-2 and -9 (Kim et al. 2001); and suppresses apoptosis by inhibiting the expression of pro-apoptotic proteins such as Bad and caspase-9 (Cardone et al. 1998; Datta et al. 1997). Each of these AKT effects can be attenuated or suppressed by the antagonistic function of PTEN. Importantly, deregulation of the ERK–c-Jun signaling axis in melanoma leads to c-Jun-mediated transcriptional upregulation of PDK1, further enhancing AKT activation (Lopez-Bergami et al. 2010). PDK1 has also been demonstrated to make critical AKT-independent contributions to tumorigenesis via activation of its substrate SGK3/CISK; this has been shown for breast cancer, among others (Vasudevan et al. 2009).

Recent studies using genetic melanoma models have substantiated the role of PDK1 in the development and progression of melanoma. Thus, melanocyte-specific inactivation of PDK1 in the $Braf^{V600E}$::$Pten^{-/-}$ mouse model delayed the formation of tumors and largely abolished the metastatic lesions commonly seen in this model. Consistent with these findings, examination of melanoma tissue microarrays revealed upregulation of PDK1 in primary melanomas compared with nevi (Scortegagna et al. 2014). Further dissection of the pathways underlying the PDK1 effects on melanoma development identified a role for the PDK1 substrate SGK3. Indeed, inhibition of SGK3 partially phenocopied the changes seen upon PDK1 inhibition. Interestingly, a synthetic lethal screen for kinases that may synergize with PDK1 in eliciting these effects identified PI3K, suggesting that concerted inhibition of the PI3K–PDK1 axis alone may suffice to inhibit growth of BRAF-mutant melanomas (Scortegagna et al. 2014, 2015).

7.3 Cell Cycle Regulation

Malignant melanoma cells are highly proliferative and often exhibit genomic instability (Hazan et al. 2002; Henrique et al. 2000; Satoh et al. 2000; Soyer 1991; Steinbeck et al. 1996; Urso et al. 1992). Such an aggressive proliferative state results from the specific expansion of transformed cells with imbalanced signal transduction favoring proliferation while deregulating normal replicative senescence and apoptotic signaling (Bennett 2008). Accordingly, the stringent cell cycle regulatory mechanisms that govern cell proliferation in normal skin tissues are frequently impaired during melanoma development. For instance, proper function of the G1/S checkpoint that restricts cell cycle progression is often lost in melanoma (Sauroja

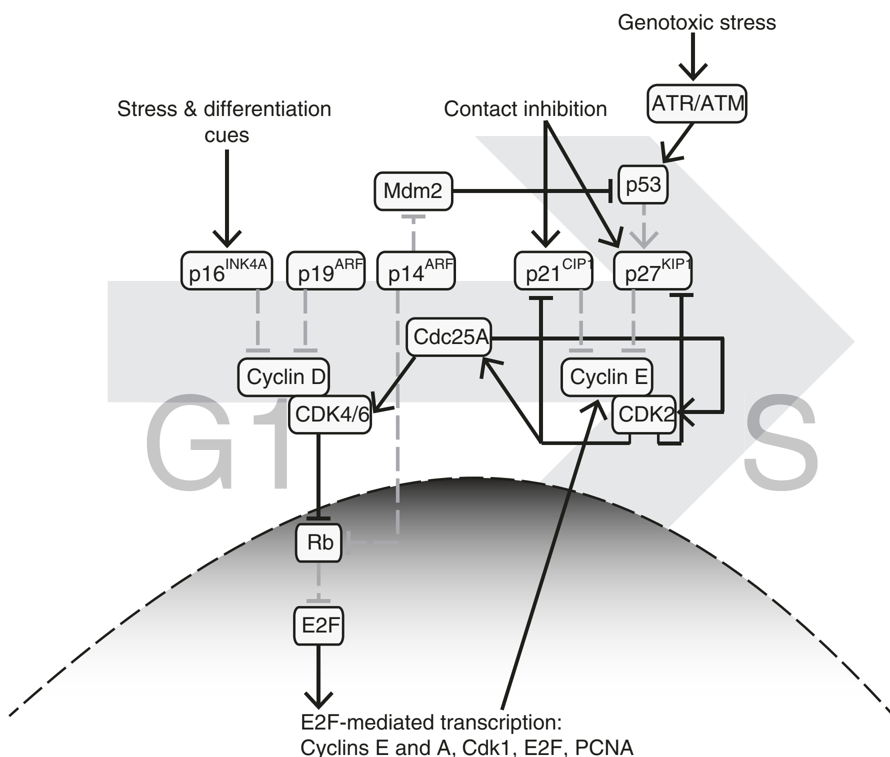

Fig. 7.5 Cell cycle control. In melanoma, loss of CDK2NA compromises major branch points in the regulatory pathways governing G1/S progression. Loss of p16INK4A, p19ARF, and p14ARF causes deregulation of Cyclin D/CDK4/6 and subsequent deregulation of Rb and p53 function, thereby promoting enhanced E2F transcriptional activity and cell cycle progression. Loss of function of other cell cycle inhibitors, such as p21CIP1 and p27KIP1, results in enhanced Cyclin E/CDK2 activity, which positively feeds back on itself and on CDK4/6 via CDC25. Branches of the cell cycle regulation that are upregulated in melanoma appear in *black*; downregulated branches appear in *dashed gray*

et al. 2000). Similar perturbations in signaling can be traced to specific familial mutations or epigenetic dysregulation that result in the downregulation of tumor suppressor genes that negatively regulate the cell cycle or the upregulation of oncogenic genes that promote cell cycle progression.

Patients afflicted with familial melanoma commonly exhibit conserved mutations in 9p21, a chromosomal locus associated with deregulation of cell cycle control. The 9p21 locus comprises the CDK2NA gene, which encodes p16INK4A and its alternate reading frames p14ARF and p19ARF. These proteins negatively regulate cell cycle progression and contribute to senescence through their control of cell cycle-promoting proteins such as Cyclin D and E and the transcription factor E2F1 (Fig. 7.5) (Bandyopadhyay and Medrano 2000; Ranade et al. 1995). Furthermore, p16INK4A binds to and inhibits the cell cycle-promoting kinase CDK4, with concomitant effects

on Rb and its control of E2F in the cell cycle. Germline mutations in CDK2NA have been reported in melanoma (Koh et al. 1995; Ranade et al. 1995) and result in amplified CDK4-mediated signaling, thereby perturbing normal cell cycle control. The increased CDK4 activity observed in melanoma can also result from mutations in the CDK4 gene (Chudnovsky et al. 2005). These perturbations impair proper cell cycle control and appropriate entry of melanocytes into senescence (Bandyopadhyay and Medrano 2000; Haferkamp et al. 2008; Rane et al. 2002).

Mutational perturbation of the alternate reading frame gene product p14ARF also promotes cellular proliferation. Indeed, mutation of ARF has been demonstrated to synergize with RAS mutations in promoting melanoma tumor development (Ha et al. 2007). p14ARF normally contributes to p53 function by targeting and suppressing the p53 negative regulator, Mdm2. Thus, although mutations in p53 are relatively rare in melanoma, its activity can be downregulated by the increased Mdm2 levels induced by mutational silencing of ARF (Freedberg et al. 2008). Of note, there have been few reports on upregulation of Mdm2 or downregulation of p53 expression or activity in melanoma (Bardeesy et al. 2001) (see also Chap. 4). Hence, the precise contribution of p53 to melanoma development remains unclear. Among the possibilities currently being explored is that p53 is partially inactivated, impairing its ability to control cell cycle arrest or apoptotic cues in melanoma.

Enhanced proliferation of melanoma cells can also be elicited by alterations in other negative regulators of cell cycle progression, including Rb. Notably, Rb is silenced in melanoma (Yang et al. 2005) as a result of nonsense mutations or of inactivating phosphorylation of the translated protein (Bartkova et al. 1996; Brantley and Harbour 2000). Loss of Rb function can also contribute to abrogation of melanocyte senescence (Haferkamp et al. 2008).

In addition to genetic mutations, alterations in the epigenetic regulation of core cell cycle and proliferation genes also contribute to melanoma development and progression. Direct modification of chromatin structure, such as by aberrant promoter hypermethylation, results in the CDK2NA silencing reported in multiple melanoma types (Straume et al. 2002; van der Velden et al. 2001). CDKN2A silencing can also be achieved via upregulation of repressor proteins. One example in melanoma is overexpression of the CDKN2A transcriptional repressor Id1 (Healey et al. 2010). Suppression of p16INK4A in melanoma is also mediated by ß-catenin (Delmas et al. 2007). The histone methyltransferase EZH2 has been implicated in the epigenetic repression of the CDK2NA locus and is upregulated in melanoma via a non-canonical NF-kB pathway. Inhibition of this pathway promotes senescence by inducing re-expression of p16INK and p21 (De Donatis et al. 2016).

Recent studies have highlighted the role played by miRNAs in the epigenetic control of melanoma progression (Jukic et al. 2010). Several miRNAs that suppress proliferation are downregulated during melanoma progression, including miR-let-7 and miR-34a. miR-let-7 targets numerous cell cycle proteins, including Cyclin D1/D3/A, and is expressed at lower levels in melanoma compared with nevi (Schultz et al. 2008). Expression of miR-34a, a transcriptional target of p53, is sufficient to induce G1 arrest/senescence and can act as a tumor suppressor by targeting c-Met. However, in melanoma, miR-34a is silenced by aberrant CpG promoter methylation

(Lodygin et al. 2008), which derepresses the cell cycle proteins Rb, CDC2, and E2F3, among others (Satzger et al. 2010; Yan et al. 2009). Deregulation of other miRNAs, including miR-210 and miR-15b, have been demonstrated to promote melanoma tumorigenesis (Satzger et al. 2010; Zhang et al. 2009) (see also the section on miRNAs and melanoma).

7.4 Therapeutic Targets

The MAPK–MEK–ERK and PTEN–P13K–AKT pathways are recognized to be critical determinants of melanoma development and progression, and an intensive effort is under way to develop inhibitors of components of these pathways (Madhunapantula and Robertson 2009; Meier et al. 2005; Russo et al. 2009). A series of specific inhibitors of $BRAF^{V600E}$ showed impressive results in early clinical trials (Kumar et al. 2004). However, the initial success was tempered by the high incidence of therapy-resistant tumors, limiting the effectiveness of these specific drugs (Flaherty et al. 2010). In recent years, extensive work on the molecular basis for this resistance has pointed to diverse mechanisms, most of which cause amplification of the downstream MAPK signaling pathway and bypass the effects of the BRAFi. One emerging approach to overcome resistance is the use of immunotherapeutic drugs that do not directly target the tumor per se, but instead unleash the anti-tumor immune response. Among these therapies are antibodies to CTLA-4 and PD-1, which overcome the drug-resistant tumor phenotype by blocking inhibitory immune checkpoints. These agents have recently been assessed as first-line therapy or as combination therapies with selective BRAFi or MEKi (Larkin et al. 2015; Menzies and Long 2013).

7.4.1 Overcoming Resistance to Targeted Therapies

To date, the use of single agents as first-line therapy has demonstrated only limited clinical efficacy. This disappointing outcome has been attributed to the unexpected plasticity of tumors, as reflected by their ability to adapt to harsh growth conditions and become resistant to initially effective drugs. The mechanisms for achieving resistance largely center on signal transduction pathways that have been rewired, either by genetic mutation or alteration in epigenetic control (Smalley et al. 2009; Emery et al. 2009).

Although BRAF inhibitors suppress tumor growth, the effect is transient, and the tumor cells eventually develop one or more “salvage” mechanisms that bypass BRAF or MEK inhibition. Such mechanisms include upregulation of activated CRAF activity (Gollob et al. 2006; Hatzivassiliou et al. 2010; Kaplan et al. 2011; Montagut et al. 2008; Paraiso et al. 2010; Tsai et al. 2008; Wellbrock and Hurlstone 2010); mutation of NRAS, which leads to CRAF activation and bypasses BRAF inhibition (Nazarian et al. 2010); upregulation of COT, which activates ERK in a MEK-dependent and RAF-independent manner (Johannessen et al. 2010); and upregulation of RTKs (Nazarian et al. 2010) (see section 7.1.1.2 on RTKs).

Melanomas may also develop resistance to BRAF-targeting therapies via upregulation of AKT3-dependent mechanisms (Shao and Aplin 2010), which is consistent with the reported cooperation between mutant BRAF and active AKT (Dankort et al. 2009). Additionally, resistance to BRAFi can be achieved by alternative splicing of BRAF (Wellbrock et al. 2004), which results in a protein lacking the RAS-binding domain due to a silent mutation in intron 8 (Salton et al. 2015). This BRAF isoform dimerizes even in the presence of low levels of RAS, conferring drug resistance through reactivation of the ERK pathway.

Combined targeting of MEK and BRAF results in additive and synergistic effects on progression-free survival of melanoma patients, with a 67 % response rate and 93 % overall survival at 6 months (Flaherty et al. 2012a; Flaherty et al. 2012b). Nevertheless, resistance to MEKi emerges through the same mechanisms seen in BRAFi resistance, including increased CRAF activity and mutation of both NRAS and MEK1 (Greger et al. 2012).

Most tumor resistance mechanisms result in increased activity of the translation initiation complex, which supports the translation of cancer-driving genes such as oncogenes and cell cycle/DNA damage response genes. Hence, partial disruption of the eIF4F complex is a potential therapeutic strategy for drug-resistant tumors, including melanoma. In support of this approach, recent studies have demonstrated efficacy in overcoming melanoma resistance to therapy by targeting eIF4F with silvestrol and several flavaglines (Boussemart et al. 2014), and by targeting a key component of the eIF4F complex, eIF4G1, with the small molecule SBI-756 (Feng et al. 2015).

7.4.2 Immunotherapy

A great deal of effort has been devoted to the use of immune-based therapies to overcome drug resistance in melanoma (Hu-Lieskovan et al. 2014; Vanneman and Dranoff 2012), but the success rate has been low and pronounced toxicity has been observed in most cases. The notion that the immune system could be manipulated to enable a global attack on tumors was initially met with skepticism, largely due to fears that uncontrolled activation would lead to autoimmunity. However, the pioneering work of Drs. Allison and Honjo introduced the immune checkpoint molecules, CTLA-4 and PD-1, respectively, as new paradigms for cancer immunotherapy (Leach et al. 1996; Okazaki et al. 2013; Peggs et al. 2006). Targeting of CTLA-4 circumvents downregulation of T-cell proliferation, whereas PD-1 blockade is likely to affect both activation of T cells and the direct anti-tumor activity of effector T cells.

Clinical trials with anti-CTLA-4 antibodies have shown unexpected success, with an overall response rate of about 20 %, albeit with notable toxicity (Attia et al. 2005; Hodi et al. 2010). Clinical trials with anti-PD-1 antibodies have achieved greater response rates (30–40 %) and significant increases in patient survival (Topalian et al. 2014). More recently, combination therapy with anti-CTLA-4 and anti-PD-1 achieved about 60 % response rate and 79 % 2-year survival rate (Topalian

et al. 2014). Other ongoing work includes the evaluation of combination therapies of immune checkpoint blockers with BRAFi or MEKi, which have shown promising results in pre-clinical experiments (Hu-Lieskovan et al. 2015).

7.5 Epilogue

Our understanding of the mechanisms underlying the development, progression, and drug resistance of melanoma has increased significantly in recent years. At the same time, we have come to appreciate that a major obstacle to achieving sustained therapeutic responses is the innate plasticity of tumor cells, which allows them to adapt to harsh conditions, withstand therapy, and acquire metastatic ability. This means that the rewired signaling observed in tumor cells could be further changed by the tumor microenvironment or by stress imposed by the chemotherapeutic drugs. Thus, we must divert from our current approach to cancer therapy—more intense targeting of a mutated pathway—to find new therapeutic modalities. These include fine-tuning the immune checkpoint machinery to enable a concerted immune attack on the tumor, and targeting the central mechanisms that provide a global advantage to the tumors. Among the latter mechanisms are the translation initiation complex and the unfolded protein response, which are cardinal nodes for tumor-driving genes and may offer a more global approach to targeting the plastic tumor. These molecular hubs have already garnered attention and we may expect an exciting new cadre of modulators to reach clinical evaluation in the coming years.

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8 Proteases in Melanoma

Paola Zigrino and Cornelia Mauch

8.1 Introduction

Activity of proteolytic enzymes expressed by tumor cells is involved in sustaining cellular growth and invasion. In addition, cross-communication of tumor cells and the stromal compartment, thereby including cellular and structural components, further contributes to cancer progression. Such interactions are particularly important for the dissociation of tumor cells from the primary tumor, the invasion of the surrounding connective tissue, and the penetration of vessel walls. While tumor cells invade and degrade the neighboring stroma, they generate a permissive microenvironment convenient for their growth, migration, and metastatic spread.

The tumor stroma, including that of melanoma, is composed of matrix proteins and a rich cellular population that includes fibroblasts, smooth muscle cells, endothelial cells, dendritic cells, macrophages, and other inflammatory cells. Several molecular modifications of the microenvironment occur by the activity of tumor cells or by stimulated stromal cells and include release of matrix or cell surface-bound factors, bioactive matrix fragments as well as cell–cell and cell–matrix contacts (see also Chap. 14). All these events are controlled by the activity of several proteolytic enzymes produced by either tumor or stromal cells (Fig. 8.1). The major proteases involved in these processes are classified into serine-, cysteine-, aspartyl-, and metalloproteinases according to structural characteristics of their active enzymatic center. In this review, we will give an overview of the expression and activity of those enzymes that have been implicated in the development of malignant melanoma and their role in the pathogenesis of this disease.

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_8

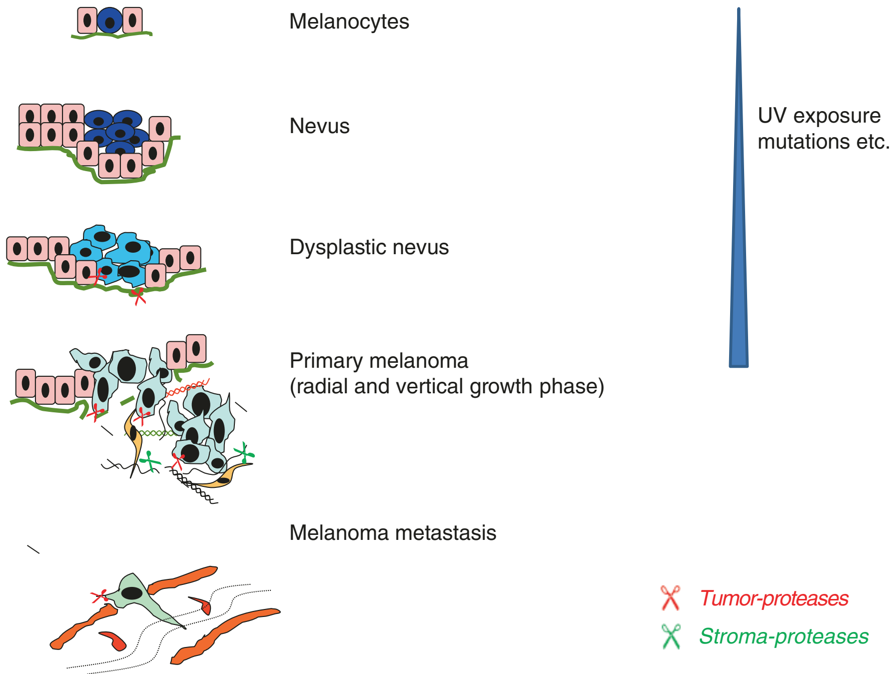

Fig. 8.1 Transformation of melanocytes or a nevus to melanoma. Benign transformation of melanocytes due to accumulation of mutations (e.g., upon UV exposure) into a nevus which progresses into a dysplastic nevus. The following step into a malignant radial and vertical growth melanoma is characterized by the activity of several proteolytic enzymes. Proteolytic enzymes either derived from tumor cells or activated stroma mediate cellular functions, leading to malignant cell progression through the tissue and contributes to the intravasation of melanoma cells into blood and lymph vessels

8.2 MMPs

Expression of matrix metalloproteinases (MMPs) in normal skin is almost undetectable; however, a low but constant expression of some MMPs is necessary for skin homeostasis. In normal skin, matrix remodeling occurs by a slow but continuous synthesis, constitutive degradation and reconstruction of matrix components. MMPs are strongly involved in the controlled degradation of connective tissue during wound repair, in the regulation of tissue architecture, and in embryonic development (reviewed in Page-McCaw et al. 2007).

Increased expression of MMPs has been observed in different malignancies and has been implicated in enhanced extracellular matrix (ECM) degradation. On the contrary, altered ECM deposition and degradation resulting from modifications of the endogenous tissue inhibitors of metalloproteinases (TIMPs) and collagenase expression such as during aging may lead to a higher cancer incidence (Lu et al.

2011) (see also Chap. 11). On a cellular level, once induced, MMPs may be either expressed on the cell surface, leading to pericellular proteolysis, or secreted, thus exerting their activity in the surrounding areas. Interestingly, membrane-associated proteases can also exert their activity in peritumoral areas as they can be associated and released through exosomes. This was observed in a proteomic profiling of exosomes derived from various melanoma cell lines. In this study, expression of MMP-14, ADAM-10, and also the inhibitor TIMP-3 was identified in these structures (Lazar et al. 2015).

In melanoma tissues, MMPs are mainly located at the tumor–stroma border and are produced by either tumor cells or stromal cells that are activated by tumor-derived soluble factors (Moro et al. 2014). In addition, growth factors and cytokines secreted by tumor-infiltrating inflammatory cells also modulate MMP expression in melanoma and stroma cells (Coussens et al. 2002).

In vitro and in vivo analysis have indicated an important role of the matrix itself for the induction of MMPs such as MMP-2/-9, and MMP-14 in invasive melanoma (Hofmann et al. 2000; Kurschat et al. 1999). Expression of MMP-14 was mainly localized in melanoma cells located at the invasive front of the tumor (Hofmann et al. 2000; Kurschat et al. 2002). This indicates that localization of both MMP-2 and MMP-14 at the interface between tumor and stroma may be required for enzyme activation and matrix degradation and highlights how the different cells cooperatively act to promote tumor progression. Indeed, the activity of MMP-2 in these peripheral areas of the tumors was suggested to be necessary to process collagen type I, to cleave fibronectin, and to promote adhesion and migration of melanoma cells (Jiao et al. 2012). Expression of the interstitial collagenases MMP-1 and −13, which are key enzymes in the cleavage of fibrillar collagens, was detected during the invasive vertical growth phase in melanoma cells thus indicating that this is a late event in melanocytic tumor progression (Airola et al. 1999). Expression of MMP-1 was also found in melanoma cells from radial growth phase (RGP) and lead to enhanced tumor growth and conferred metastatic capability in vivo (Blackburn et al. 2009). Interestingly, despite the fact that MMP-1 displays several promoter polymorphisms correlating with tumor ulceration status, it does not significantly associate with overall survival and other clinical factors (Liu et al. 2012; Wang et al. 2011).

However, even though the studies from Airola et al. (1999) show expression of both collagenolytic enzymes in tumor cells in vivo, several other in vivo and in vitro studies have indicated production of these enzymes primarily by peritumoral fibroblasts (Loffek et al. 2005; Uria et al. 1997; Wandel et al. 2000; Zigrino et al. 2009). We have recently shown that stromal expression of MMP-13 plays an important role in tumor growth of melanoma. Melanoma cells injected into the skin of MMP-13 depleted mice formed smaller tumors and developed fewer metastases as compared to wild type animals (Zigrino et al. 2009). Reduced tumor growth and metastasis is likely caused by the lack of peritumoral MMP-13, which is found to be strongly expressed in tumor-associated fibroblast-like cells. In addition, we and others could also show that MMP-13 modulates tumor vascularization by mediating the release

of matrix-bound vascular endothelial growth factor (Lederle et al. 2010). In line with these data, MMP-13 has been associated with metastasis and poor survival in melanoma patients (Zhao et al. 2015). Interestingly, in this study, processing of laminin-332 or VE-cadherin by MMP-13 was shown to accelerate metastasis, but inhibited vasculogenic mimicry in vitro. Increased production of MMP-2 and MMP-14, and deposition of the γ2 chain of laminin 332 and/or its cleavage fragments have been observed in melanoma cells in vitro (Seftor et al. 2001). This process also likely contributes to tumor progression in vivo where the cleaved form of laminin 332 was found in tumors and in tissues undergoing remodeling but not in quiescent tissues (Lohi 2001; Patarroyo et al. 2002).

Besides MMP-13, several other MMPs were shown to contribute to both pro- and anti-angiogenic processes during tumor development. Recent studies have identified a novel mechanism by which MMP-1 promotes angiogenesis. MMP-1 proteolytically activates protease activated receptor-1 (PAR1), a thrombin receptor that is highly expressed in endothelial cells (Blackburn and Brinckerhoff 2008; Boire et al. 2005).

MMP-14 can facilitate endothelial cell migration and tube formation processes in vitro and in vivo (Chun et al. 2004; Mimura et al. 2009). In contrast, the anti-angiogenic activity of MMPs has been shown by MMP-12 and MMP-9 that hydrolyze plasminogen to form the angiogenesis inhibitor angiostatin (Patterson and Sang 1997; Raza et al. 2000). The importance of MMP-14 in invasive and angiogenic processes was recently highlighted by in vivo studies showing inhibition of human tumor xenograft upon supplying a specific antibody against active MMP-14 (Devy et al. 2009). Meanwhile, various endogenous angiogenesis inhibitors were identified which are released by MMP-mediated proteolytical cleavage of plasma proteins or ECM components, such as collagen XVIII for the generation of endostatins, perlecan for that of endorepellin, and collagen IV for the generation of tumstatin (Fjeldstad and Kolset 2005; Hamano and Kalluri 2005). Thus, the balance between MMP's pro- and anti-angiogenic activities and their tissue inhibitors is likely to dictate the outcome during tumor development. Apart from its role in angiogenesis, MMP-14 expression in melanoma cells was shown to be important for the processing of Notch1, which in turn sustains melanoma growth (Ma et al. 2014). As a feedback loop, Notch1 can drive furin expression thus leading to enhanced expression and activation of MMP-14 and ADAM-10 both contributing to melanoma development (Qiu et al. 2015). Most importantly, MMP-14 can negatively regulate transcription of the tumor suppressor SPRY4 via an MMP-2/RAC1 pathway and alter melanoma cell motility, even though it is unclear whether proteolytic activity is required for this event (Shaverdashvili et al. 2015). Two further membrane-associated MMPs, the MT2-MMP (MMP-15) and MT3-MMP (MMP-16) are increased in primary and metastatic melanoma cells. In addition, MMP-16 is overexpressed in human melanoma metastases and metastatic melanoma cell lines (Ohnishi et al. 2001). Interestingly, expression of MMP-16 is associated with rapid fibrin and poor collagen invasion, suggesting that MMP-16 might be important for infiltration of melanoma cells into the perivascular space which is frequently

abundant with fibrin (Tatti et al. 2011). More recently, it has been shown that MMP-16 regulates expression of MMP-14 and L1CAM thereby supporting nodular-type growth of melanoma cells and steering the cell collectives into lymphatic vessels (Tatti et al. 2015). MT4-MMP (MMP-17) was shown to be highly expressed in two melanoma cell lines (A375 and G-361) as well as in normal skin, the precise role of MMP-17 expression has not been investigated thus far in the context of melanoma (Grant et al. 1999).

MMP-9 was also found to possess tumor-inhibiting activity. By grafting melanoma or lung carcinoma cells in either wild type or MMP-9 depleted animals it was demonstrated that this metalloproteinase functions as a metastasis suppressor but does not interfere with the growth of grafted tumors (Gutierrez-Fernandez et al. 2007). MMP-9 mediates the enzymatic cleavage of the basement membrane collagen type IV, generating the proteolytic fragment, tumstatin, that functions as an endogenous inhibitor of pathological angiogenesis and suppresses the activity of endothelial cells as well as growth of melanomas (Hamano and Kalluri 2005). In addition, using bone marrow transplantation in MMP-9 knockout mice with bone marrow-derived cells of MMP-9 control animals, peritumoral inflammatory cells were identified as the stromal source of MMP-9 during epithelial carcinogenesis and are required to sustain tumor growth (Coussens et al. 2000). Thus, to address the role of an MMP for tumor progression solely by expression studies may be insufficient, whereas addressing enzymatic activities may rule out the specific role of a proteolytic enzyme in pathological processes.

Another important role of MMPs is the shedding of cell surface receptors such as cleavage of E-cadherin (see also Chap. 9). Shedding of these cell–cell receptors has been shown to occur by the activity of MMP-7 and MMP-3, thereby regulating the invasive capacities of transformed or injured epithelial cells (McGuire et al. 2003; Noe et al. 2001). A significant increase in serum-soluble E-cadherin levels was detected in melanoma patients with advanced disease; thus suggesting that in melanoma, shedding, apart from transcriptional regulation of the receptor, contributes to render melanoma cells refractory to regulation mediated by keratinocytes (Billion et al. 2006; Hsu et al. 2000). Another example for MMP-mediated receptor shedding is that of CD44, known as the receptor for hyaluronan. Cleavage of CD44 by MMP-14 results in increased melanoma cell motility in vitro and likely plays a role for tumor progression in vivo (Kajita et al. 2001; Nakamura et al. 2004). Due to its structure as a transmembrane protein, MMP-14 was demonstrated to localize at specialized membrane areas such as lamellipodia. In agreement with this observation, high local concentrations of active MMP-14 on the cell membrane are believed to play an important role in cellular migration of melanoma cells (Friedl and Wolf 2008). It was also suggested that cancer cells may invade independently of protease activities employing an amoeboid-type of migration (Wolf et al. 2003). In amoeboid melanoma cells, this migratory “modus” would occur via regulation of actomyosin contraction by a proteolysis-independent mechanism mediated by MMP-9 (Orgaz et al. 2014). Interestingly, by using a zebrafish melanoma model, it could be shown that invasive cells switch from a protease-independent to an MMP-14-dependent

invasion mode, which allows cooperative invasion of poorly invasive cells, thus preserving heterogeneity in the tumor (Chapman et al. 2014).

It is now generally accepted that MMPs may exert both pro- and anti-tumoral activities. Thus, despite their increased expression and correlation with advanced stages of tumors, caution must be taken in interpreting expression data solely. One example is the neutrophil collagenase, MMP-8. MMP-8 ablation in mice strongly increased the incidence of skin tumors. Notably, restoring the neutrophil pool by bone marrow transplantation re-established the natural protection against tumor development in male mice (Balbin et al. 2003). In addition, in high invasive breast cancer cells, transduction with MMP-8 decreased the metastatic performance in vitro and in vivo (Montel et al. 2004). A study by Palavalli et al. (2009) has identified 23% somatic mutations of MMPs in human melanoma, and five of these were found in the *MMP-8* gene associated with the loss of its enzymatic activity. Forced expression of the wild type gene resulted in reduced melanoma growth in vivo suggesting that this MMP is a tumor suppressor (Palavalli et al. 2009). Later, Debniak et al. (2011) suggested that *MMP-8* gene variation might be associated with an increased risk of malignant melanoma (Debniak et al. 2011).

8.3 ADAMs

ADAMs (a disintegrin and metalloproteinases) are likely to contribute to the proteolytic events necessary for tumor invasion and metastasis (Bergers and Coussens 2000; Schlondorff and Blobel 1999). Most of these proteins contain a metalloproteinase, a disintegrin, a cysteine-rich domain, and a transmembrane domain, some are also found as secreted proteins (Edwards et al. 2008). Based on their domain structure, these proteins can exert modulating effects on both the migratory behavior of tumor cells as well as on the proteolytic processes with regard to tumor invasion. In support of this, their overexpression has been detected in a variety of tumors (reviewed in Murphy 2008). Extensive expression analysis has been performed on breast and pancreatic carcinomas where expression of ADAM-9, −10, −12, and −15 have been reported to be increased (Murphy 2008). However, very limited information is available on the role of the different ADAM family members in the pathogenesis of melanoma. In our own studies, we found ADAM-9 being strongly expressed in primary melanomas and low in metastases (Zigrino et al. 2005). The importance of ADAM-9 in melanoma growth was further substantiated in animals depleted of ADAM-9 expression (Abety et al. 2012; Guaiquil et al. 2009). Moreover, down-regulation of ADAM-9 synthesis (by miR-126&126*) in the tumor cell but not in the stroma of the host, resulted in reduced melanoma growth and metastasis in nude mice (Felli et al. 2013). Presently, the contribution of this protease to development, invasion, and metastasis of melanoma is unclear. Another member, ADAM-10, was also found to be regulated in melanoma. However, opposite expression levels were detected at high amounts in metastases and at low levels in primary tumors (Lee et al. 2010). Although the exact role of ADAM-10 in tumorigenesis is not understood, the very recent identification of ADAM-10 mediated constitutive

shedding of CD44 in human melanoma cells favors the idea that this protease facilitates melanoma cell proliferation (Anderegg et al. 2009). More recently, ADAM-10 has been correlated with poor progression-free survival of uveal melanoma patients. In vitro, despite cleaving the HGF receptor c-met, ADAM-10 also mediates HGF-induced uveal melanoma cell invasion (Gangemi et al. 2014).

In human tissues, ADAM-15 is expressed in melanocytes and endothelial cells of benign nevi and melanoma tissue; however, it is significantly downregulated in melanoma metastases compared to primary tumors (Ungerer et al. 2010). ADAM-15 overexpression in melanoma cells reduced invasion and growth in vitro, suggesting a tumor suppressor role for ADAM-15 in melanoma. Constitutive inactivation of ADAM-15 resulted in reduced growth and metastasis of injected melanoma cells to lungs and lymph nodes, thus suggesting a pro-metastatic role of ADAM-15 in melanoma when expressed by the host (Horiuchi et al. 2003; Schonefuss et al. 2012).

ADAM-8 has been recently described as a negative regulator of retinal vascularization and of growth of heterotopically injected B16F0 melanoma cells (Guaiquil et al. 2010) by modulating processing of several molecules involved in angiogenetic processes such as CD31, EphB4, and ephrinB2. However, in human melanoma, expression of this protease has not been investigated. Inactivation of ADAM-17 in endothelial cells significantly reduced pathological neovascularization in a mouse model for retinopathy of prematurity, and affected the growth of heterotopically injected murine melanoma cells. Surprisingly, differences in tumor growth could not be attributed to altered vascularization as no differences in CD31-positive tumor vessels were detected but rather hypothetically to the generation of factors from endothelial cells that contribute to tumor growth (Weskamp et al. 2010). However, all these data are derived from mouse models (see also Chap. 15) and whether these events may also be affected by these ADAMs during development of human melanoma is not clear yet.

Processes mediated by ADAM proteases may be relevant for melanoma growth and invasion, and these include shedding of ectodomains such as the epidermal growth factors. By shedding the ectodomains, soluble, diffusible factors are released and can carry out different biological functions as compared to their membrane-anchored protein form. An example is the EGF pathway, which is active in various cancer cell types and sustains their survival, proliferation, and motility (Edwards et al. 2008). One of the oldest examples for this is the release of the cytokine TNF-α from its inactive membrane-anchored form by ADAM-17 (Black and White 1998). In human melanoma, *ADAM-17* gene is strongly overexpressed in tumors with advanced stage and its expression correlates with the increased TNF-α expression in the blood of patients (Cireap and Narita 2013). Another shedding event relevant for cancer development is that of the ligands of the activating natural killer cell receptor, NKG2D, MHC class I chain-related molecule A and B (MICA and MICB). This shedding studied in vitro in cervix cancer cells is thought to be one of the mechanisms that promote resistance to the immunosurveillance by NK cells (Waldhauer et al., 2008). Another NK cell ligand, namely, B7-H6 is shed by ADAM-10 and −17 and, interestingly, levels of B7-H6 in blood from melanoma patients were found elevated (Schlecker et al. 2014). In other cancer cells, such as hepatocellular

carcinoma, ADAM-9 was responsible for the shedding of MICA and treatment with anticancer drug Sorafenib, causing decreased expression of ADAM-9 followed by increased MICA expression at the cell surface (Kohga et al. 2010). Recently, shedding of the NKG2D ligand MICA was shown to be mediated by MMP-14 and to occur independently from ADAMs (Liu et al. 2010). Which protease is responsible for the shedding of these NKGD2 ligands is likely the result of the expression profile in the tumor cell type involved and needs further investigation.

Shedding of surface receptors mediating cellular interactions was demonstrated for ADAM proteases. E-, N-, and VE-cadherins are substrates for ADAM-10 (Reiss and Saftig 2009) and their shedding results in profound alterations in cellular interactions, migration, and cell proliferation. ADAM-10-mediated L1 release is involved in the motility and invasion of lymphoma, lung carcinoma, and melanoma cells (Gutwein et al. 2000; Lee et al. 2010).

Apart from modulating cell adhesion by shedding events, one important aspect of ADAMs is their direct involvement in mediating adhesive events through their adhesive domains. In vitro studies have shown that ADAM-15 interacts with $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins (Nath et al. 1999), whereas ADAM-2 binds to $\alpha_6\beta_1$ integrin and ADAM-9 to $\alpha_6\beta_1$, $\alpha_v\beta_5$, and $\alpha_3\beta_1$ integrins (Almeida et al. 1995; Nath et al. 2000; Zhou et al. 2001). The interactions of ADAM proteases with cellular receptors have been proven to be of major importance in cell adhesion and fusion processes as for instance during spermatogenesis and myo- and osteogenesis (Edwards et al. 2008). In vitro studies have shown that ADAM-9 can mediate cell–cell interaction of fibroblasts and melanoma cells thereby lead synthesis of proteolytic enzymes in stromal cells (Zigrino et al. 2011). However, ADAM-9 can also be expressed in a soluble form. The secretion of a soluble splice variant of ADAM-9 by activated stromal cells is known to induce colon carcinoma cell invasion in vitro through binding to $\alpha_6\beta_4$ and $\alpha_2\beta_1$ integrins (Mazzocca et al. 2005). Recent studies have identified driver mutations in ADAMs, namely, ADAM-29 and −7, which often occur in melanoma and enhance cell migration or alter cell adhesive capacity (Wei et al. 2011). Nevertheless, limited information is available on the role of the different ADAM family members in the pathogenesis of melanoma.

8.4 ADAMTS

The ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) have also been implicated in cancer development and progression (Rocks et al. 2008). These proteins are characterized by the presence of additional thrombospondin domains which are believed to function as a sulfated glycosaminoglycan binding domain (Tang 2001). High levels of ADAMTS transcripts were detected in tumor biopsies and cell lines from osteosarcoma and melanoma (Cal et al. 2002). ADAMTS-4 exists in three forms all co-expressed in human cancer tissues (Rao et al. 2013). ADAMTS-4 and its proteolytically active N-terminal autocatalytic fragment promoted B16 melanoma growth and angiogenesis in mice, whereas its catalytically inactive mutant increased tumor cell apoptosis (Rao et al. 2013). A

single thrombospondin-type 1 repeat domain was essential and sufficient for the anti-tumorigenic activity displayed by the catalytically inactive ADAMTS-4 isoforms. Considering the diversity in the function of these various forms and their co-expression in the same tissue, it was suggested that a balance between pro- and anti-tumorigenic ADAMTS-4 isoforms would dictate the functional outcome (Rao et al. 2013). Overexpression of full-length ADAMTS-5 diminished angiogenesis, reduced proliferation, and increased apoptosis of tumor cells thus suppressing B16 melanoma growth in mice (Kumar et al. 2012). Finally, ADAMTS-18 was identified as a player in melanoma progression as mutations in the protein reduced growth-factor dependency of tumor cells, increased migration in vitro and led to increased metastasis in vivo (Wei et al. 2010).

The expression and activity of several ADAMTS proteins have not yet been carefully analyzed in melanoma. However, given the various functions attributed to these enzymes in the development of other diseases, their involvement in melanoma development is also conceivable.

8.5 Cathepsins

Cathepsins described in melanomas are both cysteine (B, H, K, and L) and aspartyl proteases (D and E). These enzymes are predominantly intracellularly localized within the lysosomal compartment. Some of these enzymes can be associated with the plasma membrane of melanoma cells in vitro or can be secreted from lysosomes upon cell contact with collagen type I (Klose et al. 2006; Moin et al. 1998). Frohlich et al. (2001) have analyzed the expression of cathepsins in normal skin, nevi, and melanoma samples to obtain information about their role and their regulation in melanoma. Activities and expression of the cathepsins B and L were found to be increased in all melanocytic lesions (Frohlich et al. 2001). In human melanoma and nevi, cathepsin B is prevalently expressed by melanoma cells, whereas cathepsin L is expressed by tumor-associated fibroblasts surrounding invading melanoma cells (Yin et al. 2012). In vitro, cathepsin B was shown to regulate expression/activity of TGF-β thereby leading to fibroblast activation and promotion of tumor invasion (Yin et al. 2012). In contrast, Humbert and Lebrun (2013) found that TGF-β strongly upregulated PAI-1 expression thus reducing plasmin activity and led to inhibition of invasion (Humbert and Lebrun 2013). The reasons for these discrepancies have not been addressed, but will need further clarifications.

Cathepsin H expression correlated inversely with the invasive potential of the lesion (Frohlich et al. 2001). The aspartyl protease cathepsin D has been detected in tumor cells as well as in the peritumoral stroma in melanoma (Kageshita et al. 1995). Expression of cathepsin D has also been inversely correlated with melanoma such that its expression is downregulated in melanoma cells as compared to melanocytes, thus suggesting a role for this enzyme in cellular transformation (Bernard et al. 2003). In vitro, cathepsin B activity was shown to be necessary for the invasion of dermal connective tissue by highly invasive melanoma cells (Dennhofer et al. 2003). Furthermore, forced expression of cathepsin L in human melanoma cells

increased their metastatic potential thus increasing their tumorigenicity (Jean et al. 1996). Neutralization of cathepsin L in vivo by lentiviral transduction of melanoma cells with the anti-cathepsin L ScFv fusion protein resulted in inhibition of the tumorigenic and metastatic phenotype of human melanoma (Rousselet et al. 2004). In contrast, the work from Matarrese et al. (2010) showed that cathepsin B but not L and D inhibition by several targeting approaches could efficiently reduce tumor growth and metastatic potential of human melanoma cells in vitro and in vivo (Matarrese et al. 2010).

Another recently described cysteine protease expressed in melanoma is cathepsin K. This enzyme has been extensively studied during bone resorption processes where it mediates the degradation of various collagens (Bossard et al. 1996). Whereas cathepsin K is not expressed in normal skin, its expression has been localized in skin fibroblasts during scar formation (Runger et al. 2007). Cathepsin K has been detected in nevi and it is increased in human primary cutaneous melanoma and metastasis (Quintanilla-Dieck et al. 2008). A potential function ascribed to cathepsin K in melanoma cells is the intracellular degradation of collagen type IV (Quintanilla-Dieck et al. 2008).

8.6 Serine Proteases

Among the group of serine proteases involved in extracellular matrix degradation, plasmin, plasminogen activators, thrombin, cathepsin G, and elastases are of particular interest.

The serine protease plasmin can activate most proMMPs in vitro (Murphy et al. 1999). However, it has not been proven whether the function as proMMP activator is also fulfilled in vivo. Their proteolytic activity has also been shown towards extracellular matrix components and cell surface receptors thereby influencing the tumor microenvironment in multiple ways (Andreasen et al. 2000).

Release of elastin fragments by the serine protease elastase was shown to enhance melanoma cell invasion through type I collagen and increase angiogenesis by the induction of MMP-14 expression (Hornebeck et al. 2005). One hypothesis advanced by Antonicelli et al. is that these peptides could catalyze the vertical growth phase transition in melanoma through increased expression of MMP-2 and MMP-14 (Antonicelli et al. 2007). Plasminogen activation by the urokinase type (uPA) or the tissue type plasminogen activators (tPA) generates plasmin whose expression is associated with ECM degradation (Li and Wun 1998). Generated plasmin can also excise the angiostatin fragment from plasminogen thus regulating angiogenic processes. uPA is bound to the surface of tumor cells by means of a specific receptor (uPAR) and engagement of this receptor can act as a survival factor for melanoma by down-regulating p53 and therefore inducing an anti-apoptotic effect (Besch et al. 2007). uPA expression has been correlated with the ability of blue nevi to transform, as respective gene ablation in mice inhibited melanoma formation (Shapiro et al. 1996). A synergism between the uPA/uPAR system and MMP-9 was shown to mediate the IFN-γ- and TNF-α-induced invasive phenotype of murine melanoma

cells (Bianchini et al. 2006). Increased uPA and MMP-2/-9 expression, accompanied by down-regulation of E-cadherin, was observed following overexpression of PKC-γ in mammary cells leading to acquired invasive abilities of those cells which became tumorigenic and able to spontaneously metastasize (Mazzoni et al. 2003). Overexpression of Hsp27 in the melanoma line A375 led to a reduced malignant phenotype by increased expression of E-cadherin and of both uPA and its inhibitor PAI-1 (Aldrian et al. 2003).

Human melanoma cells produce tPA which is bound to the cell surface (de Vries et al. 1996). In vivo, high tPA expression has been correlated with good prognosis (Ferrier et al. 2000). In these studies, lesions with more than 51% tPA-positive tumor cells were found to have the best prognosis, as compared to those with lower expression. Further, taking together tPA positivity, Breslow thickness, microscopic ulceration, and sex, showed that the extent of tPA tumor cell positivity was an independent prognostic factor for a distant metastasis-free interval and for the duration of survival (Ferrier et al. 2000). A potential reason for the better outcome is the generation of angiostatin, upon activation of plasmin and thereby cleavage of plasminogen, which may inhibit tumor-induced angiogenic processes.

Another serine protease that has also been investigated in melanoma is thrombin. Thrombin cleaves the N-terminus of protease-activated receptor-1 (PAR-1) that binds the receptor and activates signaling via G-proteins (Shapiro et al. 2000). PAR-1 can also be activated by ligands other than thrombin such as trypsin and plasmin (Kawabata and Kuroda 2000). PAR-1 is overexpressed predominantly in melanoma primary tumors and in metastatic lesions as compared with common melanocytic nevi (Massi et al. 2005). The importance of activating this pathway is described in a recent report from Melnikova et al. (2009). The authors showed that PAR-1 activation leads to the expression of melanoma cell adhesion molecule MCAM/MUC18 (MUC18) thereby mediating melanoma cell adhesion to microvascular endothelial cells, transendothelial migration, and ultimately, lung metastasis retention. The altered expression of a variety of kallikreins has also been associated with melanoma and among these increased hKLK7 was significantly associated with good prognosis and survival (Martins et al. 2011). However, KLK-7 mediated cleavage of midkine leads to reduced pro-proliferative and pro-migratory activity in melanoma cells in vitro (Yu et al. 2015). The apparent discrepancy with the in vivo human expression data may be explained in light of additional KLK-7 proteolytic activities towards, e.g., CYR61 and tenascin-C (Yu et al. 2015), which have not yet been investigated.

8.7 Summary

Although fundamental knowledge about the molecular processes of tumor progression has increased in recent years, the clinical application is still limited.

Concerns remain about the consequences of inhibiting the biological function of cell proteolytic enzymes. Several reports have contributed to gain knowledge of the regulation of proteases and their substrates and highlighted that this process is

complex and multifactorial. Experimental evidence strongly suggests that proteases of the same class may display a dual function depending on the cellular source and act as both a tumor-promoter and suppressor. Further generation of mouse models for in vivo analysis of protease depletion, will help in clarifying the importance of their activity for physiology and pathology. In addition, for more specific targeting, a deeper knowledge of the proteolytic functions is needed and the field of degradomics has already started to uncover new proteases and physiological substrates. These studies identified new and known regulatory pathways that are controlled by proteolytic processing. The problematic for targeting of protease activity (e.g., of MMPs) in vivo has been further discussed by Dufour and Overall (2013).

The direct targeting of these proteases in a cell-specific manner with, for example, monoclonal antibodies or inactivating peptides, or the inhibition of the activity of their processed substrates may be developed as potentially useful therapeutic strategies.

Acknowledgments This work was supported by the Deutsche Forschungsgemeinschaft through the SFB829 and by the Melanoma Research Network of the Deutsche Krebshilfe (Melanoma Verbund).

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Cell–Cell Contacts in Melanoma and the Tumor Microenvironment

9

Silke Kuphal and Nikolas K. Haass

Abbreviations

CDH	Cadherin
Cx	Connexin(s)
Dsc 1–3	Desmocollin
Dsg	Desmoglein
GJ	Gap junction
GJIC	Gap junctional intercellular communication

9.1 Melanoma Microenvironment

This is an update of our previous reviews on this topic (Haass et al. 2004, 2005; Kuphal and Haass 2011; Brandner and Haass 2013).

The state of a cell – quiescence, proliferation, differentiation or cell death – is under normal conditions determined by homeostasis (Bissell and Radisky 2001). A symbiotic relationship between a melanocyte and approximately 36 associated

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_9

keratinocytes, which forms the epidermal melanin unit, maintains this homeostatic balance of the human epidermis (Fitzpatrick and Breathnach 1963; Jimbow et al. 1976). Within the *stratum basale*, the melanocytes keep a lifelong stable ratio of 1:5 with the keratinocytes (Fitzpatrick et al. 1979). This balance is maintained through regulated induction of melanocyte division coordinated through intercellular communication, which can be endocrine and paracrine via soluble factors and/or by direct contact via cell–cell and cell–matrix adhesion, or gap junctional intercellular communication (GJIC) (Haass et al. 2004, 2005). Dysregulation of this homeostasis may cause an imbalance of the epidermal melanin unit and trigger uncontrolled proliferation of the melanocytes, which may lead to the development of a nevus and/or a melanoma (Haass and Herlyn 2005).

Alterations in the interaction between neoplastic cells and their immediate microenvironment play a key role in these processes (Hanahan and Weinberg 2000, 2011; Park et al. 2000). The tumor microenvironment includes (1) the tumor stroma composed of fibroblasts, endothelial cells, immune cells, soluble molecules, and the extracellular matrix (ECM); (2) the tissue where the tumor had originated from; and (3) different sub-compartments within the tumor itself. Signals to and from the stroma via cell–cell and cell–matrix contact and/or via secretion of cytokines and growth factors may lead to a remodeling of the tumor microenvironment and consequently to promotion of melanoma development, growth, and metastasis by inducing angiogenesis, invasion, and migration (Villanueva and Herlyn 2008; Zigler et al. 2011). In addition to the interaction with the tumor stroma, primary melanoma progression as well as cutaneous melanoma metastases impact on the epidermal tumor microenvironment: the multilayered epithelium of the skin (Haass et al. 2010). Finally, different microenvironmental conditions within the tumor itself are created by differential access to nutrients and oxygen (Groebe and Mueller-Klieser 1991; Minchinton and Tannock 2006; Santiago-Walker et al. 2009; Haass et al. 2014; Haass 2015).

The microenvironment is not only important for the primary tumor, but also for colonization of a secondary organ. The "seed and soil" hypothesis implies that the metastatic process depends on the tumorigenic capacity of the cells and – again – on their interactions with the microenvironment (Fidler 2003).

9.2 Adherent Junction of Cadherins

Cross-talk between benign precursor cells, malignant cells, and surrounding host cells influences tumor development. Already in 1914, Theodor Boveri recognized the importance of changes in tumor cell adhesion for the development of cancer (Boveri 1914). Among the molecules involved in this intercellular communication are cadherins, which play a critical role for the homeostasis of normal skin and also during tumor formation and progression (Fig. 9.1). The identification of cadherins in the late 1970s and early 1980s was primarily motivated by an interest in understanding the mechanisms of cell adhesion during development (Franke 2009).

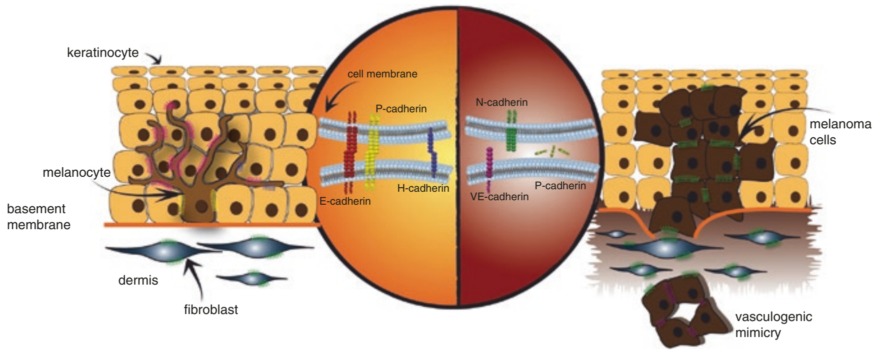

Fig 9.1 Overview of the cadherin repertoire in skin and melanoma (Illustration R.J. Bauer)

Cell–cell as well as cell–matrix adhesions are critical for cells and tissues to respond to mechanical stimuli from their environment. Both cell–cell and cell–matrix adhesions bear intrinsic mechanosensitivity, which allows them to promptly respond to stress and effectively propagate signals controlling cell shape and motility. This mechanosensitive response has been associated with pronounced changes in the size and molecular composition of specific adhesion sites and, consequently, the signals evoked by those adhesion sites. In polarized epithelia of vertebrates, the adherent junction is part of the tripartite junctional complex localized at the juxtaluminal region, which compromises the tight junction (TJ, see below), adherent junction (AJ), and desmosomes (macula adherens).

More than 80 proteins belong to the cadherin superfamily and are separated into the following "adherent junction" (AJ) subgroups in vertebrates:

1. Classical adhesive cadherins of type 1 (6 members) and type 2 (13 members), e.g., E-, N-, P-, R-, and VE-cadherin. The classical cadherin family comprises 19 members that share a common domain organization of five repetitive extracellular calcium-binding subdomains (Overduin et al. 1995). Most of these classical cell–cell adhesion molecules are connected to the actin filaments and microtubules of the cellular cytoskeleton via catenins. The four known catenins, alpha-, beta-, gamma (plakoglobin)-, and delta (p120)-catenin, are important regulatory elements either for sustained cell–cell adhesion or signaling cascades into the cell.
2. The "nonclassical" desmosomal cadherins, transmembrane proteins of desmosomes are, for example, desmocollin 1–3 (Dsc 1–3) and desmoglein 1–4 (Dsg 1–4). They are connected to intermediate filaments.
3. Finally, there are nonclassical cadherins, like the protocadherins (e.g., protocadherin 15, cadherin 23), H-cadherin, and cadherin-like molecules (e.g., Fat, Dachsous, Flamingo, or Ret) belonging to the cadherin superfamily.

The most important classical cell–cell adhesion molecules of the skin and during melanoma development are E (epithelial)-cadherin (CDH-1), N

(neuronal)-cadherin (CDH-2), and P (placental)-cadherin (CDH-3), which belong to the group of calcium-dependent glycoproteins. Certainly, this group of classical adhesion molecules can be extended with atypical VE (vascular endothelial)-cadherin (CDH-5, CD144) and the nonclassical cadherin H (heart)-cadherin (T-cadherin, CDH-13) (Fig. 9.1). In normal epidermis, melanocytes and keratinocytes are mostly connected via E-cadherin, P-cadherin, and H-cadherin (Kuphal et al. 2009; Nishimura et al. 1999; Tang et al. 1994). Whereas melanocytes in the basal layer of the epidermis seem to contain predominantly E-cadherin and H-cadherin, those residing in hair follicles are rich in P-cadherin (Nishimura et al. 1999). In contrast, N-cadherin is expressed on fibroblasts and vascular endothelial cells of normal skin (Hsu et al. 1996).

9.2.1 Loss of E-Cadherin in Tumorigenesis

E-cadherin is the major cadherin in polarized epithelial cells. Furthermore, the crosstalk between melanocytes and keratinocytes mediated by E-cadherin plays an important role in human epidermis. The normal melanocytic phenotype and controlled proliferation of melanocytes are strictly regulated by keratinocytes via E-cadherin. The E-cadherin knockout mouse is lethal in early embryonic stages (Larue et al. 1994) supporting the finding that E-cadherin has an essential role in morpho- and organogenesis. In skin development, there is evidence that E- and P-cadherin play some role in guiding melanocyte precursor cells to their final destination in the epidermis (Nishimura et al. 1999).

Malignant transformation of melanocytes is frequently attended by loss of E-cadherin expression and induction of N-cadherin (Hsu et al. 1996). This leads to the loss of the regulatory dominance of keratinocytes over melanocytes. The degenerated melanocytes/melanoma cells express N-cadherin to get into contact to fibroblasts and vascular endothelial cells during migration and invasion into the tumor stroma, dermis, lymph, and blood vessels (Hsu et al. 2000) (Fig. 9.2). The switch of the cadherin class is an interesting phenomenon of melanoma cells and in epithelial–mesenchymal transition (EMT) in general.

However, immunohistochemical examination of primary melanomas and their metastases has revealed that a proportion of melanoma cells are still E-cadherin-positive and present little, if any, N-cadherin (Danen et al. 1996; Hsu et al. 1996; Sanders et al. 1999; Silye et al. 1998). Therefore, the cadherin switch as an obligatory prerequisite of malignant behavior is still controversial and might depend on the subtype of the melanoma examined. However, immunohistochemistry data could not show whether the expressed E-cadherin is really functionally active regarding adhesion or still possesses signaling function. The general consensus is that E-cadherin is a tumor invasion suppressor.

9.2.1.1 Regulators of E-Cadherin

The mechanism by which E-cadherin expression is lost during malignancy differs between tumor entities. Loss of E-cadherin function can be caused by various genetic or epigenetic mechanisms. In patients with diffuse gastric cancer and breast

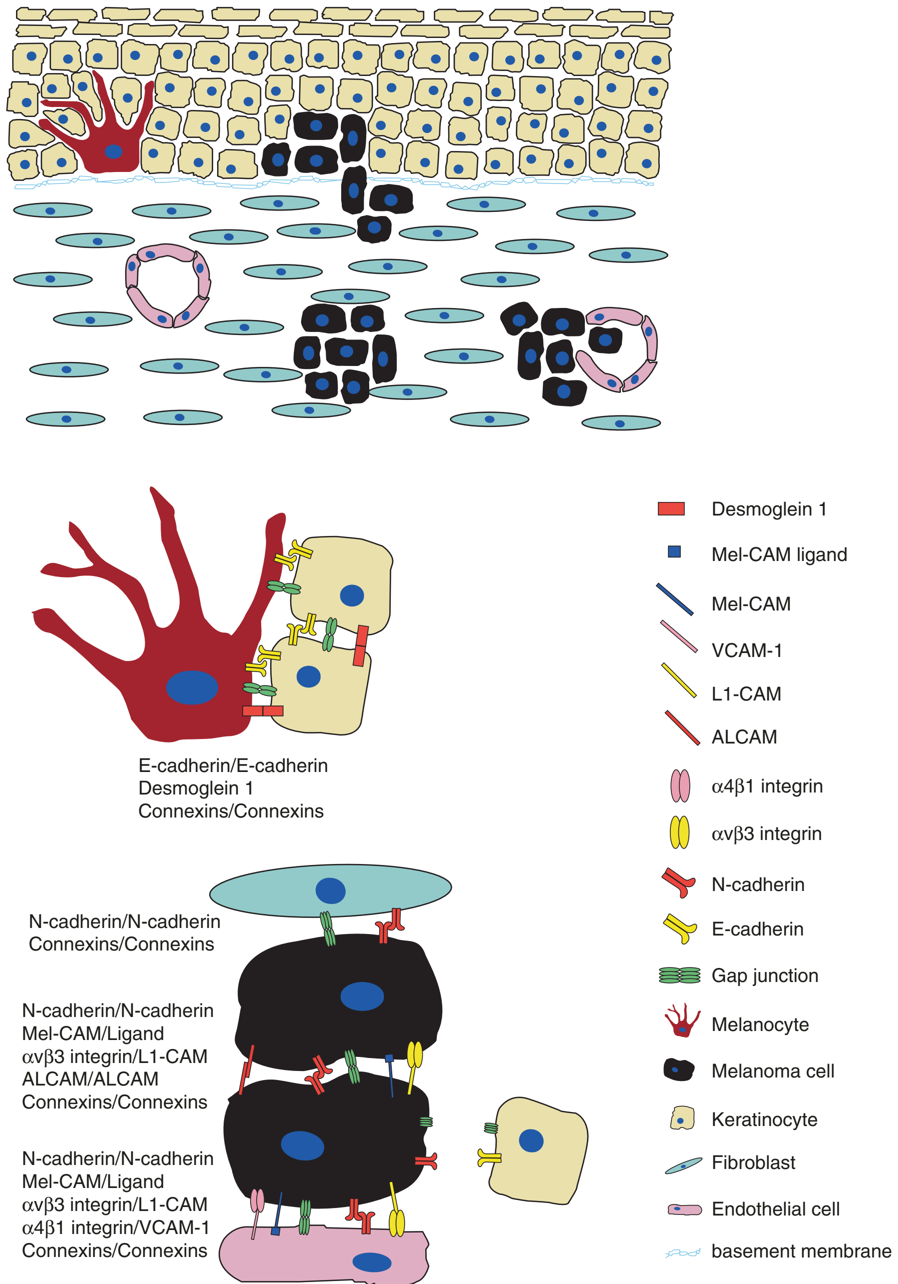

Fig. 9.2 (**a**) Cell–cell adhesion of melanocytes and melanoma cells. Melanocytes adhere to keratinocytes via E-cadherin and desmoglein, which enables them to communicate with each other through gap junctions with cells in their environment. (**b**) In melanoma cells, E-cadherin is downregulated. They interact with each other through N-cadherin, Mel-CAM/Mel-CAM ligand, αvβ3 integrin/L1-CAM, ALCAM, and connexins; with fibroblasts through N-cadherin and connexins; and with endothelial cells through N-cadherin, Mel-CAM/Mel-CAM ligand, αvβ3 integrin/L1-CAM, α4β1 integrin/VCAM-1, and connexins

cancer, the E-cadherin gene is mutated, leading to the expression of a nonfunctional protein (Strathdee 2002). The consequence is abnormal expression and abnormal subcellular localization of cadherin or the components of the cadherin-containing adhesion complex. Further, the *CDH-1* gene locus can be epigenetically silenced by hypermethylation, leading to downregulation of E-cadherin expression which is known from several cancer entities, e.g., hepatocellular carcinoma (Kanai et al. 1997), squamous cell carcinoma (Saito et al. 1998), and thyroid cancer (Graff et al. 1998).

In most cases, E-cadherin expression is downregulated at the transcriptional level. The zinc-finger containing transcriptional repressor Snail1, which is a master regulator of neural crest cell specification and melanocyte migration during development in vertebrates, is mainly responsible for the loss of E-cadherin in melanoma (Batlle et al. 2000; Poser et al. 2001). The level of Snail1 expression correlates directly with the loss of E-cadherin expression, and forces overexpression of Snail in primary melanocytes downregulates E-cadherin expression (Poser et al. 2001). Slug (Hajra et al. 2002; Bolós et al. 2003), Snail2, ZEB1 and ZEB2 (Eger et al. 2005; Caramel et al. 2013), and SIP1 (Comijn et al. 2001), also members of the zinc finger transcription factor family of Snail, are further regulators of E-cadherin in melanoma, as well as basic helix–loop–helix transcription factors E12/47 (Perez-Moreno et al. 2001) and Twist (Yang et al. 2004). Additionally, the T-box transcription factor Tbx3 is overexpressed in melanoma, which enhances melanoma invasiveness through prevention of E-cadherin expression (Rodriguez et al. 2008). Furthermore, within human melanoma lesions, GLI-2, a mediator of hedgehog signaling, is associated with loss of E-cadherin (Alexaki et al. 2010).

Proteolytic degradation of E-cadherin by matrix metalloproteinases (MMPs) is another mechanism by which E-cadherin-mediated cell–cell adhesion can be ablated. In this case, cell surface E-cadherin becomes soluble by cleavage of the extracellular domain, a process known as ectodomain shedding. For melanoma, Adam-10 is responsible for E-cadherin shedding (Billion et al. 2006) (see also Chap. 8).

A family of microRNAs, such as miR-200a, miR-200b, miR-200c, and miR-205 was reported to control the expression level of E-cadherin during the epithelial–mesenchymal transition. The microRNA targets the transcriptional repressors ZEB1 and ZEB2 of E-cadherin (Gregory et al. 2008; Hurteau et al. 2007). As one example for cancer, loss of miR-200c expression is significantly correlated with early stage T1 bladder tumor progression (Wiklund et al. 2011). Another miRNA, miR-373, induces expression of genes with complementary promoter sequences. It was found that miR-373 induces E-cadherin expression by recognizing a target site in the promoter of the *cdh-1* gene (Place et al. 2008). Liu et al. 2012 showed that miR-9 is downregulated in metastatic melanomas compared with primary melanomas. A tumor suppressor effect after re-expression of miR-9 in melanoma is mediated through its direct binding to sites within the NF-kB 3′-UTR, resulting in suppression of Snail1 and upregulation of E-cadherin. However, whether microRNAs are responsible for regulating cadherins directly and specifically in melanoma is still not known (see also Chap. 6).

9.2.2 Loss of P-Cadherin During Tumorigenesis

In human skin, P-cadherin is expressed mainly on cells of the epidermal basal layer (Furukawa et al. 1997) and those melanocytes residing in hair follicles (Nishimura et al. 1999). Concerning carcinogenesis, the effective role of P-cadherin remains an object of debate, since it can behave differently depending on the molecular context and tumor cell model studied. In melanoma cells, loss of full-length P-cadherin was reported (Bachmann et al. 2005; Van Marck et al. 2005; Jacobs et al. 2011). Therefore, P-cadherin has a similar tumor-suppressive behavior to E-cadherin. Additionally, a truncated 50 kDa form of the N-terminal part of P-cadherin was found, which appeared to be secreted from the melanoma cells. If this secreted form of P-cadherin is expressed from melanoma cells, it is responsible for cell migration and invasion (Bauer et al. 2005, 2006; Bauer and Bosserhoff 2006).

9.2.3 Loss of T-Cadherin During Tumorigenesis

T-cadherin (truncated-cadherin, cadherin 13, gene name *CDH13*) or H-cadherin, named for its strong expression in the heart, is an atypical member of the cadherin family, lacking the classical HisAlaVal recognition motif at its N-terminus, lacking the typical transmembrane and cytosolic domains and possessing a glycosylphosphatidylinositol moiety that anchors T-cadherin into the outer plasma membrane.

Immunohistochemistry of melanoma tissue samples showed positive T-cadherin staining of the endothelial cells. T-cadherin expression in endothelial cells was demonstrated to be redox sensitive (Joshi et al. 2008). The melanoma cells themselves showed loss of T-cadherin whereas healthy skin showed staining of melanocytes and keratinocytes of the basal layer of the epidermis. Loss of T-cadherin in melanoma is associated with migration and invasion of the cells (Kuphal et al. 2009). In general, the exact functional role and signaling of T-cadherin for melanoma cells itself and for the intratumoral angiogenesis are not clarified, so far. It was only shown that loss of T-cadherin in melanoma regulates AKT signaling and desensitizes for apoptosis (Bosserhoff et al. 2014). Also, a connection of loss of T-cadherin to tumor progression was speculated (Rubina et al. 2013) but not evidenced, until today.

9.2.4 N-Cadherin Expression During Tumorigenesis

N-cadherin plays a pivotal role in cell adhesion between melanoma cells and both dermal fibroblasts and vascular endothelial cells. During the cadherin class switch, loss of E-cadherin expression is accompanied by induced N-cadherin expression, which confers new adhesive properties on the cells (Fig. 9.2). The shift in cadherin profile during melanoma progression has been found not only in vitro but also in vivo (Hsu et al. 1996; Sanders et al. 1999). Experimentally, melanoma cell migration across fibroblasts is impaired upon addition of an N-cadherin neutralizing

antibody (Li et al. 2001). The functional relevance of N-cadherin is to conduct migration and invasion of melanoma cells whereas N-cadherin expression correlates with progression to advanced-stage melanoma. The cell adhesion molecule N-cadherin has been suggested to represent a melanoma progression marker (Watson-Hurst and Becker 2006).

The switch of the cadherin class from E-cadherin to N-cadherin is directly connected. The transcriptional repressor Snail not only regulates E-cadherin repression but also represses the expression of the deubiquitinating enzyme CYLD. Loss of CYLD expression in melanoma in turn led to ubiquitination of Bcl-3 which is a transcriptional regulator of N-cadherin expression (Massoumi et al. 2009).

9.2.5 VE-Cadherin Expression During Tumorigenesis

The term *vasculogenic mimicry* describes the formation of vascular-like tubular structures and patterned networks through the connection of melanoma cells. The vascular structures are essential for the supply of the tumor. Several key molecules are responsible for the formation and maintenance of the tubular networks and these molecules are also often essential in normal blood vessels. One molecule expressed during vasculogenic mimicry of melanoma cells is VE-cadherin, previously considered to be endothelial cell specific. Analyzing VE-cadherin in detail demonstrated an interaction with EphrinA2 (EphA2), a tyrosine kinase. VE-cadherin engages the membrane-bound ligand of EphA2 and becomes phosphorylated on its tyrosines at the cytoplasmic domain. The mutual impact of VE-cadherin and EphA2 results in loosening of cell–cell adhesion and allowing for an increase in cell migration, invasion, and vasculogenic mimicry. Further studies describe the role of VE-cadherin for melanoma transendothelial migration. Here, p38 MAP kinase is necessary for increased VE-cadherin-mediated junction disassembly important for the migration processes of melanoma cells (Hendrix et al. 2001, 2003; Khanna et al. 2010).

9.2.6 FAT Expression During Tumorigenesis

FAT1, FAT2, FAT3, and FAT4 are human homologs of *Drosophila* Fat, which is involved in tumor suppression and planar cell polarity (PCP). FAT molecules belong to the cadherin-like protein family. FAT1 and FAT4 undergo the first proteolytic cleavage by Furin and are predicted to undergo the second cleavage by γ-secretase to release intracellular domain (ICD). Recently, it was shown using Northern blotting that human melanoma cell lines variably but universally express FAT1 and less commonly FAT2, FAT3, and FAT4. Both normal melanocytes and keratinocytes also express comparable FAT1 mRNA relative to melanoma cells. However, in melanoma cells, the non-cleaved proform of FAT1 is also expressed at the cell surface together with the furin-cleaved heterodimer. Moreover, furin-independent processing generates a potentially functional proteolytic product in melanoma cells, a persistent 65-kDa membrane-bound cytoplasmic fragment no longer in association

with the extracellular fragment. In vitro localization studies of FAT1 showed that melanoma cells display high levels of cytosolic FAT1 protein. Such differences in protein distribution appear to reconcile with the different protein products generated by dual FAT1 processing. It was suggested that the uncleaved FAT1 could promote altered signaling, and the novel products of alternate processing provide a dominant negative function in melanoma (Sadeqzadeh et al. 2011). Among the human *FAT* gene family, *FAT4* gene is recurrently mutated in several types of human cancers, such as melanoma (40 %), pancreatic cancer (8 %), HNSCC (6 %), and gastric cancer (5 %) (Nikolaev et al. 2011).

9.2.7 Signaling of Cadherins

In contrast to integrins, evidence for cadherin-induced outside–in signaling came into focus only slowly. Over the last 10 years, a number of studies have appeared to agree that signaling cascades emanating from cadherins play an important role in confluency-dependent growth arrest, migration, invasion, and differentiation. Changes in expression or function of cell adhesion molecules can therefore contribute to tumor progression both by altering the adhesion status and by affecting cell signaling. To date, no enzymatic activity has been attributed to the cytoplasmic tails of adhesion molecules like E-cadherin or N-cadherin. The signaling capability emanates from intracellularly bound kinases and phosphatases that link to the cytoplasmic tail of adhesion receptors (Fig. 9.3).

9.2.7.1 Signaling Cascades of E-Cadherin

Four modes of E-cadherin signaling are known:

1. Modulation of receptor tyrosinase signaling (RTK) (see also Chap. 7)
2. Inhibition of the Wnt signaling pathway (see also Chap. 7)
3. Regulation of cytoplasmic β-catenin signaling
4. Regulation of signaling through Rho GTPases

One way by which E-cadherin transmits growth-inhibiting outside–in signals appears to follow a strikingly similar scheme to that of the integrins. By using an immortalized nontumorigenic keratinocyte cell line, HaCaT, as a model system, Pece and Gutkind (2000) provide evidence that the assembly of calcium-dependent adherens junctions leads to a rapid and remarkable increase in the state of activation of MAPK and that this event is mediated by E-cadherin. Furthermore, it was found in these studies about HaCaTs that E-cadherin stimulates the MAPK pathway through ligand-independent activation of receptor tyrosine kinases, in particular EGF-receptors (Pece and Gutkind 2000). They speculated that upon adherens junction formation, signals emanating as a result of the E-cadherin-EGFR interaction might be involved in maintaining the functional and structural integrity of quiescent epithelia and, as a function of the adhesion status of the cells, possibly in promoting epithelial cell differentiation rather than proliferation. In contrast, another group detected signaling

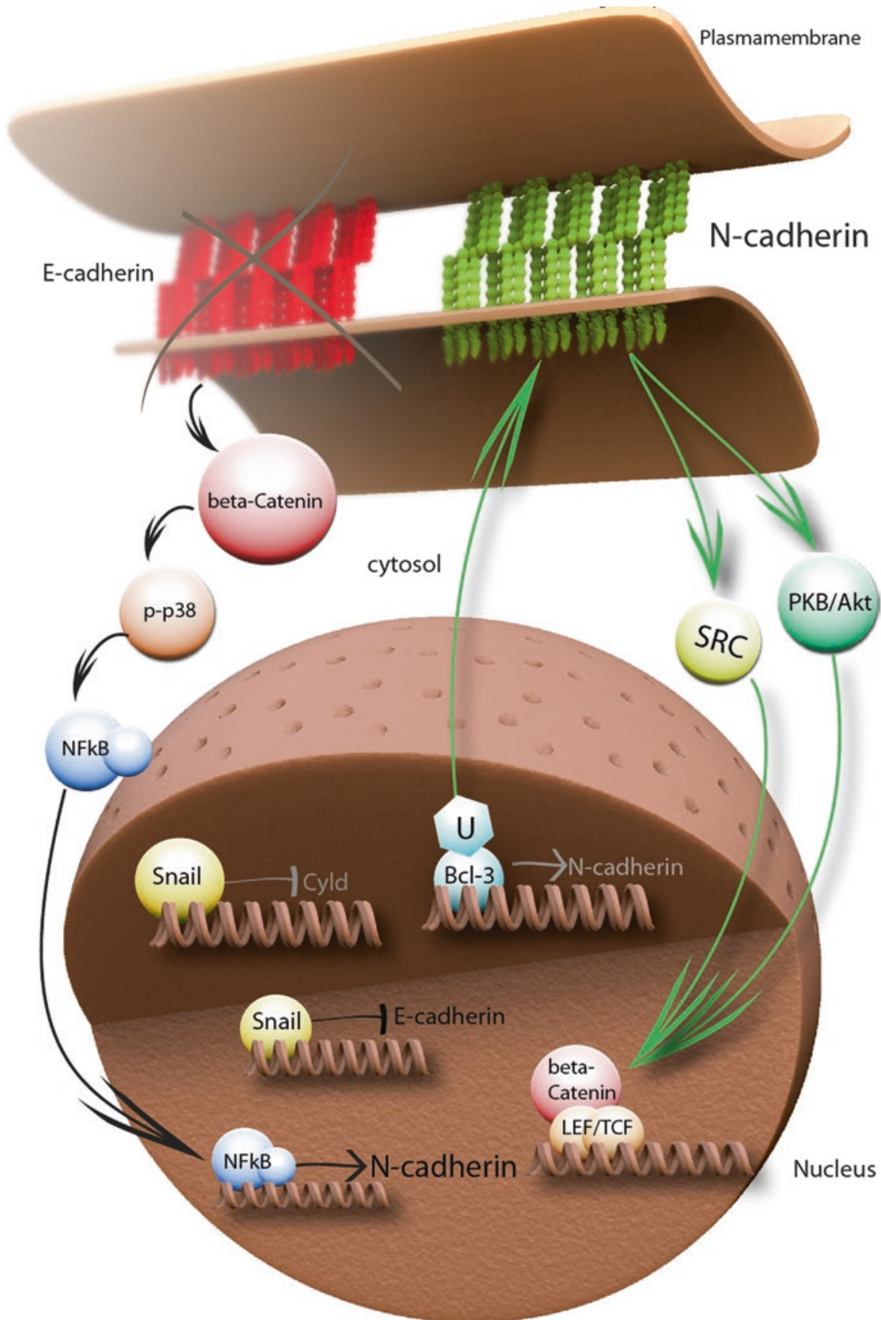

Fig. 9.3 Schematic depiction of cadherin signaling in melanoma. The transcriptional repressor Snail inactivates E-cadherin expression in melanoma. With the loss of E-cadherin cytosolic beta-catenin activates the MAP kinase p38, which stimulates the transcriptional activity of NFkappaB. NFkappaB has N-cadherin as target gene. Additionally, Snail represses the expression of the tumor suppressor Cyld, which in turn leads to ubiquitination of Bcl-3 which also has N-cadherin as target gene. The overexpression of N-cadherin activates signaling cascades of SRC and PKB/Akt which leads to tumor progression (Illustration R.J. Bauer)

cascade inhibition through EGF-receptor/E-cadherin complex formation in melanoma and breast cancer cells (Qian et al. 2004). Unfortunately, most of the literature on E-cadherin signaling does not cover melanoma. Studies on keratinocytes and other cancer cell types revealed that the E-cadherin complex associates and cooperates with an EGF-receptor family member to activate the PI3K/Akt pathway in a Src-family kinase-dependent manner (Muller et al. 2008; Perrais et al. 2007) (see also Chap. 7).

Some studies showed that homophilic ligation of E-cadherin signals directly through Rho GTPase activity (Braga 2000; Braga et al. 1997). Loss of E-cadherin in melanoma may involve changes in the organization of the cytoskeleton which is exerted by members of the Rho family. They control not only the cytoskeletal organization but also cell motility, migration, and tumor progression to malignancy at the same time. As example, E-cadherin suppresses RhoA activity in melanoma by activating p190RhoGAP (Molina-Ortiz et al. 2009). E-cadherin overexpression led to association of p190RhoGAP and p120^{ctn} on the plasma membrane where E-cadherin bounds p120^{ctn}. Recently, it was shown that E-cadherin also regulates RhoC GTPase. Here, loss of E-cadherin activates the expression of the RhoC in melanoma through upregulation of the transcription factor ETS-1, which results in increased c-Jun protein stabilization and activation (Spangler et al. 2012).

In addition to its role in adhesion, nuclear β-catenin is involved in Wnt signal transduction, and it interacts with transcription factors of the leukocyte enhancer factor (LEF)/T-cell factor (TCF) family to regulate transcription of target genes implicated in cell growth control such as cyclin D1 and c-myc (van Noort and Clevers 2002). By sequestering β-catenin at the cell surface, E-cadherin has been shown to antagonize nuclear β-catenin signaling pathways and to induce growth inhibition (Gottardi et al. 2001; Shtutman et al. 1999). Furthermore, β-catenin bound to E-cadherin inhibits phosphorylation of p38 and prevents activation of NFkappaB. Unbound cytoplasmic β-catenin activates the signaling pathway ending at transcriptional activation of N-cadherin expression in melanoma cells (Kuphal et al. 2004). In general, it was shown by Onder et al. (2008) that loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. The publication presents ~84 of 617 genes differentially expressed in shE-cadherin human breast epithelial cells (HMLE). They presented, e.g., *twist* and *TCF-8* among other 19 transcription factors as upregulated after loss of E-cadherin.

9.2.7.2 Signaling Cascades of N-Cadherin

N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells through activation of cytoplasmic signaling cascades. The Src family kinases are involved in the regulation of N-cadherin-mediated cell adhesion and signaling during, e.g., melanoma cell transendothelial migration. Src is localized at the heterotypic contacts of N-cadherin and becomes activated when melanoma cells are transmigrating across the endothelium. Activated Src has the Tyrosine-860 at the cytoplasmic domain of N-cadherin as target site for phosphorylation. The phosphorylation leads to disruption of β-catenin binding followed by nuclear translocation of this molecule to activate gene transcription of genes responsible for proliferation (Qi et al. 2006). N-cadherin mediates cell adhesion-activated antiapoptotic protein Akt/PKB and subsequently increases β-catenin and inactivates pro-apoptotic factor Bad (Li et al. 2001).

9.2.8 Desmosomes/Hemidesmosomes

Desmosomes, composed of desmogleins and desmocollins, are localized spot-like adhesions randomly arranged on the lateral sides of plasma membranes and are also members of the cadherin family. The extracellular domain of the desmosome is called the extracellular core domain (ECD) or the Desmoglea, and is bisected by an electron-dense midline where the desmoglein and desmocollin proteins bind to each other. On the cytoplasmic side of the plasma membrane, there are two dense structures called the outer dense plaque (ODP) and the inner dense plaque (IDP). In the ODP, the cytoplasmic domains of the cadherins desmoglein and desmocollin attach to desmoplakin via plakoglobin and plakophilin, while in the IDP, desmoplakin attaches to the intermediate filaments such as keratine filaments.

A number of melanoma cell lines synthesize, in the absence of desmosomes, the desmosomal cadherin desmoglein 2 (Dsg2) as a frequent plasma membrane glycoprotein that is not assembled into any junction but is dispersed over large parts of the cell surface. Indeed, in tissue microarrays, Dsg2 has been demonstrated in a sizable subset of nevi and primary melanomas (Rickelt et al. 2008). In contrast, Dsg1, Dsg3, and desmocollins 1–3, were absent in the analyzed melanoma cell lines but plakoglobin and plakophilin3 were also expressed in several melanoma cell lines (Schmitt et al. 2007). Future studies will have to clarify the diagnostic and prognostic significance of these different adhesion protein subtypes.

9.3 Integrins

Integrins are transmembrane adhesion receptors localized at cell–matrix contact sites where they link ECM (extracellular matrix) components, e.g., vitronectin, fibronectin, laminin, osteopontin, or collagen, to the actin cytoskeleton and interact with multiple structural and signaling molecules including talin, kindlin, paxillin, vinculin, α-actinin, FAK (focal adhesion kinase), ILK (integrin-linked kinase), Rho GTPases, and SHC (Berrier and Yamada 2007; Papusheva and Heisenberg 2010). The latter are important mediators downstream of integrins by which they interact either directly or indirectly to effect adhesion-dependent responses (Playford and Schaller 2004). The metastatic transformation of melanocytes is associated with altered expression of integrins, which transduce signals upon ligation to ECM proteins that regulate tumor growth and metastasis, apoptosis, differentiation as well as tumor angiogenesis. Integrin receptors are functional dimers of α- and β-integrin subunits, which each have a large ectodomain, a single transmembrane domain, and a generally short cytoplasmic tail (except for β4 integrin). The combination of different α- and β-subunits determines the substrate specificity of the dimer (Danen and Sonnenberg 2003). There are at least 18 known α-chains and 8 β-chains, allowing for at least 24 unique heterodimers.

The pattern of integrins on the cell surface is usually very specific, which makes the cell fit perfectly into its surrounding environment. Importantly, integrin expression patterns differ considerably in vitro versus in vivo. Thus, in vitro studies may not translate into the in vivo situation.

Several publications have shown that the expression levels mainly of αvβ3, α2β1, α3β1, α4β1, and α5β1 appear to increase from primary melanomas to metastatic melanoma tissue sections, whereas there was a significant decrease in α1β1, α2β1, and α6β1 expression levels in metastatic melanoma compared to primary melanoma (Friedl et al. 1998; Natali et al. 1993; Schadendorf et al. 1993). Although many integrins have been implicated in mediating melanoma growth and metastasis, perhaps none have been studied as much as the vitronectin receptor, αvβ3 (Danen et al. 1995; Mortarini and Anichini 1993; Seftor et al. 1999). αvβ3 integrin adheres to vitronectin, fibronectin, laminin, collagen, and osteopontin. Binding fibronectin and vitronectin induces the expression of MMP-2, which is able to degrade the collagen of the basement membrane (Felding-Habermann et al. 2002). Furthermore, osteopontin's RGD-sequence (Arg–Gly–Asp) has high binding affinity and specificity to αvβ3. As the aggressiveness of melanoma has been associated with high osteopontin expression (Sieg et al. 2000), this interaction of αvβ3 and osteopontin is important for melanoma progression. Interaction between αvβ3 and extracellular matrix molecules serves to promote cell attachment, spreading, and migration. αvβ3 integrin also undergoes heterophilic binding with two members of the immunoglobulin superfamily of cell adhesion molecules, PECAM-1 and L1. The αv subunit is widely expressed on melanomas regardless of disease stage. This stands in contrast to the β3 subunit, which is predominantly expressed on melanoma cells in the vertical growth phase. The onset of β3 integrin expression is one of the most specific markers of the transition from radial growth phase to vertical growth phase of melanoma (Albelda et al. 1990; Danen et al. 1995; Natali et al. 1997). Although many studies on human melanoma cell lines have correlated αvβ3 integrin expression with progression and metastasis, in vivo studies are less clear.

9.3.1 Integrin Signaling in Melanoma

Apart from being involved in the attachment of cells to the ECM, integrins are also responsible for signaling between the cells and the environment. Signaling works bidirectionally: "outside–in signaling" can control behavior, proliferation, cell polarity, cell growth, and migration. "Inside–out signaling," on the other hand, changes the integrins from a passive, weak binding state into an active, adhesive state and alters the interaction of the receptors with the extracellular environment. Integrins are receptors for cell movement in response to binding to ECM of the basement membrane or connective tissue or plasma membrane receptors expressed on endothelial cell surfaces. Additionally, integrins bind cytoplasmic adaptor proteins of the actin-myosin filaments and create a plasticity that allows the cell to move. In summary, integrins are bivalent linker proteins, binding simultaneously to extracellular ligands as well as cytoplasmic proteins including intracellular signaling molecules. They influence, for example, tyrosine kinases, serine/threonine kinases, phosphoinositides, and signaling cascades which determine the fate of a cell, letting it grow, proliferate, or die whenever it is necessary in the context of the whole organism. This paragraph introduces some of the most important and best studied proteins which are known to interact with integrins in melanoma.

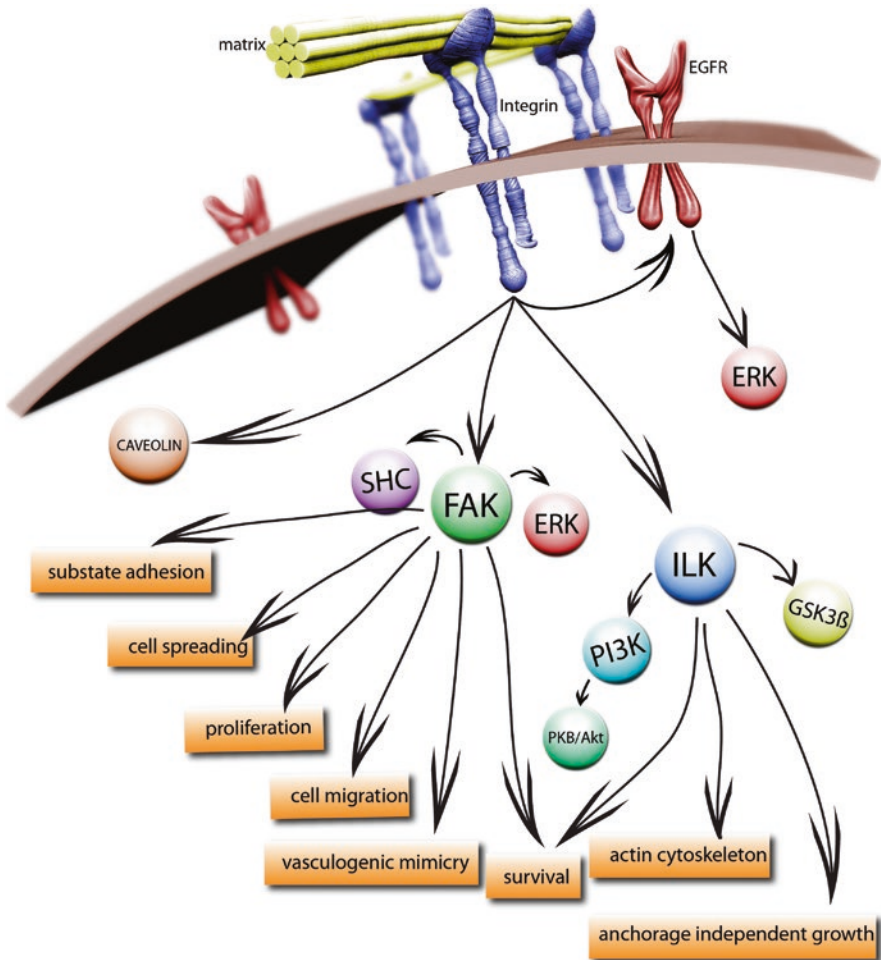

Fig. 9.4 Schematic depiction of the signaling pathways leading from integrins to focal adhesion kinase (*FAK*) and integrin-linked kinase (*ILK*), respectively, and further reactions of the cell (Illustration R.J. Bauer)

There is the non-receptor protein tyrosinase kinase FAK (focal adhesion kinase) (Fig. 9.4) that co-localizes with integrins in focal adhesions. FAK becomes phosphorylated and then controls processes like cell spreading, proliferation, motility, vasculogenic mimicry, and survival (Schaller 2001). Proteins like c-SRC, SHC, CSK, PI3K, and GRB2 are known to interact with FAK to transfer the signaling into the cytoplasm and to link FAK signaling also to MAP kinases (Chakraborty et al. 2002) (see also Chap. 7). FAK expression seems to be required in melanoma cells for substrate adhesion. It has been shown that in melanoma FAK is constitutively active and that it is essential for maintaining adhesiveness in melanoma cells (Hamamura et al. 2008; Kahana et al. 2002).

Furthermore, the integrin-linked kinase (ILK), a serine/threonine kinase, is implicated in connecting cell–extracellular matrix interaction and growth factor signaling to cell survival, cell migration, invasion, anchorage-independent growth, angiogenesis, and epithelial–mesenchymal transition. It has been shown that strong ILK expression was significantly associated with melanoma thickness, migration, and invasion (Wong et al. 2007). Increased expression of integrin-linked kinase is correlated with melanoma progression and poor patient survival (Dai et al. 2003). ILK directly phosphorylates PKB/Akt and glycogen synthase kinase-3 (GSK-3beta), which is inactivated upon phosphorylation (Delcommenne et al. 1998; Troussard et al. 1999). SHC is another protein which is implicated in integrin signaling. It is an adaptor protein capable of binding phosphotyrosine-containing sequences. So far, studies have demonstrated that SHC signaling is involved in pathways, which play a role in the development of malignancies like c-Myc activation (Gotoh et al. 1997), survival signaling (Friedmann et al. 1996; Sakai et al. 2000), cytoskeletal organization, and mitogenic signaling through RAS. It has been proposed that SHC is a substrate for FAK.

Also, the ERK/MAP kinase cascade is a pathway in which integrin-mediated adhesion is involved. In the ERK pathway, various stimuli of many important integrin signaling molecules like FAK or SHC converge and are able to influence nearly every profound cellular activity (Meier et al. 2005).

Epidermal growth factor receptor (EGFR) is also activated by integrins to generate cellular responses such as adhesion-dependent cell survival and proliferation in response to ECM. Subsequently, integrin-mediated EGFR activation induces ERK/MAP kinase signaling (Howe et al. 2002; Jost et al. 2001). Furthermore, Caveolin-1 (CAV1) is the main structural component of caveolae, which are plasma membrane invaginations that participate in vesicular trafficking and signal transduction events. Following integrin activation, B16F10 cells expressing CAV1 display reduced expression levels and activity of FAK and Src proteins. Furthermore, CAV1 expression markedly reduces the expression of integrin β3 in B16F10 melanoma cells. These findings provide experimental evidence that CAV1 may function as an antimetastatic gene in malignant melanoma (Trimmer et al. 2010).

9.4 Immunoglobulin Gene Superfamily of Cell Adhesion Molecules (CAMs)

Whereas normal melanocytes express few cell–cell adhesion receptors of the immunoglobulin gene superfamily of cell adhesion molecules (CAMs), melanoma cells show an increase in expression of melanoma cell adhesion molecule (MCAM, Mel-CAM, MUC18, CD146), L1 cell adhesion molecule (L1-CAM, CD171), activated leukocyte cell adhesion molecule (ALCAM, CD166), vascular cell adhesion molecule 1 (VCAM-1, CD106), intercellular cell adhesion molecule 1 (ICAM-1, CD54), and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1, CD66a) (reviewed in Haass et al. 2005).

9.4.1 Melanoma Cell Adhesion Molecule (MCAM, Mel-CAM, MUC18, CD146)

Mel-CAM mediates homologous and heterologous interactions between melanoma cells and endothelial cells, respectively, via a heterophilic Ca^{2+}-independent adhesion to its ligand (Shih et al. 1997a, b; Johnson et al. 1997). Recently, Laminin-411 (α4β1γ1 integrin) and Galectin-1 have been identified as Mel-CAM ligands (Flanagan et al. 2012; Jouve et al. 2013; Yazawa et al. 2015). In melanocytic cells, expression of Mel-CAM is first found in nevi, when the cells have separated from the epidermal keratinocytes and have migrated into the dermis (Shih et al. 1994; Kraus et al. 1997). With progression to malignancy, Mel-CAM expression gradually increases and is highest in metastatic melanoma cells (Xie et al. 1997; Johnson et al. 1996; Shih et al. 1994; Lehmann et al. 1987, 1989). In vitro and in vivo data supporting an important role of Mel-CAM in melanoma progression was demonstrated in several experimental studies (reviewed in Haass et al. 2005; Lei et al. 2015). Recently, the zinc finger transcription factor ZBTB7A was found to repress melanoma metastasis by directly binding to the promoter and transcriptionally repressing Mel-CAM (Liu et al. 2015).

An evaluation of tissue arrays of primary and metastatic melanomas revealed that in patients meeting the current criteria for sentinel lymph node dissection, both Mel-CAM expression positivity and intensity were independently predictive of survival and development of lymph node disease in primary melanoma over and above established markers of prognosis, such as Breslow thickness. Mel-CAM-negative patients had a 5-year survival of 92% compared with 40% for Mel-CAM-positive patients (Pearl et al. 2008). Recently, a study on 175 patients revealed that sequential molecular detection of Mel-CAM mRNA in the peripheral blood correlated with poor prognosis. The authors suggested to utilize Mel-CAM expression as a "molecular warning of progression" even in early stage patients in otherwise disease-free conditions (Rapanotti et al. 2014). However, larger trials to confirm this finding as a biomarker are still pending.

9.4.2 L1-Cell Adhesion Molecule (L1-CAM, CD171)

L1-CAM, originally described as a neuronal cell adhesion molecule, has also been detected in a number of other non-neuroendocrine tissues and in several malignant tumors, including melanoma (Nolte et al. 1999; Thies et al. 2002b). L1-CAM mediates adhesion both via homophilic (L1-CAM-L1-CAM) and heterophilic (L1-CAM-$\alpha_v\beta_3$ integrin) mechanisms (Hortsch 1996). In melanoma/melanoma cell and in melanoma/endothelial cell interactions, L1-CAM binds to $\alpha_v\beta_3$ integrin (Montgomery et al. 1996). The interaction of L1-CAM and $\alpha_v\beta_3$ integrin plays an important role in transendothelial migration of melanoma cells (Voura et al. 2001) whereas overexpression of L1-CAM promotes conversion from radial to vertical growth phase melanoma without upregulation of $\alpha_v\beta_3$ integrin expression (Meier et al. 2006). There is an increase in L1-CAM immunoreactivity in melanomas and metastases of melanoma compared to acquired melanocytic nevi (Fogel et al. 2003). A study that systematically identified novel melanoma-specific genes confirmed that L1-CAM is not expressed in normal skin and melanocytic nevi, but is highly and differentially

expressed in primary melanoma tissues and melanoma lymph node metastases (Talantov et al. 2005). Evaluation of specimens of nevi, primary melanomas, sentinel lymph nodes, and distant metastases showed that L1-CAM can serve as a highly sensitive and specific diagnostic marker for melanoma (Thies et al. 2007). A 10-year retrospective biomarker study, evaluating 100 melanoma specimens, showed that the expression of L1-CAM in human primary cutaneous melanoma is significantly associated with metastatic spread and that L1-CAM expression is an independent predictor for the risk of metastasis (Thies et al. 2002b). A recent study revealed that the CE7 epitope of L1-CAM on a variety of tumors (however, melanoma was not included in the study) may be amenable to targeting by CE7R+ T cells, making it a promising target for adoptive immunotherapy (Hong et al. 2014).

9.4.3 Activated Leukocyte Cell Adhesion Molecule (ALCAM, CD166)

ALCAM is involved in homophilic (ALCAM-ALCAM) (Degen et al. 1998) and heterophilic (ALCAM-CD6) (Patel et al. 1995) cell–cell adhesion interactions. ALCAM is expressed in metastatic human melanoma cells, whereas it is absent in non-metastatic cells (Degen et al. 1998). Immunohistochemistry on a series of common nevi, primary melanomas, and melanoma metastases revealed that ALCAM expression correlates with melanoma progression (van Kempen et al. 2000). ALCAM is therefore proposed to be a molecular melanoma progression marker. Intact cell adhesion function of ALCAM favored primary tumor growth and represented a rate-limiting step for tissue invasion, which supported the view that dynamic control of ALCAM plays an important role in progression (van Kempen et al. 2004). An immunohistochemical biomarker study, evaluating tissue microarrays showed that a significantly greater percentage of melanomas (combined primary and metastatic) than nevi contained cells that expressed ALCAM (Klein et al. 2007). Interestingly, a recent study evaluating ALCAM expression and long-term survival in melanoma patients suggested that, in primary melanomas, high ALCAM expression was a marker of negative outcome, but in regional lymph node melanoma metastases low expression of ALCAM was a feature associated with unfavorable prognosis (Donizy et al. 2015). ALCAM upregulation in metastatic melanoma cells is driven by miR-214 and depends on transcriptional mechanisms mediated by TFAP2 and posttranscriptional mechanisms mediated by miR-148b, which itself is controlled by TFAP2. Therefore, miR-214 and miR-148b have opposite effects on melanoma cell dissemination and are part of a regulatory loop (Penna et al. 2013).

9.4.4 Intercellular Adhesion Molecule-1 (ICAM-1, CD54)

ICAM-1 can be induced in a cell-specific manner by several cytokines, e.g., TNF-α (tumor necrosis factor-alpha), IL-1 (interleukin-1), and IFN-γ (interferon-gamma). The ligands of ICAM-1 are $\alpha_L\beta_2$ (lymphocyte function-associated antigen 1, LFA-1) and Mac1 on lymphocytes (van de Stolpe and van der Saag 1996). ICAM-1

correlates with melanoma progression and increased risk of metastasis (Johnson et al. 1989). Its expression in melanoma is stronger than in common nevi and increases with the Breslow index in primary melanomas (Natali et al. 1990, 1997; Schadendorf et al. 1993, 1995). The observation that stage I patients with ICAM-1-positive melanomas had a significantly shorter disease-free interval and overall survival than those with ICAM-1-negative tumors (Natali et al. 1997) and that the suppression of ICAM-1 in an animal model reduced the metastatic capacity (Miele et al. 1994), supported the role of ICAM-1 in melanoma progression and metastasis. However, the specific role of ICAM-1 in melanoma progression remains to be determined. Expression of ICAM-1 may promote aggregate formation with leucocytes, which can enhance survival in the vascular system and encourage extravasation (Aeed et al. 1988). On the other hand, ICAM-1 is shed from melanoma cells (Giavazzi et al. 1992) – possibly in a form that inhibits lymphocyte–tumor cell interaction and thus contributes to tumor survival (Becker et al. 1993). A recent study has unraveled a mechanism by which shear flow-regulated melanoma cell adhesion to the endothelium can upregulate endothelial ICAM-1 expression (Zhang et al. 2014). Elevated ICAM-1 levels may serve as receptors to recruit neutrophils and bind fibrin, which assists melanoma cell adhesion and migration. An increase of ICAM-1 expression on endothelial cells could be a result of direct ligation of tumor CD44 and endothelial E-selectin, through the PKCα-p38-SP-1 pathway. This suggests a new mechano-signaling cascade triggered by stretching E-selectin to induce ICAM-1 expression (Zhang et al. 2014).

9.4.5 Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (CEACAM-1, CD66a)

CEACAM1 is involved in intercellular adhesion and subsequent signal transduction events in a number of epithelia. In epithelial cells, CEACAM1 is believed to act as a growth suppressor, since its expression was shown to be lost or significantly down- or dysregulated in carcinomas of liver, prostate, endometrium, breast, and colon (reviewed in Haass et al. 2005). On the other hand, CEACAM1 is upregulated in non-small cell lung cancer (Sienel et al. 2003). CEACAM1 interacts with the β_3 integrin subunit via the CEACAM1 cytoplasmic domain. CEACAM1 and the β_3 integrin subunit co-localize at the tumor–stroma interface of invading melanoma masses, suggesting that CEACAM1–integrin β_3 interaction plays a role in melanoma cell migration and invasion (Brummer et al. 2001). The expression of CEACAM1 in primary melanomas is associated with the subsequent development of metastatic disease (Thies et al. 2002a). Furthermore, the overexpression of CEACAM1 in CEACAM1-negative melanocytic cells and melanoma cell lines increases the migratory and invasive growth potentials in vitro (Ebrahimnejad et al. 2004) supporting the role of CEACAM1 in melanoma progression and metastasis. Evaluation of specimens of nevi, primary melanomas, sentinel lymph nodes, and distant metastases showed that CEACAM1 can serve as a highly sensitive

and specific diagnostic marker for melanoma (Thies et al. 2007). Indeed, CEACAM1 was shown to be one of the seven plasma markers best able to identify metastatic melanoma patients (Kluger et al. 2011).

9.5 Gap Junctions/Connexins

Connexins belong to a family of transmembrane proteins that form gap junctions (GJs), cell–cell junctions that are essential for intercellular communication. Gap junctional intercellular communication (GJIC) in the skin is involved in maintenance of homeostasis, regulation of proliferation, differentiation, barrier function, and recruitment of inflammatory cells. GJIC is thus a critical factor in the life and death balance of cells (Djalilian et al. 2006; Langlois et al. 2007; Maass et al. 2004; Man et al. 2007) (reviewed in Kretz et al. 2004; Mese et al. 2007). Furthermore, GJIC is critical in keratinocyte–melanocyte interaction (Hsu et al. 2000; Satyamoorthy et al. 2001). Alternatively, connexins can form hemichannels, which allow release (e.g., ATP, NAD^+) or putative uptake of molecules and ions to and from the cellular environment (Barr et al. 2013; Chandrasekhar and Bera 2012). Finally, connexins, especially Cx43, interact with structural and signaling molecules, which may add further functions to these molecules (Herve et al. 2007).

GJs form channels between adjacent cells allowing the intercellular transport of small metabolites, second messengers, and ions (Loewenstein 1981; Spray 1994). In addition to molecular weight and size, the ability of a solute to transverse these channels depends on its net charge, shape, and interactions with specific connexins that constitute gap junctions in particular cells (Goldberg et al. 2004). Each GJ channel consists of two hemichannels called connexons, each formed by six connexins (reviewed in Richard 2000). Twenty-one connexins have been identified, 11 of which are in the skin (Di et al. 2001; Willecke et al. 2002; Zucker et al. 2013). GJs can be homotypic, heterotypic, homomeric, and heteromeric (reviewed in Richard 2000). A connexon is homomeric if it is composed of six identical connexin subunits (e.g., Cx32 only), or heteromeric if it is composed of more than one connexin species (e.g., Cx32 and Cx43 and/or others). Channels are homotypic if both connexons are homomeric of the same type, heterotypic if homomeric connexons are of different types, and heteromeric if both connexons are heteromeric. Not all connexins are equally compatible at forming a connexon – even though they may co-exist in the same cell (reviewed in Haass et al. 2004). The type of connexin-forming GJ channels influences their selectivity and thereby controls the specificity of GJIC. For example, channels formed by Cx26 prefer cations, while those formed by Cx32 prefer anions (Brissette et al. 1994; Elfgang et al. 1995; Veenstra 1996). Thus, the up- or down-regulation of a certain connexin in a tissue may change its GJIC considerably. In addition, connexins can also form hemichannels, which have been shown to be able to exchange molecules with the extracellular microenvironment. These hemichannels are relevant for signal propagation and especially for calcium homeostasis (reviewed in Evans et al. 2006).

9.5.1 Connexins Are Conditional Tumor Suppressors

Loss of gap junctional activity and/or downregulation of connexins have been reported both in cell lines as well as in tissues of many tumor types, such as hepatocellular carcinoma, gastric carcinoma, prostate cancer, lung cancer, glioma, mammary carcinoma, basal cell carcinoma, squamous cell carcinoma, and melanoma. This phenomenon was first observed half a century ago (Loewenstein and Kanno 1966) and summarized in a number of review articles (Cronier et al. 2009; Mesnil et al. 2005; Naus and Laird 2010). The type of connexins lost during tumor progression varies according to tumor type. In the 1980s and 1990s, a series of studies were published showing that reagents and/or oncogenes that promote tumor onset or progression frequently inhibit GJIC or downregulate connexin expression (Lampe 1994; Trosko et al. 1990; Atkinson et al. 1981). The role of connexins as potential tumor suppressors was also shown in gene knockdown studies (Shao et al. 2005). Correspondingly, ectopic expression of connexins in tumors restored functional communication and reduced tumor proliferation and growth both in vitro and in vivo (reviewed in Naus and Laird 2010). Importantly, ectopic expression of connexins partially differentiated transformed cells (Zhu et al. 1991; McLachlan et al. 2006; Hellmann et al. 1999; Hirschi et al. 1996). Moreover, functional abrogation of connexins, using antisense or dominant-negative mutant approaches, have demonstrated an enhancement of the malignant phenotype in several tumor types, such as Cx26 in HeLa cells (Duflot-Dancer et al. 1997), Cx32 in hepatocellular carcinoma (Dagli et al. 2004), Cx43 in lung cancer (Avanzo et al. 2004), Cx43 in glioma (Omori and Yamasaki 1998), and Cx43 in bladder carcinoma (Krutovskikh et al. 1998). Finally, Cx32 knock-out mice have an increased incidence of tumor onset when challenged with carcinogens (Temme et al. 1997; King and Lampe 2004a, b; Moennikes et al. 2000).

This may lead to the assumption that connexins are general tumor suppressors, but it appears that this is only the case in the earlier steps of cancerogenesis. In fact, the role of connexins in invasion and metastasis is very complex, and connexins might facilitate invasion, intravasation, extravasation, and metastasis (Krutovskikh et al. 1994; el-Sabban and Pauli 1991, 1994; Ito et al. 2000; Saunders et al. 2001; Lin et al. 2002; Miekus et al. 2005; Pollmann et al. 2005; Kanczuga-Koda et al. 2006; Bates et al. 2007; Li et al. 2007; Dobrowolski et al. 2008; Cotrina et al. 2008; Elzarrad et al. 2008; Ezumi et al. 2008). The following model supports both the tumor suppressor and the tumor driver theories (Cronier et al. 2009): for the step from primary to invasive tumors, there is a need for disruption of intercellular junctions including GJs, consistent with the model that connexins are tumor suppressors. In contrast, for the tumor cell dissemination and metastasis steps, increased cell contacts and communication are needed in order to enable interaction with the tumor stroma – especially between cancer cells and endothelial cells. Therefore, connexins might be better classified as conditional tumor suppressors that modulate cell proliferation as well as adhesion and migration (Naus and Laird 2010).

9.5.2 Cx43 in Cancer

Cx43 is decreased in prostate cancer (Tsai et al. 1996), mammary cancer (Hirschi et al. 1996), glioma (Huang et al. 1999), lung cancer (Jinn et al. 1998; Zhang et al. 1998), bladder carcinoma (Krutovskikh et al. 2000), cervical carcinoma (King et al. 2000), and various skin cancers including melanoma (Haass et al. 2006; Tada and Hashimoto 1997; Wilgenbus et al. 1992). Electron microscopy investigations have shown that basal and squamous cell carcinomas do not have fully developed GJs, and that Cx43 is not restricted to these poorly developed GJs but is present in the cytoplasm (Tada and Hashimoto 1997). In several cancers, Cx43 acts as a tumor suppressor gene with loss of Cx43 contributing to metastasis (Czyz 2008; Gershon et al. 2008; Shen et al. 2007). Functional abrogation of Cx43 enhances the malignant phenotype in lung cancer (Avanzo et al. 2004), glioma (Omori and Yamasaki 1998), and bladder carcinoma (Krutovskikh et al. 1998).

In contrast to other cancers, hepatocellular carcinoma is associated with an induction of Cx43, which is, however, localized in the cytoplasm, and thus is not involved in GJIC (Krutovskikh et al. 1994). The loss of GJIC might help the tumor cells to survive, as GJIC has been shown to spread cell-killing signals, most likely Ca^{2+} ions (Krutovskikh et al. 2002). In addition, downregulation of Cx43 expression or function resulted in increased proliferation and migration in primary keratinocytes, implying a contribution of Cx43 to controlling early stages of tumorigenesis (Mori et al. 2006; Wright et al. 2009; Pollok et al. 2011). Finally, increased opening of hemichannels formed by connexins resulted in cell death in cochlear supporting cells of the ear and in keratinocytes of the epidermis (Xu and Nicholson 2013).

Conversely, expression of Cx43 has also been shown to increase tumor metastasis in breast cancer, glioma as well as in melanoma through increased attachment and communication with the vascular endothelium (Bates et al. 2007; Kanczuga-Koda et al. 2006; Cotrina et al. 2008; Lin et al. 2002; el-Sabban and Pauli 1991, 1994; Pollmann et al. 2005; Elzarrad et al. 2008).

9.5.3 Cx32 in Cancer

Cx32 is downregulated in gastric carcinoma (Uchida et al. 1995), lung cancer (Jinn et al. 1998), and hepatocellular carcinoma (Eghbali et al. 1991; Loewenstein and Rose 1992; Krutovskikh et al. 1994; Yamaoka et al. 1995). In the latter case, the remaining Cx32 is localized in the cytoplasm or in the plasma membrane free from contact with other cells. In addition, it was found that there was no mutation in the coding sequence of Cx32 in hepatocellular carcinoma; instead, it appears that the aberrant localization of Cx32 is a consequence of the disruption of Cx32 gap junction plaque formation (Krutovskikh et al. 1994). Functional abrogation of Cx32 enhances the malignant phenotype in hepatocellular carcinoma (Dagli et al. 2004). Cx32 knock-out mice have an increased incidence of tumor onset when challenged with carcinogens (Temme et al. 1997; King and Lampe 2004a, b; Moennikes et al. 2000). In contrast to most other tumors, Cx32 is upregulated in some breast cancer cells (Saunders et al. 2001).

9.5.4 Cx26 in Cancer

Whereas in mammary carcinoma cells, there is a downregulation of both Cx43 and Cx26 (Hirschi et al. 1996); in human basal cell carcinoma, Cx43 is downregulated but there is an induction of Cx26 (Haass et al. 2006; Wilgenbus et al. 1992). Cx26 is also highly expressed in HeLa cells, where its functional abrogation enhances the malignant phenotype (Duflot-Dancer et al. 1997).

9.5.5 Connexins in Melanoma

Reflecting the situation in many other cancer types as discussed above, the role of connexins and GJIC is still highly controversial also in melanoma and its tumor microenvironment.

Cx43 is the most-studied connexin in melanoma. Western blotting revealed Cx43 protein expression in foreskin-derived melanocytes and several melanoma cell lines (Hsu et al. 2000). This was confirmed by immunofluorescence detecting Cx43 expression in human melanoma cell lines (Lin et al. 2010). While neither study quantified the Cx43 protein expression levels, a qRT-PCR and immunofluorescence study demonstrated lower Cx43 expression levels in human melanoma cell lines compared to human melanocytes (Schiffner et al. 2011). Also, a microarray study revealed that Cx43 was expressed at low levels in human melanoma cell lines and, importantly, that its overexpression suppressed anchorage-independent growth in colony-forming efficiency assays, suggesting a tumor-suppressor role of Cx43 in melanoma (Su et al. 2000). By qRT-PCR, no expression for Cx26, Cx30, Cx31.1, Cx36, and Cx37; low expression for Cx30.3 and Cx31; and higher expression levels for Cx32, Cx40, Cx43, and Cx45 were detected in human melanoma cell lines (Zucker et al. 2013). Surprisingly, Western blotting showed much higher Cx43 expression levels in migrating than in non-migrating cells (Zucker et al. 2013). Consistently, high levels of Cx43 protein expression were found in human metastatic melanoma cell lines (Villares et al. 2009). Loss of protease-activated receptor-1 (PAR-1) expression resulted in the loss of Cx43 and, correspondingly, overexpression of PAR-1 contributed to melanoma metastasis via upregulation of Cx43 (Villares et al. 2009, 2011). Interestingly, while initial levels of Cx43 were low in B16 mouse melanoma cells, Cx43 protein levels increased after infection with bacteria or treatment with interferon-γ (Saccheri et al. 2010). This was followed by the transfer of preprocessed antigenic peptides from melanoma cells to dendritic cells, which then presented those peptides on their surface and activated cytotoxic T cells against the tumor antigen. Correspondingly, melanoma cells in which Cx43 had been silenced, failed to elicit a cytotoxic antitumor response after infection with bacteria (Saccheri et al. 2010).

In addition to the discussed in vitro data, there are also a number of studies on human melanoma tissue. Using immunofluorescence on human tissue samples, we did not detect Cx43 (nor Cx26 and Cx30) in nevi, primary melanomas, or cutaneous melanoma metastases, while the internal controls (adjacent epidermis) were positive

in the expected layers (Haass et al. 2006, 2010). In contrast, using immunohistochemistry, other groups reported Cx43 expression in human melanoma tissue, higher than in human nevi (Rezze et al. 2011; Sargen et al. 2013). However, neither of these studies provided high magnification images to confirm the subcellular localization nor did they show appropriate positive and negative controls. Indeed, in both studies, Cx43 expression in melanoma cells appeared to be cytoplasmic and hence would argue for a cell–cell or cell–matrix communication-independent role of these connexins. This would not support the mechanism for melanoma survival in brain metastasis proposed by Lin and colleagues, who showed that reactive astrocytes protect metastatic melanoma cells in the brain from chemotherapy by sequestering intracellular calcium through direct cell–cell communication (Lin et al. 2010). Moreover, in the Rezze and Sargen studies, the expression pattern of Cx43 in nevi and different melanoma stages appeared very variable and the typical Cx43 staining in the epidermis was missing (Rezze et al. 2011; Sargen et al. 2013). An Oncomine analysis of human tissue showed that increased Cx43 (and Cx26) gene expression in primary lesions correlated with metastasis and poor patient survival (Stoletov et al. 2013).

Cx26 and Cx30 are much less studied. Cx26 was found to be upregulated in the highly aggressive BL6 sub-line of B16 mouse melanoma cells compared to the less aggressive F10 sub-line (Ito et al. 2000). F10 cells transfected with wild-type Cx26 exhibited similar metastatic behavior to the BL6 cells. Correspondingly, BL6 cells transfected with a dominant-negative Cx26 mutant showed the less aggressive behavior characteristic of F10 cells. Cx26 was not found to be expressed in human melanoma in situ but was upregulated in invasive melanomas (Ito et al. 2000). However, in this study, Cx26 staining in both melanoma cells and epidermal keratinocytes was cytoplasmic. Moreover, the study did not distinguish between Cx26 and Cx30. In contrast, we showed in immunofluorescence studies on human melanoma tissue samples, that all areas of melanocytic nevi, primary melanomas, and cutaneous melanoma metastases lacked Cx26 and Cx30 expression (Haass et al. 2006, 2010) – similar to our findings in Merkel cell carcinoma (Haass et al. 2003a). This was confirmed by other groups who did not detect Cx26 in melanoma using immunohistochemistry on human tissue samples (Sargen et al. 2013) or did not find Cx26 and Cx30 expression in human melanoma cell lines using qRT-PCR (Zucker et al. 2013). Contrastingly, a positive correlation between Cx26 expression and metastatic potential was reported using Cx26 shRNA in B16 mouse melanoma cells (Stoletov et al. 2013). This was supported by an Oncomine analysis of human tissue, which showed that increased Cx26 expression in primary lesions correlated with metastasis and poor patient survival (Stoletov et al. 2013).

Interestingly, loss of Pannexin 1, a channel-forming glycoprotein remotely related to connexins, attenuated melanoma progression by reversion to a melanocyte-like phenotype (Penuela et al. 2013).

The Oncomine data (Stoletov et al. 2013) do not seem to match the data on primary melanomas in other studies; however, it would be interesting to re-analyze these data more in detail. As there appears to be a correlation to tumor thickness, is there no or little expression on thin tumors and a differential expression pattern in different areas of thick melanomas?

The discrepancies between the different studies in Cx43, Cx26, and Cx30 in melanoma may be due to the following reasons:

1. Several studies investigated the molecules on mRNA level only. The presence of mRNA does not necessarily mean that the respective protein is present.
2. In tissues, it is difficult to separate between connexins present in melanoma cells and those present in epidermal, mesenchymal, or endothelial tissues enclosed by the tumor.
3. Immunohistochemistry is often dependent on staining conditions and can result in false-positive and false-negative results. Appropriate positive and negative controls showing the sensitivity and specificity of the antibody are indispensable for the interpretation of these results. For example, the Cx26 antibody used in some of the discussed studies shows cross-reactivity with Cx30.

Importantly, most of the apparent discrepancies in this paragraph can be explained by a model, which implies that connexins are tumor suppressors during early melanomagenesis but tumor drivers during metastasis (Cronier et al. 2009). During early melanomagenesis, the respective connexins are typically located in the cell membranes indicating that they are functioning through GJIC. In contrast, in advanced stages, connexins are typically located in the cytoplasm indicating a different function – possibly through interaction with signaling molecules.

9.5.6 Connexins in the Epidermal Tumor Environment of Melanoma

Keratinocytes communicate with melanocytes but not with melanoma cells via GJIC; instead, melanoma cells communicate among themselves and with fibroblasts and endothelial cells (Hsu et al. 2000). This switch in communication partners coincides with the E- to N-cadherin switch, suggesting that the gain of N-cadherin with the concurrent loss of E-cadherin facilitates GJ formation with fibroblasts and endothelial cells (Hsu et al. 2000). Additionally, GJ formation in human melanoma cell lines appears to require MCAM (Satyamoorthy et al. 2001). This switch will allow melanoma cells to de-couple from the epidermal microenvironment and to communicate with cell types important for their metastatic spread. Several studies have suggested that connexins may promote metastasis in melanoma and other tumors by forming intercellular connections between cancer cells and vascular endothelium that are able to initiate tumor cell diapedesis (Hsu et al. 2000; Villares et al. 2009; el-Sabban and Pauli 1991, 1994; Saito-Katsuragi et al. 2007; Pollmann et al. 2005). Melanoma cells expressing higher levels of Cx43 show increased coupling to vascular endothelial cells (el-Sabban and Pauli 1991) and the ability of tumor cells to metastasize appears to correlate with the ability of tumor cells to communicate with endothelial cells (Pollmann et al. 2005). Also, Cx26 may contribute to the metastasis of melanoma by facilitating communication between melanoma cells and their surrounding endothelial cells (Saito-Katsuragi et al. 2007).

Cx26 expression is associated with lymphatic vessel invasion and poor prognosis in human breast cancer (Naoi et al. 2007).

Melanoma brain metastases are surrounded and infiltrated by astrocytes, and these astrocytes can play a role similar to their established ability to protect neurons from apoptosis (Lin et al. 2010). In co-culture experiments, astrocytes reduced apoptosis in human melanoma cells treated with various chemotherapeutic drugs. This chemoprotective effect was dependent on physical contact and GJIC between astrocytes, which express high levels of Cx43, and tumor cells. Moreover, the protective effect of astrocytes resulted from their sequestering calcium from the cytoplasm of tumor cells. These data suggest that brain metastases can harness the neuroprotective effects of reactive astrocytes for their own survival (Lin et al. 2010). In a chick embryo model, B16 mouse melanoma cells, which express Cx26 but not Cx43, colonized the chicken brain forming numerous microtumors invading along the preexisting vasculature (Stoletov et al. 2013). In contrast, Cx26 knockdown B16 cells formed significantly fewer and less invasive tumors, suggesting that in metastatic melanoma cells Cx26 expression enhances microtumor formation in the brain in association with the existing vasculature (Stoletov et al. 2013).

While these studies demonstrate the interaction of melanoma cells with the stroma and the role of connexins and/or GJIC in the early and late steps of melanomagenesis, interactions between melanoma and the epidermal tumor microenvironment (ETM) – the multilayered epithelium of the skin – are poorly understood. In this regard, we have demonstrated the induction of Cx26 and Cx30 in the epidermis adjacent to malignant tumors (e.g., melanoma and Merkel cell carcinoma), but not in the epidermis adjacent to benign tumors (e.g., melanocytic nevi and angiomas) (Haass et al. 2003a, 2006). Subsequently, we found correlation between (a) tumor thickness (Breslow index) and vertical Cx26 and Cx30 expression in the ETM, (b) tumor thickness and horizontal Cx26 dissemination in the ETM, (c) metastasis and horizontal Cx26 expression in the ETM, and (d) vertical epidermal expression patterns of Cx26 and Cx30 and the proliferative index in the ETM. We thus provided evidence for the association of ETM alteration with tumor malignancy and progression (Haass et al. 2010). The results of this study, which included dysplastic nevi as well as thin melanomas which are often difficult to distinguish (reviewed in Haass and Smalley 2009), suggest that membrane expression of Cx26 and Cx30 in the epidermal tumor microenvironment may be a useful diagnostic aid for the distinction of melanomas and melanocytic nevi (Haass et al. 2010). As neither Cx26 nor Cx30 are expressed in the melanoma itself, but both are induced in its tumor microenvironment, they may be useful complementary melanoma markers.

Cx26 and Cx30 upregulation in the epidermal tumor microenvironment did not correlate with the proliferative index of the melanoma cells, but correlated significantly with the proliferative index in the epidermis. In transgenic mice expressing Cx26 ectopically, proliferation was increased in the epidermis (Djalilian et al. 2006), suggesting that Cx26 influences keratinocyte proliferation and not vice versa. Interestingly, Cx26 overexpressing mice showed a delay in wound healing, which needs to be explored with regards to ulceration, a biomarker associated with very poor prognosis for melanoma patients (Balch et al. 2001). In our study, all

melanomas with ulceration showed Cx26 (and Cx30) expression in all layers of the epidermal tumor microenvironment (Haass et al. 2010). Induction of angiogenesis by the hyperplastic epithelium could stimulate growth and progression of melanoma (McCarty et al. 2003). This suggests a positive feedback mechanism: tumor cells induce alterations in keratinocytes, which results in the production of growth factors which, in turn, stimulate tumor survival via endothelial cells. The induction of Cx26 and Cx30 in the epidermis adjacent to melanoma putatively leading to GJIC or signaling via hemichannels may play a role in this feedback mechanism by inducing proliferation and other functions. An interruption of this vicious circle may provide a novel therapeutic approach.

9.6 Tight Junctions

In simple epithelia and endothelia, tight junctions (TJs) are responsible for the formation and maintenance of the tissue barrier between distinct compartments by controlling the paracellular pathway ("barrier function") (reviewed in Stevenson and Keon 1998; Tsukita et al. 2001). Subsequently, the involvement of TJs in the barrier function of a complex epithelium, the epidermis, was shown (Pummi et al. 2001; Brandner et al. 2002, 2003; Furuse et al. 2002; Langbein et al. 2002). In addition, TJs prevent the diffusion of membrane proteins and lipids from the apical to the basolateral side of an epithelial cell sheet, helping to maintain cell polarity ("fence function") (reviewed in Mitic and Anderson 1998; Tsukita et al. 2001). Therefore, TJs are crucial for the epithelium to generate chemical and electrical gradients that is necessary for vectorial transport processes such as absorption and secretion (reviewed in Martin and Jiang 2009). Moreover, TJ molecules act as intermediates and transducers in cell signaling, thus playing a role in the processes of polarity, cell differentiation, cell growth, and differentiation. Finally, TJs act as cell–cell adhesion molecules and as a barrier to cell migration (reviewed in Martin and Jiang 2009).

TJs are composed of integral transmembrane proteins (claudin 1–24, occludin, and junctional adhesion molecules A-C, 4 (JAMs)), peripheral plaque proteins (zonula occludens (ZO) proteins 1–3, MAGI 1–3, MUPP-1, PAR-3, PAR-6, AF-6, CASK, and CAROM), and associated proteins (symplekin, ZONAB, cingulin, Rab-13, Rab-3B, c-src, α-catenin, PKA, ZAK, and Rho GTPases). The molecular composition of TJs is highly complex and varies according to the cell type and degree of differentiation. TJ molecules from neighboring cells associate and form paired strands which seal the paracellular pathway and which contain aqueous pores or paracellular channels, explaining the ion and size selectivity for passaging molecules of TJ (Tsukita and Furuse 2000).

In cancer, disruption of TJs should occur in three critical steps: (1) detachment of the tumor cell from the primary tumor, (2) intravasation of the tumor through the endothelium, and (3) extravastion of the circulating tumor cell (reviewed in Martin and Jiang 2009). Early studies have shown a correlation between lack of TJs and tumor differentiation and there is evidence that TJs need to be overcome by cancer

cells in order to metastasize (reviewed in Martin and Jiang 2001, 2009). Cancer cells frequently exhibit deficiencies in TJ function, as well as decreased differentiation and cell polarity (Weinstein et al. 1976; Soler et al. 1999). Loss of TJ integrity may be particularly important in allowing the diffusion of nutrients and other factors necessary for the survival and growth of the tumor cells (Mullin et al. 1997). In addition, decreased polarity and differentiation may be important for the metastatic phenotype, where individual cells must leave the primary site and enter the blood vessels to reach distant sites (Ren et al. 1990).

Electron microscopy studies in human thyroid tumors showed that TJs decrease in number and are attenuated during carcinogenesis, which is associated with loss of tumor differentiation (Kerjaschki et al. 1979). Expression of TJ proteins is decreased in some cancer types, e.g., ZO-1 and occludin in gastrointestinal adenocarcinoma (Kimura et al. 1997), occludin in epithelial-derived tumors (Li and Mrsny 2000), claudin 3 in glioblastoma multiforme (Wolburg et al. 2003), claudin 1 in sporadic and hereditary breast cancer (Kramer et al. 2000), and claudin 7 in ductal carcinoma of the breast (Kominsky et al. 2003). On the other hand, some TJ molecules appear to be upregulated in some cancers. We found protein expression of claudins 3, 4, and 5, occludin, and ZO-1 in Merkel cell carcinoma cells (Haass et al. 2003b). Strikingly, expression of some claudin family members is highly elevated in various human cancers, e.g., claudin 7 in two breast cancer cell lines (Nacht et al. 1999), claudin 1 in colorectal cancer (Miwa et al. 2000), and claudins 3 and 4 in ovarian (Hough et al. 2001; Rangel et al. 2003) and prostate cancer (Long et al. 2001).

The expression of TJ proteins in melanoma tissues and cultured melanoma cells was described on RNA and on protein level (Cohn et al. 2005; Smalley et al. 2005; Leotlela et al. 2007; Schmitt et al. 2007; Morita et al. 2008). In a tissue array study, Claudin-1 was found to be significantly reduced in metastatic melanoma (Cohn et al. 2005). These data were, however, directly contradicted by another study (Leotlela et al. 2007). In this study Claudin-1 appeared to contribute to melanoma cell invasion, as transient transfection of melanoma cells with Claudin-1 increased metalloproteinase 2 (MMP-2) secretion and activation, and subsequently, motility of melanoma cells as demonstrated by wound-healing assays. Conversely, knockdown of CLDN1 by siRNA resulted in the inhibition of motility, as well as decreases in MMP-2 secretion and activation (Leotlela et al. 2007).

In contrast to most cancers, where levels of ZO-1 are typically downregulated, leading to increased motility, we found that ZO-1 expression is upregulated in melanoma cells and is located at adherens junctions between melanoma cells and fibroblasts (Smalley et al. 2005). Immunofluorescence and co-immunoprecipitation studies showed co-localization of ZO-1 with N-cadherin. Downregulation of ZO-1 in melanoma cells through RNA interference produced marked changes in cell morphology – leading to a less dendritic, more rounded phenotype. Consistent with a role in N-cadherin-based adhesion, RNAi-treated melanoma cells were less adherent and invasive when grown in a collagen gel. These data provided the first evidence that increased ZO-1 expression in melanoma contributes to the oncogenic behavior of this tumor and further illustrated that protein products of genes, such as

ZO-1, can function in either a pro- or anti-oncogenic manner when expressed in different cellular contexts (Smalley et al. 2005).

In summary, while it appears that functional TJs may be tumor suppressors, the upregulation of certain TJ proteins can contribute to oncogenic behavior. The relationship between TJ protein overexpression and cancer initiation or progression is thus unclear at present, but may be explained by the lack of functional TJs and that the upregulated TJ proteins therefore likely function through TJ-independent mechanisms.

Acknowledgment NKH is a Cameron Fellow of the Melanoma and Skin Cancer Research Institute, Australia, and a Sydney Medical School Foundation Fellow. NKH also thanks the German Research Foundation (DFG, HA26801), Cancer Council NSW (RG 09–08, RG 13–06), Cancer Australia/Cure Cancer Australia Foundation (570778), Cancer Institute New South Wales (08/RFG/1-27), and the National Health and Medical Research Council Australia (1003637, 1084893) for contributing grant support.

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Regulation of Apoptosis in Melanoma Cells: Critical Targets for Therapeutic Strategies

10

Jürgen Eberle and Lothar F. Fecker

10.1 Introduction: Critical Roles of Apoptosis Deficiency for Melanoma

While the incidence of most solid tumors has decreased or stabilized in the last decades, melanoma incidence continued to rise with approximately 3 % per year (Siegel et al. 2014). The until recently almost unbroken high mortality resulted from an early dissemination associated with pronounced chemotherapy resistance. In the past, neither chemotherapy regimens nor biotherapy (IL-2 and IFN-α) or vaccination protocols could significantly improve the fatal situation of metastasized melanoma patients (Fang et al. 2008; Garbe et al. 2012). This has changed recently with the development of selective BRAF inhibitors and targeted immune modulators such as anti-CTLA4 and anti-PD1, which for the first time could significantly improve the overall survival (Menzies and Long 2014; Hughes et al. 2015). Nevertheless, after initial tumor reduction and clinical improvement, tumor relapse and therapy resistance often follow within only a few months (Chen and Davies 2014), thus challenging for combination therapies that may further improve the clinical outcome.

Although different cellular mechanisms may contribute to therapy resistance in cancer, apoptosis deficiency appears as a major cause. This is explained by the fact that the final elimination of cancer cells by proapoptotic programs represents a

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_10

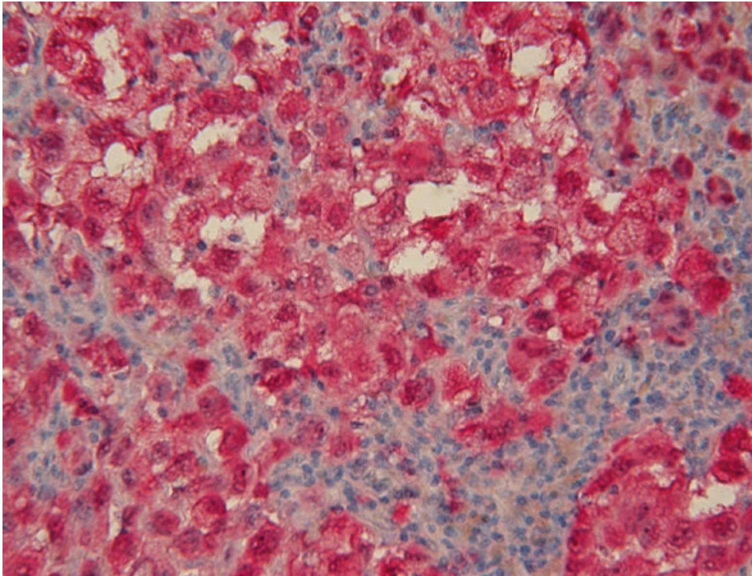

Fig. 10.1 Immunogenicity of melanoma. Massive lymphocytic tumor infiltrates are seen in a section of a primary melanoma. Lymphocytes: *gray* with small nuclei. Melanoma cells: *stained red* by S100 antibody

common final path of most anticancer therapies. For example, different chemotherapeutic drugs cause cellular or DNA damage, which induces cell-intrinsic proapoptotic pathways, and also, BRAF inhibitors have been related to an induction of apoptosis or sensitization for proapoptotic programs (Eberle et al. 2007b; Beck et al. 2013; Berger et al. 2014). Furthermore, immune stimulation results in cytotoxic T-lymphocytes, which express death ligands to trigger extrinsic proapoptotic pathways in target cancer cells (Martinez-Lostao et al. 2015).

Proapoptotic pathways serve as essential safeguard mechanisms preventing the growth of abnormal tumor cells in multicellular organisms (Eberle et al. 2007a). Thus, intrinsic proapoptotic pathways are activated by different kinds of cellular stress situations and dysfunction, also including typical characteristics of cancer cells as oncogene activation. Furthermore, the elimination of cancer cells in an immune response is based on the extrinsic induction of apoptosis. Thus, resistance to apoptosis appears as a prerequisite of early cancer growth, and in the second run, it may enable tumor cells to survive anticancer therapy (Hanahan and Weinberg 2011).

Melanoma is a particular example for pronounced chemotherapy resistance and large resistance to immune cells. The latter is suggested by the frequently high immunoreactivity in initial tumor stages and high numbers of tumor-infiltrating lymphocytes (Fig. 10.1). Nevertheless, the tumor grows and may finally form metastases. Thus, the sensitization of melanoma cells for apoptosis may support the therapeutic effects both of BRAF inhibitors (intrinsic proapoptotic pathways) and of immunotherapy due to a strengthening of extrinsic proapoptotic pathways (Fig. 10.2).

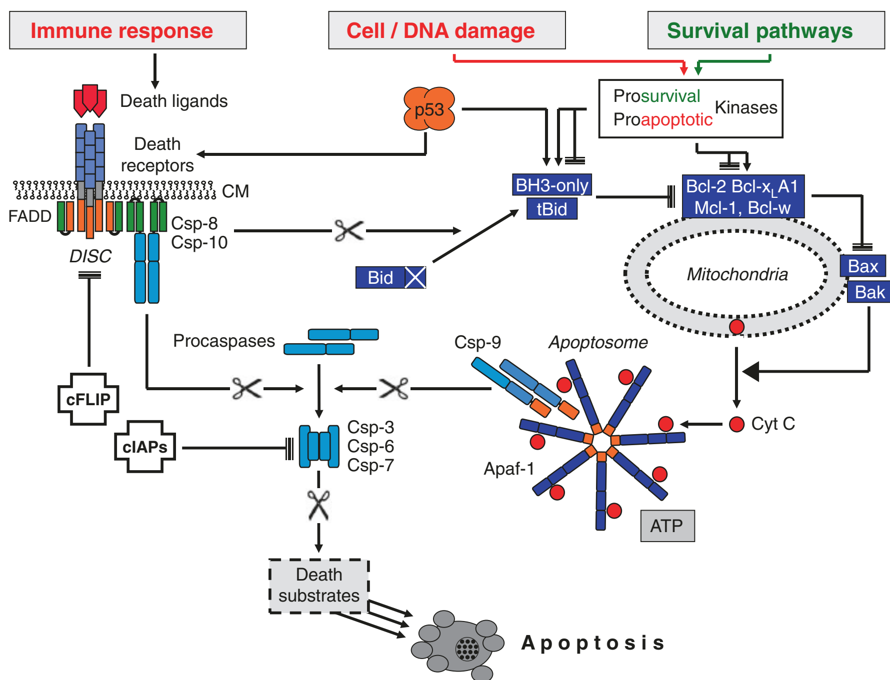

Fig. 10.2 Signaling pathways for regulation of apoptosis. Extrinsic proapoptotic pathways (*left*) are triggered by death ligands released from immune cells in an immune response, whereas intrinsic proapoptotic pathways (*right side*) are initiated upon cellular damage via p53 activation or via proapoptotic kinases. Kinases are affected in the opposite way by survival pathways, e.g., the BRAF-initiated MAP kinase pathway. Prosurvival and proapoptotic kinases in particular control the activity of antiapoptotic Bcl-2 proteins (Bcl-2, Bcl-x$_L$, A1, Mcl-1, and Bcl-w) as well as the proapoptotic BH3-only proteins. Two multiprotein complexes, the death-inducing signaling complex (DISC) and the apoptosome are employed for caspase activation. Caspase 8/10 binding to the DISC is mediated by the adaptor protein FADD and the interaction of death domains (*orange*) and death effector domains (*green*), while binding of caspase-9 to the apoptosome is mediated by the caspase recruitment domains (*orange*). The caspase-8/-10 homolog cFLIP inhibits activation of the initiator caspases at the DISC, while cIAPs block effector caspases (-3, -6, -7). *FADD*, Fas-associated death domain; *Csp*, caspase; *tBid*, truncated Bid; *APAF-1*, apoptotic protease-activating factor-1; *ATP*, adenosine triphosphate; *Cyt C*, cytochrome C; *BH3-only*, BH3-only proteins. *c-FLIP* cellular homolog of FLICE-inhibitory protein, *c-IAPs* cellular inhibitor of apoptosis proteins, *CM* cytoplasmic membrane. Scissors indicate protease activity

Apoptosis deficiency is based on an inactivation of proapoptotic factors or the activation of antiapoptotic factors. Both options are linked to activated survival pathways. In normal tissues, homeostasis is maintained by a well-balanced equilibrium of cell proliferation and cell death. In this way, programmed cell death (apoptosis) represents an active cellular process, whose physiological endpoint is phagocytosis by macrophages or neighboring cells (Kerr et al. 1972). Due to this key function, apoptosis pathways are tightly regulated. Various cellular options for counter-regulation, necessary for the survival of normal cells, abet tumor cells to escape from apoptosis control.

10.2 Intrinsic Apoptosis Pathways

Intrinsic proapoptotic pathways can be induced by different kinds of cellular stress situations as by DNA damage, hypoxia, oncogene activation, or other intrinsic problems. They can be mediated by the tumor suppressor and transcription factor p53 or by proapoptotic kinases (Fig. 10.2). P53 is negatively regulated by ubiquitin ligation through HDM-2 resulting in its early degradation. In response to cellular stress situations, it is stabilized by different kinds of protein modifications as by phosphorylation. Apoptosis by p53 is related to transcriptional upregulation of death receptors and proapoptotic Bcl-2 proteins as Bax, Noxa, Puma, Bik/Nbk, and Bid (Zuckerman et al. 2009).

The large family of pro- and antiapoptotic Bcl-2 proteins control mitochondria-mediated apoptosis pathways. Bcl-2 proteins are characterized by up to four conserved Bcl-2 homology domains (BH1–BH4) (Fig. 10.3). Whereas most antiapoptotic proteins as Bcl-2 share all four domains, proapoptotic proteins subdivide in multidomain proteins as Bax, Bak, and Bok which enclose BH1, BH2, and BH3 as well as the large subfamily of BH3-only proteins (Chipuk et al. 2010). Other proteins reveal further domain combinations. Thus, Bcl-x_S (BH3, BH4) and Bcl-x_{AK} (BH2, BH4), which derive from the *Bcl-x* gene by alternative splicing, showed characteristic proapoptotic functions in melanoma cells by activating the Bak or the Bax and Bak pathway, respectively (Plötz et al. 2012a, b).

Multidomain proapoptotic proteins as Bax and Bak are believed to induce pores in mitochondrial membranes for release of mitochondrial factors, such as cytochrome C. They are negatively controlled by antiapoptotic Bcl-2 family members,

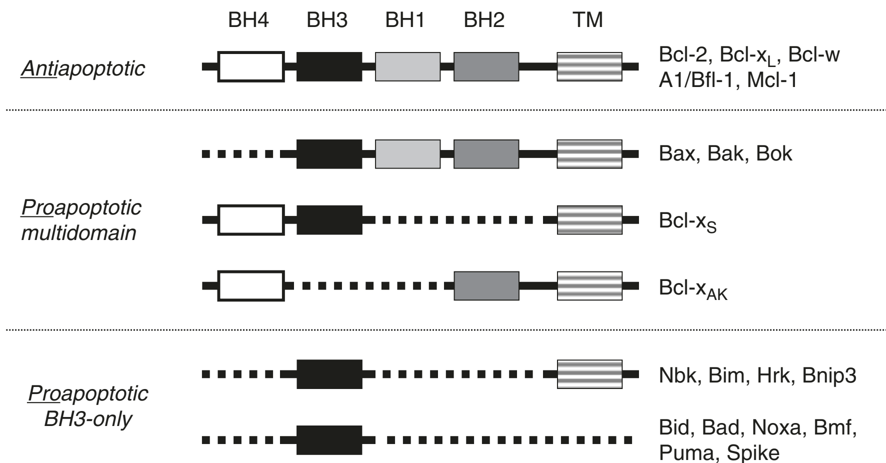

Fig. 10.3 Structure of pro- and antiapoptotic Bcl-2 proteins. A schematic view is given of the principle structures of Bcl-2 proteins. The presence of up to four Bcl-2 homology domains (*BH*) is indicated. Unlike the schematic view, Bcl-2 proteins have variable lengths. Besides the main groups of multidomain antiapoptotic, multidomain proapoptotic (Bax/Bak group), and BH3-only proteins, two proapoptotic splice variants of Bcl-x with an unusual domain structure are shown. BH3-only proteins may have a transmembrane domain (*TM*) or not

which neutralize proapoptotic Bcl-2 proteins through heterodimerization, thus protecting the mitochondrial membrane integrity. Finally, BH3-only proteins function as sensors in apoptosis control. When activated by cellular stress signals, they bind to antiapoptotic Bcl-2 proteins thus to free Bax and Bak. Some were even reported to activate Bax in a direct way as Bid and Bim (Chipuk et al. 2010).

Bax and Bak are mutually exchangeable, seen in mice deficient for either one of the two. These mice are viable, whereas the double knockout strongly impairs developmental apoptosis, resulting in perinatal death. Also cultured cells deficient for both proteins are largely apoptosis-resistant (Lindsten et al. 2000). Deficiency of Bcl-x_L is also lethal, whereas Bcl-2 knockout mice are viable, but reveal developmental defects particularly concerning increased apoptosis in melanocyte precursors, thus underlining the particular role of Bcl-2 for the melanocytic lineage (Korsmeyer 1999).

The balance between pro- and antiapoptotic Bcl-2 proteins controls the permeability of the outer mitochondrial membrane. Once a certain threshold is reached, a rapid release of mitochondrial intermembrane factors is induced, which exert specific proapoptotic functions in the cytoplasm, as reported for cytochrome C, endonuclease G, AIF (apoptosis-inducing factor), Smac/DIABLO, and HtrA2/Omi. Thus, cytochrome C released into the cytosol induces formation of the apoptosome, a protein complex consisting of Apaf-1, ATP, cytochrome C, and the initiator caspase-9 (Chipuk et al. 2010). Here, caspase-9 is activated by induced proximity, which initiates a subsequent caspase signaling cascade. Caspases (aspartate-specific cysteine proteases) represent hallmarks in apoptosis. They are synthesized as inactive zymogens and activate each other by proteolytic processing. Proapoptotic caspases separate in initiator caspases (2, 8, 9, and 10) and effector caspases (3, 6, and 7), which have a large number of cellular target proteins (Pop and Salvesen 2009) (Fig. 10.2).

10.3 Extrinsic Apoptotic Pathways

Cytotoxic T-lymphocytes and natural killer cells employ death ligands as tumor necrosis factor (TNF-α), CD95L/FasL, and TNF-related apoptosis-inducing ligand (TRAIL), which trigger extrinsic apoptosis in target cells (Fig. 10.2) (Chan and Housseau 2008). They bind to four death receptors (TNF-R1, CD95, TRAIL-R1/DR4, and TRAIL-R2/DR5), whereas decoy receptors (DcR 1–3 and OPG) do not forward the proapoptotic signal (Fig. 10.4). Characteristic for the proapoptotic program is receptor oligomerization upon ligand binding and formation of membrane-bound, death-inducing signaling complexes (DISC). Here, attached initiator caspases (8 and 10) become activated due to induced proximity and/or autocatalytic proteolysis. Besides their proapoptotic function, death receptors may also activate NF-kB and MAPK-related pathways, and NF-kB is also activated by TNF-R2, which does not trigger caspases (Guicciardi and Gores 2009) (Fig. 10.4). The NF-kB-mediated transcriptional activation of antiapoptotic proteins as c-FLIP, Bcl-x_L, and c-IAPs may enable a balanced response to death signals (Karin 2006).

Thus, both extrinsic and intrinsic pathways result in the activation of initiator caspases, which in turn promote a caspase cascade leading to active effector

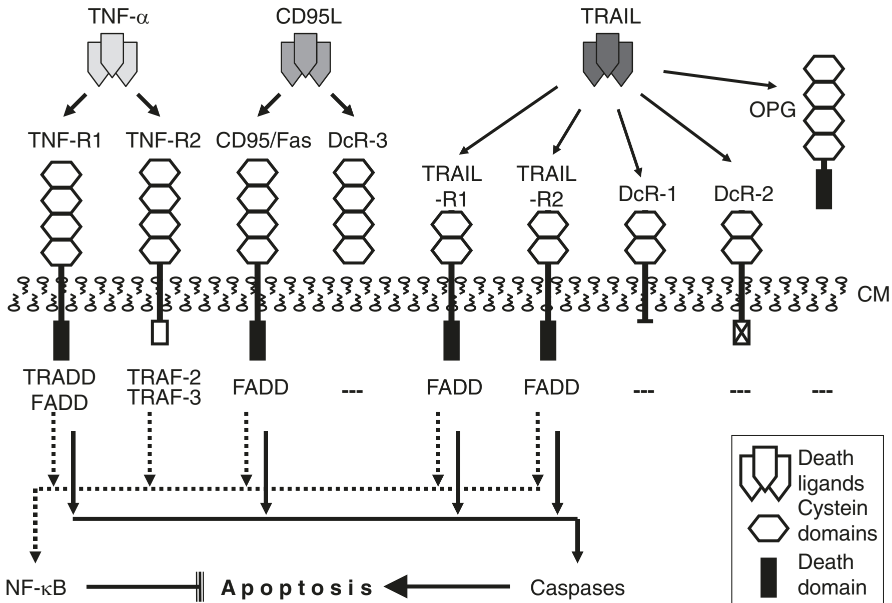

Fig. 10.4 Death receptors and death ligands. Receptors of TNF-α, CD95L/FasL, and TRAIL are shown. They enclose four death receptors, characterized by the death domain (black), four decoy receptors (DcR, OPG), and TNF-R2. While the death domain is lacking or not functional in DcR-1 and DcR-2, respectively, DcR-3 and OPG lack a functional transmembrane domain. Finally, the cytoplasma domain of TNF-R2 does not trigger DISC formation but a complex to activate NF-kB. The four death receptors can activate both pathways (caspases and NF-kB). *FADD*, Fas-associated death domain; *TRADD*, TNF receptor-associated death domain; *TRAF*, TNF receptor-associated factor; *CM*, cytoplasma membrane. Outside the cytoplasma membrane, the receptors have 2–4 cysteine-rich domains (hexagons)

caspases. These cleave a large subset of cellular proteins (death substrates) including enzymes for DNA repair and modification as well as signaling and structural proteins, in this way reprogramming the cell for apoptosis (Fischer et al. 2003). Crosstalk between the pathways may lead to mutual enhancements, e.g., transactivation of death receptors by p53 or cleavage and activation of the proapoptotic Bcl-2 protein Bid by caspase-8. Truncated Bid is a BH3-only protein and involved in activation of the mitochondrial pathway (Fig. 10.2).

10.4 Less Obvious Roles of p53 in Melanoma

The tumor suppressor gene p53 is mutated in around 50 % of human cancer cases but only in 10–20 % of human melanoma (Mar et al. 2013). Nevertheless, the tumor-suppressive function of p53 in melanoma cells is largely lost. The important role of p53 for suppression of melanoma growth is suggested by the rapid proliferation of BRAFV600E-mutated melanocytes on loss of p53 function (Yu et al. 2009). Inactivation

of the p53 pathway may also result from overexpression/amplification of its antagonists MDM2 and MDM4 as well as from mutation/deletion of CDKN4, which encodes the cell cycle inhibitor p16 as well as the MDM2 inhibitor p14^{ARF} (Lu et al. 2014).

In addition, p53 transcriptional activity may be modulated. Thus, p53 frequently found accumulated in the nucleus of melanoma cells seems to exhibit only little transcriptional activity, likely indicative for a post-translational inactivation. Accordingly, melanoma cell lines with wild-type p53 did not respond to exogeneous p53 overexpression, whereas apoptosis was induced in p53-mutated melanoma cells by overexpression of a functional p53 (Satyamoorthy et al. 2000). Transcriptional activity of p53 is critically modulated by the evolutionarily conserved ASPP family of proteins. Thus, phosphorylated nuclear iASPP was found in 90 % of wild-type p53-expressing melanoma cell lines. Compared to unphosphorylated cytoplasmic iASPP, phosphorylated nuclear iASPP binds p53 better and is more potent in inhibiting p53's apoptotic function. Furthermore, high levels of phosphorylated nuclear iASPP are associated with poor survival in melanoma patients (Lu et al. 2013).

10.5 Role of Caspase Downregulation and Inhibitor of Apoptosis Proteins

The initiator caspases 8 and 10, which are essential for death ligand-induced apoptosis (Fig. 10.2), are frequently downregulated in tumors, which may result from gene silencing by DNA methylation. In melanoma cell lines, both caspases are highly expressed but are downregulated in cells selected for TRAIL resistance (Kurbanov et al. 2007).

Many proapoptotic pathways merge at the level of effector caspases, which are suppressed by cIAPs (Nachmias et al. 2004). In melanoma, expression of survivin, livin/ML-IAP, and X-linked IAP (XIAP) correlated with drug resistance, progression, and survival, and their downregulation increased melanoma cell chemosensitivity in vitro and in xenotransplants (Yan et al. 2006). The antiapoptotic function of XIAP in melanoma cells, which is antagonized by Smac release, is suggested by an incomplete processing of caspase-3 upon TRAIL treatment. The role of the Smac/XIAP rheostat in TRAIL-induced apoptosis was proven by XIAP overexpression and/or by Smac knockdown, which both protected melanoma cells from TRAIL-induced apoptosis (Hornle et al. 2011; Quast et al. 2012; Berger et al. 2013). High XIAP activities thus appear as one of the major barriers for efficient apoptosis induction in melanoma (Quast et al. 2014).

10.6 The Role of Bcl-2 Proteins in Melanoma Apoptosis Resistance

The mitochondrial, proapoptotic pathway is critically controlled by three groups of Bcl-2 proteins (Figs. 10.2 and 10.3). The expression of both antiapoptotic proteins as Bcl-2, Bcl-x_L, and Mcl-1 as well as many proapoptotic proteins as Bax, Bak, Bid,

Bad, PUMA, and Noxa was reported in melanoma cells. A high Bcl-2/Bax ratio correlated to apoptosis resistance, and exogeneous Bcl-2 overexpression almost abrogated melanoma cell sensitivity for many strategies as CD95L, TRAIL, ceramide and kinase inhibitors (Eberle et al. 2007b; Quast et al. 2014). Expression and activity of antiapoptotic Bcl-2 proteins is controlled by different survival pathways (Fig. 10.2), e.g., Bcl-2 is controlled by microphthalmia-associated transcription factor (MITF) or Bcl-x_L is controlled by NF-kB (Eberle et al. 2007b; Karin 2006).

Despite the significant role of Bcl-2 in melanocyte cell survival, its contribution to chemoresistance of metastasized melanoma remains unclear because Bcl-2 levels did not correlate with prognosis in primary melanoma and even reduced expression was found in metastases, whereas Bcl-x_L and Mcl-1 were upregulated (Zhuang et al. 2007). On the other hand, several proapoptotic Bcl-2 proteins may be upregulated in course of chemotherapy or kinase inhibition. Thus, taurolidine-induced apoptosis in melanoma cells correlated with enhanced Bax and reduced Bcl-2 expression (Sun et al. 2007), or Puma and Bim were upregulated by BRAF inhibition (Beck et al. 2013; Berger et al. 2014). Particularly, the expression of proapoptotic Bcl-2 proteins as Bax and Bak appears of prognostic value, as their downregulation in primary melanomas was correlated with unfavorable prognosis (Fecker et al. 2006), and inactivation of Bax appeared as a critical step in TRAIL resistance of melanoma cells (Quast et al. 2014).

Thus, high expression of antiapoptotic Bcl-2 proteins in melanoma and/or low activity of proapoptotic family members may elevate the threshold for an activation of the mitochondrial pathway and may thus critically contribute to chemoresistance. In agreement, significant induction of apoptosis was enabled by overexpression of different proapoptotic Bcl-2 proteins in melanoma (Eberle et al. 2007b; Plötz et al. 2012a, b; Plötz et al. 2013).

10.7 Blockage of Death Receptor–Mediated Pathways in Melanoma

Despite a high immunoreactivity, proliferating melanomas withstand apoptosis induction by immune cells (Fig. 10.1). Expression of TNF-α, CD95L, and TRAIL was proven for melanoma-infiltrating lymphocytes (Thomas and Hersey 1998). This indicates a high selective pressure for melanomas to acquire death ligand resistance. Accordingly, molecular changes in the death receptor-mediated pathways are frequently seen. Thus, melanoma cell lines often reveal resistance to CD95 activation, and cultures of metastases were more resistant than those of primary tumors. Loss of CD95 expression and missense mutations in the death domain as well as high Bcl-2 or Mcl-1 expression have been related to resistance to CD95 (Chetoui et al. 2008; Eberle et al. 2007b).

Also, resistance to TNF-α and loss of TNF-R1 have been reported for melanoma cells, which may be correlated to promoter hypermethylation (Kaminski et al. 2004). In contrast, neither mutations of TRAIL death receptors nor involvement of

decoy receptors (DcR-1, DcR-2) have been identified in melanoma cells so far. However, expression of TRAIL-R1, which mediates high TRAIL sensitivity in melanoma cells when expressed, was frequently lost. In contrast, TRAIL-R2 is constitutively expressed, but the downstream pathways may be inactive as shown in melanoma cell lines (Kurbanov et al. 2005). Inactivation of TRAIL-R2 may result from its decreased glycosylation or from high c-FLIP expression (Wagner et al. 2007; Geserick et al. 2008).

Whereas no clear correlation was found to the expression of antiapoptotic factors, TRAIL resistance in TRAIL-selected melanoma cells clearly correlated with downregulation of proapoptotic regulators as initiator caspases, DR4 and BH3-only proteins (Kurbanov et al. 2007). Furthermore, melanoma resistance to TRAIL was explained by three major steps, namely, high levels of antiapoptotic Bcl-2 proteins, high levels of inhibitor of apoptosis proteins (cIAPs), and suppressed Bax activity (Quast et al. 2014).

10.8 Roles of MAP Kinase Pathways in Apoptosis Resistance

Central signaling pathways downstream of growth factor receptors as of MAPKs, PI3K/Akt, and NF-kB are not only critically implicated in enhanced proliferation of cancer cells but also in their apoptosis resistance (see also Chap. 13). As seen by BRAF mutations, these survival pathways are activated in melanoma. They contribute to the control of a number of apoptosis regulators either by affecting the transcriptional level through regulation of respective transcription factors or by regulating the activity of apoptosis factors directly through phosphorylation (Eberle et al. 2007b).

High activity of the canonical MAPK pathway via RAF, MEK, and ERK in melanoma is mainly attributed to the activating mutations in *B-Raf* and *N-Ras* (Davies et al. 2002). Downstream are a multitude of transcription factors that also control the expression of many apoptosis regulators. For example, the transcription factor MITF plays a particular role in apoptosis resistance of melanoma cells due to its upregulation of Bcl-2 as well as the cIAP Livin (Dynek et al. 2008). Further transcription factors involved in apoptosis resistance have been identified within the Ets and CREB/ATF families, which may contribute to upregulation of also Bcl-2 or of Bcl-x_L downstream of MAPK activation (Eberle et al. 2007b) (see also Chap. 7). An example for the direct regulation of apoptosis factors by kinases is the inactivation of the BH3-only proteins, Bad, Bim, and Puma, through phosphorylation by MAPKs (Inamdar et al. 2010; Beck et al. 2013; Berger et al. 2014). Thus, selective BRAF inhibitors induced apoptosis and/or sensitized for proapoptotic strategies, e.g., for TRAIL (Beck et al. 2013; Berger et al. 2014). Similarly, targeting of the MAPK pathway downstream of BRAF as by MEK inhibitors induced apoptosis, sensitized melanoma cells for TRAIL, and reduced the growth of lung metastases in mice (Inamdar et al. 2010; Berger et al. 2014). These effects have been related to upregulation or activation of the BH3-only proteins Puma, Bim, and Bmf, downregulation of Mcl-1, as well as activation of Bax (VanBrocklin et al. 2009; Wang et al. 2007;

Quast et al. 2014). Thus, clinical efficiency of MAPK inhibitors appears as strongly related to enhanced apoptosis sensitivity, which may be further improved by combination therapies.

10.9 Targeting of the PI3K/Akt/mTOR Pathway

A central role in cell survival has been ascribed to the phosphoinositide 3-kinase (PI3K)/AKT pathway, which is related to increased chemoresistance in many tumors (see also Chap. 16). Akt may directly phosphorylate and inactivate several proapoptotic proteins such as Bax, Bad, and caspase-9, and may further inactivate proapoptotic transcription factors such as FoxO and p53. In addition, AKT is linked to the survival pathway of mTOR (mammalian target of rapamycin), which inhibits cell death pathways as apoptosis and autophagy (Stiles 2009). Melanomas reveal high immunoreactivity for activated, phosphorylated AKT, which may partly be related to mutations of its inhibitor phosphatase and tensin homolog on chromosome 10 (PTEN). Antiapoptotic activities of AKT have been described for melanoma cells, such as inactivation of Bad and activation of IKK (I-kB kinase) leading to a cross-activation of the NF-kB pathway. The critical role of the AKT survival pathway in melanoma was demonstrated by expression of a dominant negative AKT mutant, by its siRNA downregulation as well as by overexpression of PTEN, which all triggered apoptosis (Robertson 2005).

By using small molecule approaches, inhibition of PI3K-reduced melanoma growth in mice, particularly in combination with inhibition of MEK (Inamdar et al. 2010), and both AKT and mTOR inhibition consistently enhanced apoptosis and chemosensitivity of melanoma cells (Sinnberg et al. 2009). Also, the farnesyl transferase inhibitor lonafarnib, which inhibits mTOR signaling, enhanced sorafenib-induced apoptosis in melanoma cells, which was related to induction of ER stress and downregulation of Mcl-1 (Niessner et al. 2011). However, inhibitors of AKT as well as of mTOR did not result in significant clinical responses in metastatic melanoma patients, when used as monotherapy (Eberle et al. 2007b). But PI3K and AKT inhibitors showed synergistic enhancement of apoptosis in combination with TRAIL, which was explained by a novel so far not well described pathway that is based on the generation of reactive oxygen species (ROS) (Quast et al. 2013). Thus although inhibition of this pathway may not be sufficient by itself, combination strategies may be an option.

10.10 New Proapoptotic Strategies by BH3 Mimetics

To further improve the clinical efficiency of melanoma therapy, combinations of survival pathway inhibitors with proapoptotic strategies appear as highly promising. Frequently, chemotherapeutics were used in combinations, which cause cellular stress situations as DNA damage to trigger apoptosis via intrinsic pathways. A selective induction of apoptosis in cancer cells may, however, be more efficient and better tolerated.

When considering the important role of mitochondrial proapoptotic pathways in melanoma, approaches that target the Bcl-2 protein family appear of particular interest. Thus Bcl-2 antisense strategies have been applied, which revealed in vitro and in mouse models significant apoptosis induction and sensitization for chemotherapy. However, in clinical trials in melanoma, the combination of Bcl-2 antisense with chemotherapy did not prove as effective enough (Bedikian et al. 2006). This may be related to the finding that Bcl-2 may be even downregulated in metastatic melanoma and may be substituted by other antiapoptotic Bcl-2 proteins (Zhuang et al. 2007). Thus, the simultaneous targeting of several antiapoptotic Bcl-2 proteins may be necessary. Alternatively, proapoptotic Bcl-2 proteins may be overexpressed to trigger the rheostat of pro- and antiapoptotic Bcl-2 proteins from the other side. The efficiency of such strategies has been demonstrated by plasmid or adenovirus-mediated overexpression of Bax, Bik/NBK, Bcl-x_S, Bcl-x_{AK}, and Bim (Eberle et al. 2007b; Plötz et al. 2012a, b, 2013).

Of particular interest are effectors that mimic the BH3 domain of proapoptotic Bcl-2 proteins. BH3 is required for the interaction between proapoptotic Bcl-2 proteins and the hydrophobic pocket of antiapoptotic Bcl-2 proteins formed by BH1, BH2, and BH3. These are peptides or small molecules structurally related to BH3 domains of different proteins and may have the potential to block different antiapoptotic Bcl-2 proteins (Adams and Cory 2007; Plötz and Eberle 2014).

There are several preclinical reports on the effects of BH3 mimetics in melanoma. Thus, the natural BH3 mimetic gossypol, a compound of cotton seeds, induced cell death in melanoma cells with even higher efficacy than some chemotherapeutics (Shelley et al. 1999). Apoptosis resistance due to high levels of antiapoptotic Bcl-2 proteins was overcome by the BH3 mimetic TW-37 when used in combination with the MEK inhibitor U0126 (Verhaegen et al. 2006). A synergistic induction of mitochondrial apoptosis has been reported in melanoma cells by combination of the BH3 mimetic ABT-737 and the proteasome inhibitor MG-132, which resulted in simultaneous upregulation of Noxa (Miller et al. 2009). Efficient induction of apoptosis has also been described for the combination of ABT-737 and a Mcl-1 knockdown (Keuling et al. 2009). A combination of obatoclax, a BH3 mimetic with inhibitory activity against Mcl-1, with an ER stress-inducing compound resulted in marked induction of apoptosis in melanoma cells, which was dependent on Mcl-1 inhibition and again induction of Noxa (Jiang et al. 2009).

New BH3 mimetics designed by computer-based modeling are presently developed and more effective ones, preferentially targeting several antiapoptotic Bcl-2 proteins, may be expected in the future. Considering the high dependency of apoptosis induction in melanoma cells on the mitochondrial pathway, BH3 mimetics provide hope for an efficient targeting of melanoma (Plötz and Eberle 2014; Liu et al. 2015). The complex mutual regulation of Bcl-2 proteins, however, needs further clarification in melanoma to optimize these approaches.

10.11 TRAIL as a Combination Partner

As suggested by the high immunoreactivity of melanoma (Fig. 10.1) and by its frequent resistance to death ligands (Kurbanov et al. 2007), strategies for enhancing death ligand sensitivity and for overcoming resistance appear as also highly promising. In contrast to chemotherapy, death ligands trigger apoptosis independently of p53 and thus may overcome drug resistance related to p53 inactivation. Although CD95 agonistic antibodies and TNF-α have shown efficiency in mouse xenotransplants, their application in patients is largely prevented due to systemic inflammation (TNF-α) or liver toxicity (CD95L) (Kelley and Ashkenazi 2004; Trauth et al. 1989). Their selective expression in gene therapeutic approaches, however, remains an alternative strategy. Thus, expression of CD95L prevented growth of melanoma xenotransplants in mice (Eberle et al. 2003), and its selective expression via melanoma-selective replication-competent adenoviral vectors appeared as an efficient approach for targeting melanoma cells (Fecker et al. 2010).

As compared to TNF-α and CD95L, systemic application of TRAIL is much better tolerated. Induction of apoptosis and eradication of xenotransplants were demonstrated in a variety of tumor models, whereas toxicity for normal cells remained at a low level. Both TRAIL receptors (DR4 and DR5) may be targeted by recombinant TRAIL or by selective agonistic antibodies, which showed efficiency and synergistic effects with chemotherapy in preclinical studies. TRAIL also demonstrated antitumor activities in clinical trials; it appeared, however, not as efficient enough when used as monotherapy, as shown in a phase III trial with agonistic DR4 antibodies for therapy of patients with refractory colorectal cancer (Newsom-Davis et al. 2009; Trarbach et al. 2010).

All melanoma cell lines express TRAIL-R1/DR5, which may or may not mediate TRAIL sensitivity. However, all melanoma cell lines that have TRAIL-R1/DR4 in addition revealed pronounced sensitivity to TRAIL. Importantly, the majority of primary melanomas are DR4-positive, as proven by immunohistochemistry, thus suggesting principle TRAIL sensitivity (Kurbanov et al. 2005). However, DR4-positive melanoma cells respond to TRAIL treatment with an inducible TRAIL resistance that was correlated to downregulation of DR4 and initiator caspases as well as to an inactivation of Bax (Kurbanov et al. 2007; Quast et al. 2014). Chemotherapy, which may upregulate death receptors (Singh et al. 2003), proteasome inhibitors, which may prevent death receptor degradation (Kurbanov et al. 2007) or IFN-γ, which may upregulate initiator caspases (Fulda and Debatin 2006), may thus be helpful in countering TRAIL resistance. Furthermore, several signal pathway inhibitors showed high efficiency in combination with TRAIL as they mediated Bax activation (Quast et al. 2014).

Indeed, melanoma cells could be sensitized for TRAIL by multiple different strategies, e.g., resveratrol (Ivanov et al. 2008), chemotherapeutics (Fecker et al. 2011), the multi-kinase inhibitor indirubin (Berger et al. 2011), the ion channel inhibitor TRAM-34 (Quast et al. 2012), IKK inhibition (Berger et al. 2013), PI3K or AKT inhibition (Quast et al. 2013) as well as BRAF and MEK inhibition (Berger et al. 2014). A common principle of TRAIL sensitization appeared to be the

inhibition of the cell cycle by different inhibitors and the activation of Bax (Quast et al. 2015). Due to the multiple strategies that could sensitize melanoma cells for TRAIL, this ligand appears as an ideal combination partner for melanoma therapy.

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Senescence 11

Helen Rizos, Sebastian Haferkamp, and Lyndee L. Scurr

11.1 Introduction

Cellular senescence is regarded as an intrinsic stress response mechanism that limits the proliferative lifespan of cells. Senescence is induced by various signals, including telomere attrition (a response often referred to as replicative senescence), activated oncogenes (a process known as oncogene-induced senescence), DNA damage, oxidative lesions, and suboptimal culture conditions (reviewed in Collado and Serrano 2006). Irrespective of the initiating trigger, the hallmark of cellular senescence is permanent proliferative arrest, and whereas quiescent cells can be stimulated to resume proliferation, senescence cells cease to respond to mitogenic stimuli.

The limited proliferative capacity of normal human cells was recognised by Hayflick and Moorehead more than five decades ago (Hayflick and Moorhead 1961). They reported that primary human fibroblasts ceased proliferating after serial cultivation in vitro and although these arrested fibroblasts remained metabolically active for many weeks, they did not initiate DNA replication despite adequate culture conditions. Replicative senescence has since been described in many normal somatic human cells, including epidermal keratinocytes and melanocytes as well as

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_11

in cells derived from rodents, birds, and several other species (Campisi 2001; Kim et al. 2002). The Hayflick limit is often used to refer to the maximum number of population doublings for any given cell population.

11.2 Characteristics of Senescent Cells

Senescent cells have been identified both in vitro and in vivo using a series of phenotypic features and markers that are not exclusive to the senescent state but act as powerful predictors of senescence when used in combination (reviewed in Collado and Serrano 2006; Campisi and d'Adda di Fagagna 2007). Increased activity of acidic ß-galactosidase, termed senescence-associated ß-galactosidase (SA-ß-gal) is the most widely accepted marker of senescent cells (Dimri et al. 1995). The expression of this

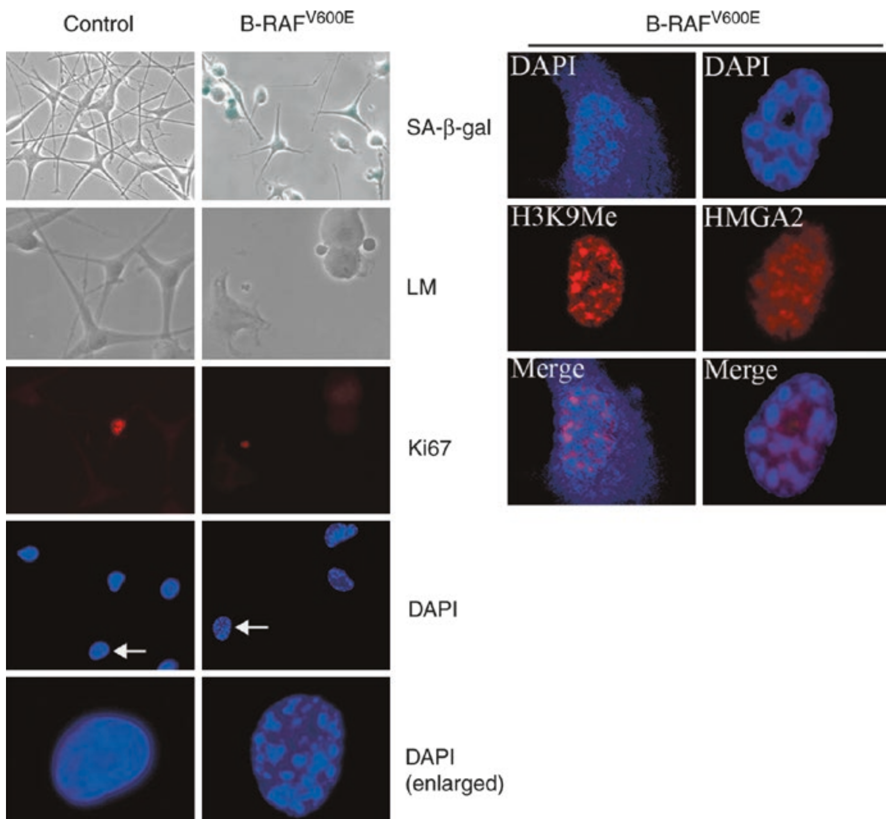

Fig. 11.1 Senescence is associated with positive SA-ß-gal activity and appearance of condensed chromatin. B-RAF^{V600E}-induced senescence in human melanocytes is characterised by rapid cell cycle arrest (reduced Ki67), appearance of SAHF (DAPI foci), and increased SA-ß-gal activity (*left panel*). Cells enlarged to show DAPI-stained SAHF foci are indicated with arrows. SAHF are enriched for markers of heterochromatin, including H3K9Me and HMGA2 (*right panels*)

enzyme correlates strongly with the senescence state (Fig. 11.1), although it can also be induced by cellular stresses such as serum withdrawal and prolonged cell culture (Severino et al. 2000). SA-ß-gal activity derives from residual lysosomal ß-galactosidase activity at the suboptimal pH 6.0 (pH 4.5 is optimal) and reflects the increased lysosomal content of senescent cells (Kurz et al. 2000; Lee et al. 2006). The appearance of DAPI-stained heterochromatic regions, known as senescence-associated heterochromatic foci (SAHF) (Fig. 11.1), which promote the stable repression of certain E2F target genes involved in proliferation, is also associated with senescence (Narita et al. 2003). SAHF occur preferentially with oncogene-induced DNA replication stress, and although global heterochromatin marks can co-exist with cellular proliferation, the accumulation of repressive chromatin marks at the promoters of E2F-target genes is a marker of oncogene-induced senescence (Di Micco et al. 2011). Each SAHF contains portions of a single condensed chromosome, which is enriched for common markers of heterochromatin, including heterochromatin protein 1γ, histone H3 methylated at lysine 9 (H3K9Me), and the non-histone chromatin protein, HMGA2 (Fig. 11.1) (Narita et al. 2003; reviewed in Adams 2007).

Several other markers of senescence have also been described and validated, including accumulation of the cyclin-dependent kinase (CDK) inhibitors $p16^{INK4a}$, $p15^{INK4b}$, an anti-apoptotic bcl-2 member Mcl-1, and the transcription factor Dec1 (Collado and Serrano 2005). Levels of the p53 transcription target and CDK inhibitor $p21^{Waf1}$, the plasminogen activator inhibitor-1 (PAI-1) protein and miR-34 family of microRNAs are also elevated in senescent cells (Goldstein et al. 1994; Wong and Riabowol 1996; He et al. 2007). Finally, morphological changes such as cell enlargement with a concomitant increase in nuclear size, vacuolisation, and cell flattening are typical of senescent cells in vitro (reviewed in Sharpless and Sherr 2015).

11.3 Senescent Cells In Vivo

The existence of senescence cells in vivo has important implications for multicellular organisms; the onset of stress-induced premature senescence may prevent the development of malignant cancer, but the accumulation of damaged senescent cells may eventually compromise tissue integrity and prove detrimental.

11.3.1 Cellular Senescence and Cancer

The observation that aberrant activity of many oncogenes, including RAS, c-MYC, and B-RAF, triggers senescence in vitro (reviewed in Gorgoulis and Halazonetis 2010) suggested that oncogene-induced senescence may act as a barrier to tumourigenesis. Compelling evidence for the tumour suppressor role of oncogene-induced senescence stems from studies in mouse cancer models and human tumours. For instance, pre-malignant lesions in the lung (which developed in a conditional knock-in mouse model expressing oncogenic $K\text{-}RAS^{G12D}$) contained many cells expressing markers of oncogene-induced senescence including $p16^{INK4a}$, $p15^{INK4b}$, SAHF, and

SA-ß-gal, whereas lung adenocarcinomas were almost completely devoid of cells positive for these markers (Collado and Serrano 2005). Similarly, constitutively active N-RAS^{G12D} prevented lymphomagenesis by triggering potent senescence in murine lymphocytes (Braig et al. 2005) and H-RAS^{G12V} triggered a dose-dependent senescence response in mammary epithelia (Sarkisian et al. 2007). Further, inactivation of the PTEN tumour suppressor, which acts as a phosphoinositide 3-kinase (PI3K) pathway inhibitor, in mouse prostate triggered an acute senescence response that suppressed the development of invasive adenocarcinoma (Chen et al. 2005). Finally, consistent with tumour suppression, oncogene-induced senescent cells exhibit a metabolic shift favouring mitochondrial oxidative phosphorylation, rather than the abnormal glycolytic metabolism common to cancer (Kaplon et al. 2013; Li et al. 2013).

Senescence markers are also abundant in human pre-malignant lesions of the skin, colon, prostate, and nervous system, whereas they are almost completely absent in malignant tumours (Chen et al. 2005; Michaloglou et al. 2005; Bartkova et al. 2006; Courtois-Cox et al. 2006; Kuilman et al. 2008). Perhaps the most compelling data come from studies with human naevi (moles), which are small benign tumours of melanocytes that frequently harbour oncogenic mutations in the B-RAF kinase (Pollock et al. 2003). Naevi remain growth arrested for decades and rarely become melanomas (Kuwata et al. 1993; Maldonado et al. 2004), presumably because aberrant B-RAF signalling induces potent senescence (Michaloglou et al. 2005; Gray-Schopfer et al. 2006; Dankort et al. 2009; Dhomen et al. 2009; Goel et al. 2009). Human naevi display some features of oncogene-induced senescence, including intact telomeres (the repetitive sequences at the ends of each chromosome), increased p16^{INK4a} expression, and positive SA-ß-gal activity (Miracco et al. 2002; Michaloglou et al. 2005; Mooi and Peeper 2006), although the expression of senescence-associated features, including SA-ß-gal, that should distinguish human naevus cells from primary and transformed melanocytes remains controversial (Cotter et al. 2007, 2008; Michaloglou et al. 2008; Tran et al. 2012). There is also some discord regarding the clonality of B-RAF^{V600E} mutations in melanocytic naevi, although recent data using sensitive droplet digital PCR in combination with immunohistochemistry support the notion that oncogenic B-RAF initiates melanocytic hyperplasia (Ichii-Nakato et al. 2006; Lin et al. 2009; Yeh et al. 2013).

11.3.2 Cellular Senescence and Aging

Evidence that replicative (telomere-associated) senescence reflects organismal aging and may contribute to age-related decrements in tissue structure and function is more contentious. Certainly, telomeres progressively shorten with each cell division and DNA damage foci marking telomere dysfunction increase with age to approximately 20 % in very old primates (Herbig et al. 2006). Further, cells with a senescent phenotype are prevalent at sites of age-related diseases, such as osteoarthritis and atherosclerosis (Chang and Harley 1995; Price et al. 2002) and mice lacking the mitotic regulator BubR1 or the p53 homologue, p63, developed age-related pathologies that were associated with cellular senescence (Baker et al. 2004; Keyes et al. 2005).

SAHF-positive senescent fibroblasts also increase with age in primate skin (Herbig et al. 2006) and SA-ß-gal-positive senescent cells were found to accumulate in the skin of elderly people (Dimri et al. 1995; Dekker et al. 2009) although this latter result was not reproduced (Severino et al. 2000). Conversely, in some 13 studies involving 79 patients with accelerated aging disorders, such as Werner and Hutchinson–Gilford syndromes, the replicative capacity of fibroblasts was consistently lower than fibroblasts derived from age-matched controls (reviewed in Davis et al. 2007; Maier and Westendorp 2009). Early reports described a weak inverse relationship between replicative lifespan of fibroblasts in vitro and the chronological age of the donor, although there was large variability in the data and recent studies using larger cohorts have found no significant association between age and replicative lifespan (Cristofalo et al. 1998; reviewed in Maier and Westendorp 2009). It is worth considering that the majority of these studies utilise mass cell cultures, so that the Hayflick limit, which is comparable in young and older individuals, reflects the cells with the longest proliferative capacity. This does not preclude the possibility that overall replicative capacity declines with age, in fact the replicative lifespan of individual human fibroblast clones were related to donor age (McCarron et al. 1987).

The accumulation of senescent cells may contribute to the aging process by depleting the regenerative potential of stem cells or by altering tissue structure and function. Many studies suggest that the expression of p16^{INK4a}, which increases during senescence and with age in many rodent, baboon, and human tissues (Zindy et al. 1997; Melk et al. 2003; Krishnamurthy et al. 2004; Herbig et al. 2006; Edwards et al. 2007; Liu et al. 2009), limits the proliferative capacity of self-renewing stem cells and thus impairs tissue regeneration. Mice lacking p16^{INK4a} retain stem cell division and tissue regeneration with advancing age (Janzen et al. 2006; Krishnamurthy et al. 2006; Molofsky et al. 2006; Signer et al. 2008). In contrast, p16^{INK4a} inhibits the reprogramming of differentiated human cells into induced pluripotent stem cells. Reprogramming can be likened to a rejuvenation process and there is an age-related decline in reprogramming efficiency that can be reversed by inactivation of the *p16INK4a* gene (Banito et al. 2009; Li et al. 2009).

In spite of the substantial evidence linking p16^{INK4a} expression with senescence and molecular aging in humans, a recent study suggested that p16^{INK4a}, and its homologue p15^{INK4b}, may have anti-aging functions. This work was based on a mouse model genetically engineered to carry two copies of the *INK4a/ARF* locus, which encodes the p16^{INK4a}, p19ARF, and p15^{INK4b} tumour suppressor proteins. These mice were less susceptible to cancer, showed increased median longevity and a lower incidence of aging-associated kidney lesions and DNA damage response (Matheu et al. 2009). Although, the additional p15^{INK4b} and p19ARF genes complicate interpretation of these data, it is conceivable that the role of p16^{INK4a} in aging reflects its expression. The progressive upregulation of p16^{INK4a} may delay aging by reducing cell proliferation and maintaining stem cell reserves. In contrast, the acute, mitogen-driven upregulation of p16^{INK4a} may promote aging by initiating senescence and permanently preventing stem cell proliferation and tissue regeneration.

Senescent cells may also contribute to aging by altering tissue structure and function; senescent cells secrete many extracellular matrix associated factors and

inflammatory proteins. Many of these secreted factors, including TGFß, IL-6, and PAI1, accumulate with aging. Some, including PAI1 and matrix metalloproteinases, may damage or alter tissue integrity (reviewed in Campisi and d'Adda di Fagagna 2007; Kuilman and Peeper 2009).

Taken together, these findings suggest that irreversibly growth-arrested senescent cells can act as a barrier against tumour formation in young organisms, but their net accumulation may reach a point that compromises tissue function and stem cell renewal leading to the development of deleterious phenotypes with age (Campisi and d'Adda di Fagagna 2007).

11.4 Critical Pathways in Cellular Senescence

Although diverse stimuli can induce a senescence response, they appear to converge on two pathways that initiate and maintain this programme. These pathways are regulated by the tumour suppressor proteins p53 and the retinoblastoma protein (pRb), both of which are frequently lost in human cancer cells (Sherr 1996). Importantly, although cancer cells have partially lost the capacity to initiate senescence, the senescence response can be re-engaged by restoring the p53 and pRb pathways, and tumour regression through senescence may be achieved. It has been shown, for instance, that re-instating p16^{INK4a} in human tumour cells resulted in the establishment and maintenance of a senescence response (Sugrue et al. 1997; Haferkamp et al. 2008).

The senescent states induced by the p53 and pRb pathways may be distinct and whether cells engage one or the other pathway appears to reflect the type of stress signal, the tissue and species of origin. Recent data also reveal senescence-inducing pathways that appear to be independent of the p53- and pRb-pathways. For instance, neither p53 nor p16^{INK4a} were required for H-RAS-induced senescence in melanocytes and senescence induced by B-RAF^{V600E} or N-RAS^{Q61R} did not depend on p16^{INK4a} or p53 (Denoyelle et al. 2006; Zhuang et al. 2008; Haferkamp et al. 2009a, b).

11.4.1 The p53 Pathway and Senescence

p53 engages a formidable proliferative arrest by transactivating genes, such as p21^{Waf1} and the miR-34 class of microRNAs (Smith et al. 1996; Brown et al. 1997; He et al. 2007) primarily in response to DNA-damage checkpoint signals triggered by telomere dysfunction and activated oncogenes (Ramirez et al. 2001; Herbig et al. 2004; Bartkova et al. 2006; Di Micco et al. 2006). Shortened dysfunctional telomeres are recognised as DNA double-strand breaks and lead to the activation of the DNA damage checkpoint (d'Adda di Fagagna et al. 2003). Aberrant oncogenic activity also triggers DNA replication stress and a DNA damage response, possibly via the suppression of RRM2, a key rate-limiting regulatory unit in dNTP synthesis (Bartkova et al. 2006; Aird et al. 2013). Thus, senescent cells are characterised by persistent DNA damage foci, termed DNA segments with chromatin alterations reinforcing senescence (DNA-SCARS). DNA-SCARS are associated with

promyelocytic leukaemia protein nuclear bodies and the accumulation of activated DNA damage response mediators, including ataxia telangiectasia mutated (ATM) and checkpoint-2 (Chk2) kinases (Rodier et al. 2011).

Dampening the DNA damage checkpoint via the inactivation of p53-regulators (including ATM and Chk2) or p53 itself can overcome oncogene-induced and telomere-dependent senescence in human fibroblasts (d'Adda di Fagagna et al. 2003; Di Micco et al. 2006). Similarly, inactivation of the upstream p53 activator, ARF, overcame oncogene-induced senescence in mouse embryo fibroblasts (MEFs) (Serrano et al. 1996; Kamijo et al. 1997), while the loss of $p21^{Waf1}$ caused cells to bypass telomere-dependent replicative- and oncogene-induced senescence in normal human fibroblasts and MEFs, respectively (Brown et al. 1997; Pantoja and Serrano 1999; Wei and Sedivy 1999).

Despite the substantial evidence that p53 promotes senescence, recent data indicate that physiological p53 signalling promotes longevity and favours quiescence over senescence (Matheu et al. 2007, 2008; Demidenko et al. 2010). Mice engineered to express mildly elevated levels of wild-type p53 display strong resistance to tumourigenesis, normal longevity but decreased levels of aging-associated damage (Garcia-Cao et al. 2002, 2006). In contrast, mice expressing constitutively activate forms of p53 showed accelerated aging and an increase in the proportion of senescent cells in vivo (Dumble et al. 2004; Hinkal et al. 2009). It is likely that the cellular context of the p53 response dictates whether quiescence or senescence is triggered. For instance, inhibition of the growth-promoting mTOR pathway by p53 favours quiescence, whereas the simultaneous activation of p53 signalling along with mTOR (i.e., via oncogenic RAS, loss of PTEN) promotes senescence (Fig. 11.2) (Alimonti et al. 2010; Galluzzi et al. 2010; Korotchkina et al. 2010).

11.4.2 The $p16^{INK4a}$/pRb Pathway

Although inactivation of the p53 pathway can reverse the senescence arrest in some cells, there is an emerging consensus that this reversal is blocked in cells with an activated $p16^{INK4a}$/pRb pathway (Sakamoto et al. 1993; Beausejour et al. 2003; Herbig et al. 2004). Active, hypophosphorylated pRb interacts with E2F transcription factors and facilitates chromosome condensation at E2F target promoters. The reorganisation of chromatin leads to the formation of SAHF and the stable repression of proliferation-promoting genes, resulting in the irreversible growth arrest associated with senescence (Narita et al. 2003).

$p16^{INK4a}$ is a positive regulator of pRb and is crucial in generating SAHFs (Narita et al. 2003). Not surprisingly, $p16^{INK4a}$ also acts as a tumour suppressor and is frequently inactivated in established human tumours and inactivating melanoma-associated mutations in $p16^{INK4a}$ are inherited in melanoma-dense kindreds (Goldstein et al. 2006). In fact, $p16^{INK4a}$-deficient human melanocytes, derived from melanoma affected individuals, show an extended lifespan and can be immortalised by ectopic expression of telomerase reverse transcriptase, whereas normal melanocytes display neither of these features (Bennett 2003; Sviderskaya et al. 2003).

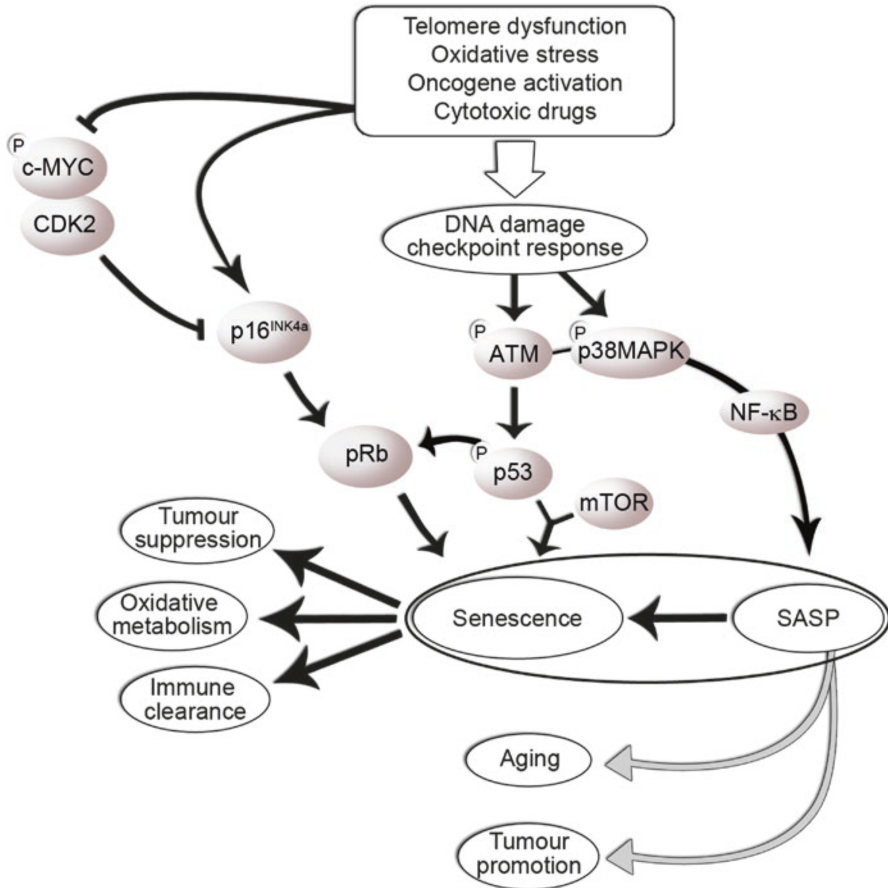

Fig. 11.2 Diverse stress stimuli activate cellular senescence via the DNA damage checkpoint response. Persistent DNA damage leads to the activation of the p53-signalling cascade, which favours senescence when the mTOR growth-promoting pathway is switched on. p53 also signals through the pRb tumour suppressor pathway via the transcriptional induction of the CDK inhibitor $p21^{Waf1}$ (not shown). pRb activation is reinforced by the CDK inhibitor, $p16^{INK4a}$, which is induced by oncogenic signalling, in part by inhibition of c-MYC-CDK2 transcription activity. Sustained damage to DNA also promotes the secretion of a large number of proteins, a phenomenon known as the senescence-associated secretory phenotype (*SASP*), via ATM and p38MAPK-mediated activation of NF-kB. SASP can have different effects on cancer and aging; it can suppress cancer by reinforcing the senescent state and inhibiting mitogenic signals, it can activate the innate immune response leading to improved clearance of tumour cells, it can impair the function of stem cells and promote aging, and it can stimulate the proliferation of neighbouring tumour cells

Furthermore, replicative- and oncogene-induced senescence are accompanied by accumulation of $p16^{INK4a}$ in primary human cells (Alcorta et al. 1996; Hara et al. 1996; Serrano et al. 1997), while ectopically expressed $p16^{INK4a}$ initiates a senescence programme characterised by cell cycle arrest, senescence-associated changes in cell morphology, increased SA-ß-gal activity, and the appearance of SAHF (Dai and Enders 2000; Haferkamp et al. 2008).

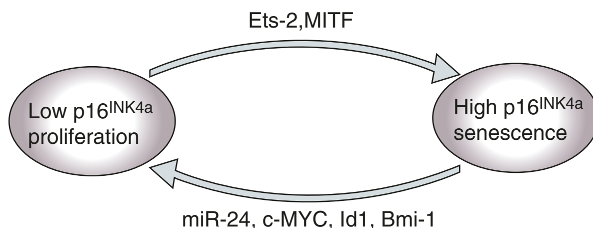

Fig. 11.3 Acute activation of the CDK inhibitor p16^{INK4a} favours senescence. Aberrant oncogenic activity promotes p16^{INK4a} accumulation by suppressing the MYC, Id1, and Bmi-1 transcription factors, while inducing the accumulation of the Ets-2 and MITF transcription regulators. As cells near replicative senescence the p16^{INK4a}-inhibitory microRNA, miR24, is depleted and p16^{INK4a} translation is restored

p16^{INK4a} expression is maintained at low levels prior to senescence by miR-24, a negative-regulator microRNA that suppresses p16^{INK4a} translation. As cells near senescence, miR-24 expression diminishes and p16^{INK4a} protein accumulates (Lal et al. 2008). Oncogenic signalling also induces p16^{INK4a} expression by reducing the levels of the p16^{INK4a} transcriptional repressors Bmi-1 and Id1 (Jacobs et al. 1999a; Ohtani et al. 2001) and increasing the activity of the p16^{INK4a} transcription enhancers, Ets-2 and MITF (Fig. 11.3) (Ohtani et al. 2001; Loercher et al. 2005). Importantly, Bmi-1 is a direct transcription target of c-MYC, and reduced c-MYC signalling is associated with oncogenic N-RAS and B-RAF activity in melanocytes (Zhuang et al. 2008). Thus, decreased c-MYC activity leads to senescence by regulating Bmi-1 and p16^{INK4a} (Jacobs et al. 1999b; Guney et al. 2006). Further, Bmi-1 deficiency inhibits stem cell renewal via p16^{INK4a} induction in mice and Bmi-1 downregulation was associated with increased p16^{INK4a} expression in human keratinocytes derived from older individuals (Molofsky et al. 2003, 2005; Park et al. 2003; Cordisco et al. 2010).

11.4.3 The Senescence-Associated Secretory Network

Senescent cells secrete a complex range of chemokines, interleukins, proteases, and growth factors that are associated with inflammation and malignancy. Collectively, these senescence-associated soluble factors have been termed the senescence-associated secretory phenotype (SASP) and the senescence-messaging secretome (SMS) (Coppe et al. 2008; Kuilman and Peeper 2009; Gorgoulis and Halazonetis 2010). Detailed lists of SASP factors have been provided in several recent articles and are briefly summarised in Table 11.1 (Kuilman and Peeper 2009; Coppe et al. 2010; Gorgoulis and Halazonetis 2010, reviewed in Lasry and Ben-Neriah 2015). This review will focus on the role of SASP in senescence and its relationship with DNA damage response, p53 and pRb pathways.

SASP is not triggered by senescence but is induced in response to persistent DNA damage signalling and via activation of the stress-inducible kinase p38MAPK,

Table 11.1 The senescence-associated secretory phenotype

Soluble signalling factors	
Interleukins	IL-6, −7, −1a, −1b, −13, −15
Chemokines	IL-8; GROa, −ß; MCP-2, −4; HCC-4; cotaxin-3; MIP-3a, −1a; IGFBP-2, −3, −4, −5, −6, −7; G-CSF; GM-CSF
Growth factors	EGF; bFGF; HGF; IGF, SCF; TGFß, NGF; VEGF
Non-protein factors	Nitric oxide, reactive oxygen species
Serine proteases	
	MMP-1, −3, −10, −12, −13, −14; TIMP-1, −2; PAI-1, −2
Secreted insoluble factors	
	Fibronectin, collagen, laminin

A subset of factors significantly altered during senescence

both of which lead to the phosphorylation and activation of the transcription factor target, NF-kB. DNA-damaged induced ATM and p38MAPK synergistically phosphorylated and activated the NF-kB transcription factor, which induced the majority of SASP factors in senescent human fibroblasts (Freund et al. 2011). SASP relies on upstream elements of the DNA damage response cascade, including NBS1 (a sensor of DNA damage), ATM kinase and its target Chk2, but does not require the downstream DNA damage signalling target p53. Thus, cells induced to senescence in response to p16^{INK4a}, in the absence of DNA damage, do not initiate SASP. Similarly, oncogenic RAS promotes senescence with limited SASP in ATM-deficient cells (Rodier et al. 2009). Although SASP is not restricted to the senescence programme, it can reinforce senescence growth arrest. For instance, human fibroblasts depleted for IL-6 bypassed B-RAF^{V600E}-induced senescence and showed a strong suppression of other inflammatory regulators including IL-8, IL-1α, and IL-1β (Kuilman et al. 2008). Similarly, the disruption of SASP via inhibition of NF-kB confirmed the importance of SASP in stabilising cell cycle arrest and promoting immune clearance (Chien et al. 2011). SASP molecules IL-8 and the chemokine receptor 2 (CXCR2) ligands support senescence by boosting the DNA damage response, while IGFBP7, IL-6, and PAI-1 contribute to senescence by inhibiting proliferative and mitogenic pathways (Kortlever et al. 2006; Acosta et al. 2008; Kuilman et al. 2008; Wajapeyee et al. 2008; Acosta and Gil 2009). Thus, the onset of a protracted DNA damage response controls senescence by initiating a rapid p53- and/or pRb-dependent proliferative arrest, followed by induction of a DNA damage responsive cytokine secretory response (Fig. 11.2).

The secretion of soluble factors into the extracellular environment can also have pro-tumourigenic effects. This probably reflects a complex combination of cellular and genetic context along with the level of SASP activity. SASP factors such as IL-6 and IL-8 can promote cancer progression by stimulating proliferation, angiogenesis, invasiveness, and inducing epithelial–mesenchymal transition (Ancrile et al. 2007; Coppe et al. 2008; Kuilman et al. 2008; Rodier et al. 2009). Others, such as matrix metalloproteinases, promote breast tumourigenesis by altering the differentiation of epithelial tumour cells and increasing tumour cell migration by weakening

tissue integrity (Liu and Hornsby 2007; Acosta and Gil 2009). The combined loss of p53 with the aberrant activation of RAS amplifies and accelerates the development of SASP and this coincides with the potent stimulation of growth, invasiveness, and epithelial–mesenchymal transition of nonaggressive human cancer cells (Coppe et al. 2008). In vivo cytokine secretion is a feature of preneoplastic lesions of the colon and breast, which display markers of DNA damage and senescence, while IL-6 expression correlates with ATM (DNA damage sensor) kinase activity in invasive ductal breast carcinomas (Kuilman et al. 2008; Rodier et al. 2009). Conversely, the selective suppression of SASP factors with the mTORC1 inhibitor rapamycin, which also inhibited IL1A-NF-kB signalling, diminished the ability of senescent fibroblasts to stimulate prostate tumour growth in mice (Laberge et al. 2015).

Finally, the innate immune system can be activated by SASP inflammatory cytokines and can effectively restrict oncogenic SASP activity by clearing damaged and senescent cells (Xue et al. 2007). The accumulation of inflammatory cytokines is associated with several age-associated diseases, and it is not surprising that accumulation of SASP factors such as IL-6, TGFß, PAI, and fibronectin also correlate with aging (Goldstein et al. 1994; Rasoamanantena et al. 1994; Ershler and Keller 2000; Carrieri et al. 2004; Herbig et al. 2006; Rossi et al. 2007). Although the precise impact of the SASP in vivo remains unresolved, these data indicate that damaged senescent cells mount a complex cytokine response that communicates with neighbouring cells, modifies the tissue microenvironment, and leads to multiple pathologies. In particular, cytokines may reinforce senescence in surrounding non-malignant cells (Acosta et al. 2013), generate a potent immune response to clear damaged cells, affect the function of stem cells, or even promote tumourigenesis of high-grade pre-malignant and malignant cells in a paracrine manner (Fig. 11.2).

11.4.4 p53- and p16^{INK4a}-Independent Oncogene-Induced Senescence in Melanocytes

Recent reports have shown that pro-oncogenic forms of the H- and N-RAS GTPases, and their downstream kinase B-RAF, are not functionally equivalent and each may induce senescence via distinct programmes that do not require pRb or p53. Certainly, the inactivation of p16^{INK4a}, pRb, or p53 did not prevent cell cycle arrest, DNA damage signalling, or SA-ß-gal activity in human melanocytes transduced to express these oncogenes (Michaloglou et al. 2005; Denoyelle et al. 2006; Zhuang et al. 2008; Haferkamp et al. 2009a, b). One alternative pathway appears to involve the endoplasmic reticulum-associated unfolded protein response (UPR). Ectopic expression of H-RAS^{G12V} in human melanocytes induced the UPR and inhibition of this response (by silencing expression of several UPR proteins) suppressed H-RAS-induced senescence (Denoyelle et al. 2006). The RAS-mediated activation of the PI3K pathway was required for UPR induction and this was most potently achieved by oncogenic H-RAS, as UPR was poorly induced by N-RAS and not activated by B-RAF. Consistent with these data, Spitz naevi expressing mutated H-RAS showed a significantly greater expansion of the endoplasmic reticulum and activation of the

UPR compared to benign naevi expressing mutated B-RAF or N-RAS (Denoyelle et al. 2006). It has been suggested that UPR-induced senescence may account for the bias against H-RAS mutations in melanoma (Denoyelle et al. 2006), although it should be noted that the efficiency of UPR signalling declines during aging (Naidoo 2009a, b) and melanomas seem to have adapted to the presence of extreme endoplasmic reticulum stress (Jiang et al. 2007; Hersey and Zhang 2008).

Another alternative senescence pathway has been proposed for oncogenic B-RAF in human melanocytes. Ectopic expression of the oncogenic transcription factor c-MYC was found to partially rescue B-RAF^{V600E}-induced senescence, and more weakly, N-RAS^{Q61R}-induced senescence of human melanocytes. c-MYC did not influence the UPR pathway and this may account for the inability of c-MYC to overcome N-RAS-induced senescence (Zhuang et al. 2008). Suppression of c-MYC has been shown to induce senescence in several mouse tumour models, including lymphoma, osteosarcoma, and hepatocellular carcinoma (Wu et al. 2007), and in human melanoma cells expressing B-RAF^{V600E} or N-RAS^{Q61R} (Zhuang et al. 2008). The requirement for p16^{INK4a} or p53 in senescence caused by c-MYC depletion remains controversial; intact p16^{INK4a}, pRb, and p53 were required in mouse tumours for the initiation of senescence by c-MYC inactivation (Wu et al. 2007), whereas c-MYC depletion led to senescence of p16^{INK4a}-null and p53-null human melanoma cells (Zhuang et al. 2008). The impact of p16^{INK4a} and p53 expression on c-MYC activity in human melanocytes remains to be investigated. Importantly, c-MYC is frequently upregulated in human tumours and is up to sevenfold higher in metastatic melanomas compared to benign naevi (Zhuang et al. 2008).

The anti-senescence function of c-MYC requires phosphorylation of MYC at Ser-62 by cyclin E/CDK2. CDK2 acts as a c-MYC transcription co-factor altering the MYC-dependent regulation of genes, such as Bmi-1, p16^{INK4a}, and p21^{Waf1}, all of which participate in senescence control. Inhibition of CDK2 leads to the upregulation of p16^{INK4a} and p21^{Waf1} and the repression of Bmi-1. Thus, MYC can induce senescence in MEFs lacking CDK2, but not in wild-type MEFs (Hydbring et al. 2010; Hydbring and Larsson 2010). This has important implications, as the inhibition of CDK2 (via pharmacological inhibition or induction of the CDK2 inhibitor p27^{Kip1}) can drive MYC-transformed cells into senescence and prevents c-MYC from bypassing RAS-induced senescence (reviewed in Hydbring and Larsson 2010). Accordingly, depletion of CDK2 delayed MYC-induced B-cell lymphomas, −neuroblastoma cells, −breast cancers, and -melanoma cell lines (Du et al. 2004; Deans et al. 2006; Molenaar et al. 2009; Campaner et al. 2010) and deletion of Skp2 (an oncogenic E3 ligase that targets p27^{Kip1} and MYC for degradation) suppressed tumourigenesis through senescence (Lin et al. 2010).

11.5 Re-establishing Senescence Mechanisms

The concept of re-instating senescence as a cancer therapy is being approached with caution because the retention of damaged senescent cancer cells that are metabolically active and capable of secreting cytokines and growth factors could be harmful. It is important to consider, however, that many current cancer treatments cause

senescence as a result of acute DNA damage (te Poele et al. 2002; Roberson et al. 2005), and the presence of senescent cells in colon carcinomas contributed to improved overall outcome to therapy (Haugstetter et al. 2010). The existence of sporadic senescent cells in tumours may indicate a retained susceptibility to senescence induction and this may translate to sensitivity to senescence-inducing therapies.

Senescence can be triggered by reactivating tumour suppressor molecules or targeting oncogenes. For instance, reactivation of p53 induces a potent senescence response in sarcomas and liver carcinomas that were subsequently cleared by the innate immune system (Ventura et al. 2007; Xue et al. 2007). Presumably, SASP molecules were responsible for the activation of the immune cells. Targeting the oncogenic E3-ubiquitin ligase, Skp2, triggered senescence in H-RAS^{G12V} expressing or PTEN-deficient prostate cancer cells (Lin et al. 2010). Likewise, deleting CDK2 induced senescence in c-MYC expressing MEFs (Campaner et al. 2010). Importantly, senescence was also triggered in these oncogenic model systems with small molecule inhibitors of Skp2 and CDK2 function (Lin et al. 2010). Similarly, the selective inhibition of B-RAF and MEK triggered senescence in B-RAF^{V600E}-mutant human melanoma cells and inhibition of MEK sensitised melanoma cells to radiotherapy (Haferkamp et al. 2013; Schick et al. 2015). Intriguingly, melanoma resistance to B-RAF inhibition commonly involves the hyperactivation of MAPK signalling, often via mutational activation of NRAS, MEK, or *B-RAF* amplification, and this appears to confer a fitness deficit to tumour cells by promoting senescence (Das Thakur et al. 2013). These data coincide with genetic evidence that RAS and B-RAF mutations are mutually exclusive in cancer, and recent evidence confirming that co-expression of mutant KRAS and B-RAF resulted in elevated MAPK activity, increased p16^{INK4a} production, and promoted SA-ß-gal in MEFs (Cisowski et al. 2016).

The pharmacological induction of senescence has been shown to suppress the in vivo growth of prostate cancer cells (Alimonti et al. 2010; Lin et al. 2010), and therapy-induced senescence is detectable in tumour biopsies after neoadjuvant chemotherapy (Roberson et al. 2005). Mouse models of chemotherapy have also shown that MYC-initiated lymphomas responded to cycloheximide by inducing tumour cell senescence, which contributed to better prognosis (Schmitt et al. 2002). This suggests that delivery of chemotherapy to cells undergoing senescence could be used to augment senescence or to transform cell cycle arrest into cell death.

Heterochromatin may also prove a useful target in oncogene-expressing senescent and proliferating tumour cells. The condensation of chromatin hinders access of DNA damage response proteins to DNA lesions and this diminishes DNA damage response signalling (Di Micco et al. 2011). Perturbation of heterochromatin, using histone deacetylase (HDAC) inhibitors, augmented DNA damage response activity, induced apoptosis and tumour regression in a K-RAS-driven colorectal cancer xenograft model (Di Micco et al. 2011). HDAC inhibitors have shown encouraging anti-tumour activity with manageable toxicities in clinical trials, and several have been granted approval by the US Food and Drug Administration for cancer treatment (West and Johnstone 2014).

Despite the contribution of senescence to tumour suppression, the possibility that drug-induced senescent cancer cells may accumulate and contribute to drug resistance and tumour recurrence must be considered. As described above, the secretion of inflammatory factors can stimulate tumour cell proliferation and angiogenesis, and escape from drug-induced senescence in breast cancer cells resulted in the generation of aggressive tumour clones with stem cell-like properties (Achuthan et al. 2011).

Conclusions

Together, these data confirm that senescence plays an important role in suppressing tumourigenesis and possibly predicting treatment outcome. The long-term impact of senescent tumour cells in vivo remains largely unknown and much more work is needed to resolve the complex regulation of the pro- and anti-tumourigenic activities of senescent cells. The finding that the senescent phenotype can be uncoupled from cell cycle arrest suggests that it may be possible to minimise the detrimental aspects of senescence (Dulic et al. 2000; Wang et al. 2004; Rodier et al. 2009). For instance, p53 activity and the miR-146 class of microRNAs suppress excessive SASP activity (Coppe et al. 2008; Bhaumik et al. 2009) and ectopic expression of CDK inhibitors, including $p16^{INK4a}$ and $p21^{Waf1}$, can induce senescence without SASP (Coppe et al. 2011). Disrupting SASP, however, weakened the proliferative arrest and diminished the immune recognition and clearance of senescent Eμ-MYC lymphoma cells in vivo (Chien et al. 2011). Thus, in order to maximise the therapeutic potential of senescence, we may need to establish a new equilibrium that favours immune-mediated clearance of permanently arrested preneoplastic cells, while limiting the deleterious paracrine effects that disrupt tissue homeostasis.

Acknowledgements Our work is supported by the National Health and Medical Research Council of Australia (NHMRC), the Cancer Council of New South Wales. HR is a NHMRC Research Fellow.

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Melanoma Stem Cells

12

Tobias F.I. Schatton and Markus H. Frank

12.1 Introduction

The hypothesis that cancers and tissue stem cells might share several biological traits is many decades old (Bruce and Van Der Gaag 1963; Hamburger and Salmon 1977; Park et al. 1971). Indeed, the defining stem cell traits of (a) self-renewal, (b) differentiation, and (c) potential to proliferate nearly indefinitely are commonly observed among cancer subpopulations (Reya et al. 2001). Moreover, interactions with stromal elements and signaling pathways that govern physiologic stem cell behavior were also found to play important roles during tumor development (Frank et al. 2010). Together, these observations have led to the cancer stem cells (CSCs) theory of tumor initiation and growth, which postulates that the tumorigenic process relies on a reservoir of self-renewing, aggressive cells that confer clinical virulence, that is, CSCs (Reya et al. 2001). Such tumorigenic minority populations or CSCs have since been characterized in a growing number of tumor entities (Schatton et al. 2009), including in human melanoma (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Schatton et al. 2008).

In this chapter, we will review the current knowledge of CSCs and their biological features with particular emphasis on human malignant melanoma and discuss controversial aspects of the CSC theory. Furthermore, we will explore the

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_12

implications of the findings of melanoma as a CSC-driven disease for the development of more effective treatment modalities for melanoma patients.

12.2 What Is a "Cancer Stem Cell"?

It has long been established that tumors comprise multiple phenotypically and functionally distinct populations of cancer cells (Hanahan and Weinberg 2000). Several theories have been put forward to account for the occurrence of such tumor heterogeneity. According to the classic view of tumorigenesis, tumor heterogeneity can be explained by both intrinsic factors (i.e., progressive accumulations of genetic alterations over time) and extrinsic stimuli (e.g., distinct cues from the tumor microenvironment, Hanahan and Weinberg 2000). The so-called stochastic theory of tumor initiation postulates that all malignant cells within a cancer, regardless of their phenotype, possess equivalent capacities to proliferate, form new tumors, and cause relapse (Nowell 1976). The CSC hypothesis, on the other hand, provides an alternative explanation for tumor heterogeneity (Reya et al. 2001): It posits that cancers, like physiologic tissues, are organized as developmentally defined hierarchies of cells with divergent differentiation features and disparate capabilities for self-renewal and neoplastic proliferation. The CSC concept thus proposes that only a subpopulation of tumor cells within a cancer, namely CSCs, bears the competence to fuel tumor growth by continuously undergoing self-renewal and differentiation, whereas the bulk of differentiated cancer components lacks the capacity for tumor initiation and unlimited proliferation (Murphy et al. 2014) (Fig. 12.1).

According to a consensus definition (Clarke et al. 2006), a CSC is a cell within a tumor that possesses the capacity to undergo both self-renewing cell divisions that expand the CSC pool, and cell divisions that result in more differentiated cancer cell progeny. Therefore, CSCs can only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor (Clarke et al. 2006).

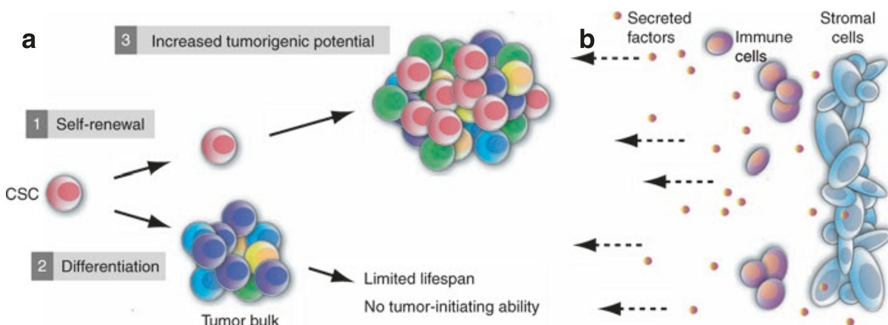

Fig. 12.1 *The cardinal features of CSCs*. Illustrated are (**a**) the defining features of CSCs, that is, (*1*) self-renewal capacity, (*2*) differentiation capacity, and (*3*) increased tumorigenic potential. (**b**) TME characteristics, that is, secreted factors, host immunity, and stromal cell interactions also regulate CSC-dependent tumor growth

Accordingly, the experimental characterization of a putative CSC population relies on the use of an in vivo model system that allows for a rigorous confirmation of the traits used to define CSCs. The gold standard assay that fulfills this criterion for identification of human CSC populations is serial xenotransplantation at limiting dilution of marker-defined clinical cancer subpopulations into an orthotopic site of immunocompromised mice (typically NOD/SCID), which although imperfect is considered the best experimental system to evaluate CSC activity (Clarke et al. 2006). Using this approach, CSCs capable of sustained self-renewal and tumor propagation were first described in cancers of the hematopoietic lineage (Bonnet and Dick 1997). These initial studies demonstrated that it is possible to isolate from a single tumor sample two distinct cancer subpopulations that differ in their cell surface antigen profile and their tumor-seeding properties: (1) a CSC-enriched subset, as defined by its exclusive ability to self-renew as well as differentiate into nontumorigenic cancer cell progeny and its competence to seed new tumors upon serial xenotransplantation, and (2) the bulk of tumor cells that lack the capacity to generate tumors in animal hosts (Bonnet and Dick 1997). Subsequent studies extended these findings to a variety of additional hematological malignancies and solid tumor entities (Al-Hajj et al. 2003; Castor et al. 2005; Chan et al. 2009; Cox et al. 2004, 2007; Dalerba et al. 2007; Hermann et al. 2007; Ishikawa et al. 2007; Li et al. 2007; O'Brien et al. 2007; Prince et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004; Suva et al. 2009; Yang et al. 2008; Zhang et al. 2008), including malignant melanoma (Boiko et al. 2010; Schatton et al. 2008). Hierarchical tumor organization has been confirmed in syngeneic mouse models in which only fractions of murine tumor cells possessed the fundamental CSC features of extensive self-renewal, differentiation, and enhanced tumorigenic capacity (Cho et al. 2008; Deshpande et al. 2006; Held et al. 2010; Wu et al. 2008).

Despite these advances in our understanding of functional tumor heterogeneity, the CSC model has represented a topic of considerable controversy (Hill 2006; Jordan 2009). Some of this controversy appears to arise from uncertainty regarding the term "CSC." For instance, the term CSC has been interpreted to mean that the cellular precursors of such tumorigenic subpopulations were originally physiologic stem cells, which accumulated genetic alterations resulting in cancerous transformation. While this may be the case in some malignancies (Barker et al. 2009; Zhu et al. 2009), CSCs in other cancers may originate from more differentiated cells that reacquired stem-like properties through a series of mutagenic events (Huntly et al. 2004; Jamieson et al. 2004; Krivtsov et al. 2006). Additionally, differentiation in the context of CSC biology does not refer to multipotent differentiation plasticity as it occurs during organogenesis or physiologic tissue regeneration, but rather to the ability of CSCs to give rise to cancer cells that lack tumor-initiating capacity (Clarke et al. 2006). Furthermore, in contrast to physiologic stem cells, which represent only a small cellular fraction of a particular tissue, CSCs may represent larger relative proportions of a total cancer cell population, depending on tumor type, variance of genetic alterations, and stage of disease progression (Gupta et al. 2009). In support of this notion, the frequency of leukemic CSCs varied more than hundred-fold between distinct patient specimens (Bonnet and Dick 1997). CSC representation

may also vary within a single cancer specimen, in which undifferentiated regions may harbor larger numbers of CSCs compared to more differentiated tumor areas (Gupta et al. 2009). Importantly, the number of cells needed to initiate a tumor is not part of the CSC definition (Reya et al. 2001; Schatton et al. 2009). Hence, a larger relative proportion of tumorigenic cells does not contradict the CSC model of tumor initiation and growth. Given the potential confusion associated with the term "CSC," many investigators in the field refer to them as tumor-initiating or tumor-propagating cells (Clarke et al. 2006).

The determination of relative CSC frequencies is influenced by the experimental model system used to assess cancer "stemness" (Boiko et al. 2010; Bonnet and Dick 1997; Lapidot et al. 1994; O'Brien et al. 2007; Quintana et al. 2008, 2010; Ricci-Vitiani et al. 2007; Schatton et al. 2008; Shmelkov et al. 2008). In this regard, it has been established for some time that biological aspects of the tumor microenvironment, including growth factor availability, extracellular matrix (ECM) composition, or the degree of vascularization, as well as host immunocompetence can control the tumorigenic potential of cancer subpopulations (Scadden 2006) (Fig. 12.1). Given this dependence of a defining CSC feature on microenvironmental factors and the immune status of the host, it is not surprising that animal models that offer a more hospitable microenvironment for tumor growth – that is, through the exogenous addition of ECM factors (e.g., Matrigel) (Quintana et al. 2008) and/or the use of more severely immunocompromised mice (Bonnet and Dick 1997; Quintana et al. 2008) – can yield higher relative CSC counts compared to CSC frequencies assessed in the absence of cografted stromal factors in more immunocompetent mouse models (Lapidot et al. 1994; Quintana et al. 2008; Schatton et al. 2008). Based on these considerations, more permissive xenotransplantation conditions might be inadequate for the accurate assessment of CSC biology as it occurs in humans, as they could enable non-CSCs to initiate and maintain experimental tumor growth (Gupta et al. 2009; Murphy et al. 2014).

The tumor biospecimens used for the isolation and characterization of putative CSC populations also influence the assessment of CSC frequency (Gupta et al. 2009). For instance, utilizing tumor cell isolates from tumor xenografts that have been passaged in vivo for extended periods of time Quintana et al. (2008, 2010) in lieu of fresh patient-derived tumor samples likely obscures the accurate assessment of CSC frequencies and biological functions (Boiko et al. 2010). Indeed, Boiko and colleagues demonstrated that both in vitro and in vivo passaging of melanoma cells can result in the emergence of tumorigenic subclones that drive experimental tumor growth independent of their immunophenotype (Boiko et al. 2010). Similarly, tumor cell lines may have lost the hierarchical structure of the primary tumor from which they originated (Zhou et al. 2009). Variations in tumor dissociation, isolation, and/or inoculation techniques could also account for differences in calculated CSC frequencies between different laboratories (Shackleton 2010). Moreover, the use of tumor specimens from patients with more advanced disease (Quintana et al. 2008), which have been demonstrated to contain elevated CSC numbers compared to primary tumors (Schatton et al. 2008), could yield higher estimated CSC frequencies (Boiko et al. 2010).

Clearly, a considerable variability has been observed in the course of CSC identification efforts with regard to estimated frequencies of tumorigenic cells depending on the mouse model used. While the relative frequency of CSCs is not directly relevant for their identification as it is not a CSC-defining feature, it is nevertheless important to define the factors responsible for this variability because such studies could help identify additional CSC-specific functions (Schatton et al. 2009). For example, the tumor microenvironment, including that of an immunocompromised host, may govern not only the tumorigenic potential of cancer subpopulations but also additional CSC-defining traits, including differentiation and self-renewal (Postovit et al. 2006). It is thus possible that a very permissive milieu for tumor growth may not only enable non-CSCs to seed cancers but could also facilitate the "de-differentiation" of such nontumorigenic bulk populations into CSC phenotype-expressing cells (Hoek and Goding 2010). The conversion of non-CSCs into CSC-like cells may, however, not occur in more hostile cancer environments observed in patient tumors, which are typically characterized by low nutrient availability, marked levels of hypoxia, necrosis, and inflammation, and the presence of antitumor immune responses. Only a minority of tumor cells, that is, CSCs, might possess the ability to survive and fuel the tumorigenic process under these selective pressures. In support of this hypothesis, hypoxia-inducible factors (HIFs) are preferentially expressed in CSCs, and blockade of HIF2α on glioma CSCs attenuated their potential to initiate experimental tumors (Li et al. 2009b). Furthermore, viable glioma CSCs preferentially localize to perivascular niches in both clinical and experimental cancer specimens (Calabrese et al. 2007). The bulk of tumor cells within cancerous lesions that have reached a significant size, on the other hand, is often necrotic or undergoes apoptosis (Cotter 2009), indicating that their conversion into apoptosis-resistant stem-like cancer cells does not commonly occur under these conditions.

The complexity of CSC biology and its implications for the type of animal model utilized for their characterization is further highlighted by the identification of novel CSC functions (Frank et al. 2010), including their intrinsic property to evade or actively modulate antitumor immune responses (Chan et al. 2009; Di Tomaso et al. 2010; Majeti et al. 2009; Schatton and Frank 2009; Schatton et al. 2010; Todaro et al. 2009; Wei et al. 2010a, b). Such mechanisms, which may confer selective growth advantages to the CSC pool, need to be taken into consideration when designing biologically relevant assays for their characterization. These findings highlight the importance of establishing translationally relevant assays for the study of CSCs, which accurately mimic the environmental factors found in clinical cancers (e.g., presence of human stromal cells, low-nutrient levels, necrosis, hypoxia, and relatively intact antitumor immunity) rather than further deviating from the naturally occurring microenvironmental characteristics. Current xenotransplantation protocols and animal models for the study of tumorigenic subpopulations that do not accurately reflect the pathological environment of spontaneously occurring human malignancies might favor niche-independent tumor growth (Quintana et al. 2008). Similarly, the so-called tumor sphere culture assay, which favors anchorage-independent in vitro passaging of cancer cells, and which has been proposed to serve as a surrogate tool for the

identification of tumor-initiating cells (Fang et al. 2005; Keshet et al. 2008; Perego et al. 2010), represents an inadequate stand-alone assay for the relevant study of CSC biology in the absence of an in vivo confirmation of CSC-defining traits of a marker-defined tumor subset (Schatton and Frank 2010).

Although much work is still required to fully characterize CSCs and to reach consensus about clinically relevant model systems for their study, efforts directed toward identifying strategies that effectively target tumorigenic minority populations could significantly enhance current treatment modalities. In the following sections, we will discuss how the study of MMICs can yield new insights into melanomagenesis that could potentially lead to the development of improved melanoma treatment strategies.

12.3 Melanoma Stem Cells: Evidence for Their Clinical Relevance

The study of CSCs in human malignant melanoma has recently elicited considerable interest. Given its marked heterogeneity, pronounced resistance to conventional anticancer therapy, and highly aggressive behavior, it has been speculated for some time that melanoma, like numerous additional solid cancers, may follow the CSC model of tumor initiation and growth (Hendrix et al. 2003a; Schatton and Frank 2008). Melanoma remains the most lethal form of skin cancer and melanoma incidence is increasing faster than any other cancer worldwide (Jemal et al. 2010). Although the survival rate of patients diagnosed with melanoma has improved over the past decades (Linos et al. 2009), and greatly accelerated to improve in recent years with the advent of novel molecular targeted therapies and checkpoint blockade immunotherapies (Ugurel et al. 2016), there continues to exist a clear need for novel strategies to eradicate the melanoma subpopulation in which clinical virulence and therapeutic resistance reside, which appear to coincide with MMICs.

Initial support for the potential existence of MMICs came from the demonstration that CSC phenotype-expressing cancer subpopulations are present in melanoma cell lines and clinical melanomas (Frank et al. 2005). Specifically, this study showed that a number of stem cell markers, including the prospective CSC determinant, *CD133* (Hermann et al. 2007; O'Brien et al. 2007; Ricci-Vitiani et al. 2007; Singh et al. 2004; Suva et al. 2009), were over-expressed by chemoresistant tumor subsets among heterogeneous malignant melanoma cultures and within clinical melanoma biospecimens (Frank et al. 2005). Subsequently, Monzani and colleagues showed that $CD133^+$ melanoma fractions had the preferential ability to initiate primary tumor formation in NOD/SCID mice compared to $CD133^-$ melanoma bulk populations (Monzani et al. 2007), demonstrating the existence of tumorigenic melanoma subpopulations. The authors of the study did not examine, however, whether $CD133^+$ melanoma subsets were capable of self-renewal and differentiation in serial xenotransplantation experiments (Monzani et al. 2007). In the absence of stringent in vivo assays aimed at dissecting these key CSC-defining features, no definitive conclusions can be made about the potential relationship of $CD133^+$ or other

melanoma minority populations to MMICs. This limitation also applies to a study characterizing melanoma subpopulations based on their ability to grow in spheroid bodies in vitro (Fang et al. 2005). Melanoma cells isolated from such "melanospheres" preferentially expressed the marker of mature B cells, *CD20*, and were more tumorigenic when grafted to immunodeficient mice compared to melanoma cells grown as adherent in vitro cultures (Fang et al. 2005). While the results by Fang and colleagues (Fang et al. 2005) did not establish the existence of MMICs, they suggested, however, that melanomas might comprise functionally distinct subpopulations with divergent tumorigenic capacities.

Unequivocal evidence for the existence of melanoma CSCs, capable of long-term self-renewal and differentiation, was first provided when the chemoresistance mediator (Cheung et al. 2011; Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004; Yang et al. 2010) and cell surface molecule, *ABCB5*, was established as a prospective biomarker of MMICs (Schatton et al. 2008). This study demonstrated that ABCB5$^+$ melanoma fractions, but not ABCB5$^-$ melanoma bulk populations, isolated from patient biopsies were capable of initiating primary and secondary neoplasms upon serial xenotransplantation at limiting dilution into NOD/SCID mice (Schatton et al. 2008). In addition, ABCB5$^+$ melanoma cells were capable of generating phenocopies of the original patient tumors, indicating their ability to both self-renew (i.e., to maintain the ABCB5$^+$ cell pool by generating more copies of themselves) and differentiate (i.e., to give rise to more differentiated, ABCB5$^-$ tumor cell progeny, incapable of initiating experimental tumor growth) upon serial xenotransplantation (Schatton et al. 2008). These results thus established that ABCB5$^+$ melanoma subpopulations represent MMICs (Zabierowski and Herlyn 2008) as per the CSC consensus definition (Clarke et al. 2006) outlined above.

To further confirm the selective capacity of ABCB5$^+$ MMICs for enhanced tumorigenic growth, self-renewal, and differentiation, genetic lineage tracking was performed in human melanoma to NOD/SCID mouse xenotransplantation experiments employing genetically encoded DsRed (red fluorescent protein) and EYFP (enhanced yellow–green fluorescent protein) labeling of MMICs and melanoma bulk populations, respectively (Schatton et al. 2008). Xenotransplantation of ABCB5$^+$DsRed$^+$ melanoma cells and ABCB5$^-$EYFP$^+$ tumor bulk components, reconstituted at naturally occurring ratios, resulted in markedly increased relative frequencies of DsRed$^+$ cells of ABCB5$^+$ origin in melanoma xenografts (Schatton et al. 2008), confirming the enhanced tumorigenic capacity of ABCB5$^+$ MMICs. In addition, these lineage-tracing experiments revealed a tumor hierarchy, in which ABCB5$^+$ cells had the ability to generate both ABCB5$^+$ and ABCB5$^-$ tumor progeny, whereas ABCB5$^-$ melanoma cells exclusively gave rise to more copies of themselves (Schatton et al. 2008). In more recent work, the increased tumorigenic capacity of ABCB5$^+$ vis-a-vis ABCB5$^-$ melanoma subpopulations has been independently confirmed not only in human to NOD *scid* gamma (NSG) mouse xenotransplantation experiments (Kupas et al. 2011), but also for murine melanomas in syngeneic tumor transplantation models (Zhang et al. 2016). Thus, two fundamentally different cell types with divergent tumorigenic potentials exist within melanomas, only

one of which is capable of self-renewal and differentiation – that is, ABCB5+ MMICs. To further establish that the melanoma subpopulation marked by ABCB5 is required for efficient tumor growth, as anticipated by the MMIC model, our laboratory examined whether selective ablation of MMICs could inhibit tumor development (Schatton et al. 2008). Indeed, administration of an anti-ABCB5 monoclonal antibody into nude mice xenografted with human melanomas impaired tumor initiation and slowed tumorigenic growth via antibody-dependent cell-mediated cytotoxicity (ADCC) directed at ABCB5+ MMICs (Schatton et al. 2008). These results provided proof-of-concept for the potential therapeutic utility of targeting MMICs and provided evidence that melanoma is a CSC-driven disease.

In the same study, using an established melanocytic tumor tissue microarray (Kim et al. 2006), a positive correlation between ABCB5+ melanoma cell frequency and clinical melanoma progression was established (Schatton et al. 2008). Specifically, there was higher ABCB5 expression in primary melanomas versus benign nevi, and in metastatic melanomas versus primary melanomas, showing that ABCB5 correlates with neoplastic progression (Schatton et al. 2008). A role for ABCB5 in clinical melanoma initiation/progression has recently also been established by additional laboratories (Gazzaniga et al. 2010; Sharma et al. 2010), and these findings have been extended to acral melanomas (Vasquez-Moctezuma et al. 2010) and in vivo human melanoma model systems (Fukunaga-Kalabis et al. 2010; Ma et al. 2010). ABCB5 expression also correlates with neoplastic progression in additional malignancies, including hepatocellular carcinoma, where it serves as a major independent clinical biomarker of poor survival (Cheung et al. 2011). The potential importance of ABCB5 as a biomarker of metastatic melanoma progression and disseminated disease was initially further supported by an additional study from our laboratory (Ma et al. 2010): ABCB5 mRNA could be detected in peripheral blood mononuclear cell preparations from human stage IV melanoma patients but not healthy controls (Ma et al. 2010). Similarly, human ABCB5 mRNA expression could also be detected among peripheral blood mononuclear cells isolated from NOD *scid* gamma (NSG) recipients of subcutaneous human melanoma xenografts (Ma et al. 2010), indicating that circulating melanoma-initiating cells might be present in both murine models and stage IV melanoma patients. Indeed, viable circulating tumor cells (CTCs), isolated from the blood circulation of tumor-bearing hosts, were capable of primary melanoma and metastasis formation in serial xenotransplantation experiments (Ma et al. 2010). Strikingly, CTCs capable of melanoma initiation demonstrated significantly increased ABCB5 expression levels compared to primary tumor xenografts and metastatic lesions (Ma et al. 2010). These results provided initial evidence that circulating melanoma cells are tumorigenic and capable of metastasis formation, and showed that CTCs are enriched for MMICs. Indeed, subsequent clinical studies showed that ABCB5 subpopulations are also more common among CTCs than in solid tumors in melanoma patients (Gray et al. 2015) and that ABCB5 mRNA levels in the peripheral circulation of melanoma patients had significant prognostic value in inferring disease recurrence (Reid et al. 2013). Together, these results highlighted that ABCB5 identifies clinically important

disseminated melanoma subpopulations that warrant future research investigations regarding their potential role as a novel diagnostic and therapeutic biomarker.

Additional support for the CSC properties of ABCB5$^+$ melanoma fractions has also come from syngeneic melanoma mouse models (Ehira et al. 2010). Specifically, a tumorigenic B16 murine melanoma cell line variant demonstrated increased Abcb5 expression upon in vivo tumor formation (Ehira et al. 2010) alongside with other markers previously shown to enrich for murine MMIC-like cells with increased clonogenic and tumorigenic potentials (Dou et al. 2007). Taken together, these findings indicate that murine Abcb5$^+$ B16 melanoma subpopulations, like human ABCB5$^+$ MMICs, represent CSC-like cells that amplify during the tumorigenic process (Ehira et al. 2010). Similarly, the ABCB5 gene was also preferentially expressed by human melanomas with high in vivo tumorigenic capacity in human to murine xenotransplantation experiments Hoek et al. (2004, 2008). Consistent with these findings, human melanoma subpopulations with enhanced in vitro clonogenic and self-renewal capacities demonstrated increased ABCB5 expression levels (Keshet et al. 2008). In a separate study, induction of terminal differentiation of human melanoma cells resulted in a significant decrease in ABCB5 expression concomitant with reduced proliferation and enhanced susceptibility to chemotherapeutic agents (Botelho et al. 2010). Thus, both in vitro and in vivo substantiation of the CSC properties of ABCB5$^+$ melanoma fractions was established in settings that are independent of the foreign host milieu given in human to mouse xenotransplantation assays (Botelho et al. 2010; Ehira et al. 2010; Keshet et al. 2008).

Recently, additional evidence that melanoma follows the CSC model of tumor initiation and growth was provided in serial human melanoma to mouse xenotransplantation experiments (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Civenni et al. 2011). Boiko et al. and Civenni et al. demonstrated that MMICs can be prospectively isolated from melanoma patient specimens based on their expression of the nerve growth factor receptor (NGFR) *CD271* (also known as p75) (Boiko et al. 2010; Civenni et al. 2011). Serial xenotransplantation of CD271$^+$ melanoma subsets into engrafted human skin or bone in severely immunocompromised, T-, B-, and natural-killer deficient Rag2$^{-/-}\gamma^{-/-}$ mice resulted in experimental tumor growth in 70 % of injected animals (Boiko et al. 2010). In contrast, only 7 % of mice inoculated with CD271$^-$ melanoma cells developed tumors (Boiko et al. 2010). In addition, CD271$^+$ melanoma subpopulations demonstrated both self-renewal and differentiation capacity through serial in vivo passaging (Boiko et al. 2010), consistent with the existence of a functional melanoma hierarchy (Dirks 2010). Importantly, CD271$^+$ melanoma cells were also capable of metastasis in vivo (Boiko et al. 2010), suggesting a critical role for MMICs not only in tumor initiation but also in neoplastic progression. Remarkably, we found the MMIC markers ABCB5 (Schatton et al. 2008) and CD271 (Boiko et al. 2010) preferentially coexpressed on the same tumor subpopulation in human melanoma specimens (Schatton et al. 2010). The preferential coexpression of both markers has also been similarly documented by others (Civenni et al. 2011), indicating significant overlap of these MMIC populations.

In a separate study, the detoxifying enzyme, aldehyde dehydogenase (ALDH), was used to select for tumorigenic melanoma cell fractions (Boonyaratanakornkit

et al. 2010). Of note, ALDH expression identifies CSCs in human breast cancer (Ginestier et al. 2007) and colon cancer (Carpentino et al. 2009; Huang et al. 2009). Similarly, high ALDH activity enriched for tumorigenic cells with sustained self-renewal capacity in human malignant melanoma was determined in both NOD/SCID and NSG recipients mice (Boonyaratanakornkit et al. 2010), confirming that not all melanoma cells are equally adept at tumor initiation, including in NSG recipients. Consistently, $ALDH^+$ cells were found to give rise to $ALDH^-$ melanoma progeny, while the conversion of $ALDH^-$ into $ALDH^+$ melanoma cells was rarely observed in a separate study (Prasmickaite et al. 2010), suggesting a preferential self-renewal ability of the $ALDH^{high}$ cell pool. However, in this study ALDH activity did not enrich for melanoma subsets with enhanced tumorigenic potential (Prasmickaite et al. 2010), pointing to potential limitations of ALDH as a universal MMIC identifier. The relationship of ALDH expression to ABCB5 and/or CD271 is currently unknown and deserves further investigation, especially given the importance of the ALDH enzyme for conferring chemoresistance to alkylating agents (Vasiliou et al. 2004).

In summary, several recent studies independently demonstrated that not all melanoma cells possess equal capacities to initiate and maintain tumor growth in immunodeficient hosts (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Schatton et al. 2008; Civenni et al. 2011). Rather, a distinct subpopulation of MMICs capable of self-renewal and differentiation exists (Boiko et al. 2010; Schatton et al. 2008). Both the ABCB5 (Schatton et al. 2008) and the CD271 (Boiko et al. 2010) surface molecules have been established as prospective biomarkers of MMICs, and important links of these unique melanoma subpopulations to neoplastic progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010) and melanoma chemotherapy (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004) and immunotherapy resistance (Boiko et al. 2010; Schatton et al. 2010) have emerged. Taken together, these findings highlight the clinical relevance of MMICs and emphasize the potential importance of research investigations directed at targeting these tumorigenic melanoma subpopulations to achieve better response rates in patients with advanced stage disease.

12.4 Melanoma Stem Cell Assays

Despite increasing knowledge regarding MMICs and their established relationship to melanocytic tumor progression and therapy resistance, a range of opinions exists with regard to the most relevant assay systems for CSC identification and frequency determination. MMIC frequencies in human melanoma to NOD/SCID mouse xenotransplantation experiments have been estimated to be approximately 1 in 10^6 cells among unfractionated melanoma populations when tumor formation was assessed 8 weeks post melanoma cell inoculation (Quintana et al. 2008; Schatton et al. 2008). Determination of experimental tumorigenicity in NOD/SCID recipients at 32 weeks post melanoma cell inoculation yielded a higher estimated frequency of

tumorigenic melanoma cells of approximately 1 in 10^5 cells (Quintana et al. 2008). The use of more immunocompromised NSG hosts for assessment of tumor formation under otherwise equal experimental conditions yielded a further increase in estimated MMIC frequencies to approximately 1 in 5.5×10^3 cells among unsegregated patient-derived melanoma populations (Quintana et al. 2008). An even greater enrichment of tumorigenic cells was observed when melanoma cells were co-injected into NSG mice with matrigel (Quintana et al. 2008). Under these modified conditions, an average of 1 in 9 melanoma cells formed tumors at 8 weeks post melanoma cell inoculation (Quintana et al. 2008). While this study did not directly address CSC-defining features, such as sustained self-renewal and differentiation capacity in serial xenotransplantation experiments, the results support the view that alterations of the tumor environment can govern MMIC behavior.

Specifically, host environments characterized by absence of immune selective forces could permit tumor bulk populations, which do not normally initiate tumors and may not possess CSC-specific self-renewal and differentiation capacity to also cause experimental tumor growth. MMICs, on the other hand, might possess the preferential capacity to evade host immunosurveillance and initiate tumor growth (Schatton and Frank 2009), which would explain findings of lower estimated MMIC frequencies in more immunocompetent hosts. Indeed, several mechanisms by which MMICs evade antitumor immunity have recently been identified (Boiko et al. 2010; Schatton et al. 2010). For example, MMICs were found to express low to absent levels of immunogenic tumor-associated antigens (TAAs) (Boiko et al. 2010; Schatton et al. 2010), suggesting evasion from antitumor immune responses directed at TAAs. Furthermore, $ABCB5^+$ MMICs also preferentially blocked production of the proliferative cytokine IL-2 by cocultured lymphocytes (Schatton et al. 2010). Additional recently unraveled immunomodulatory functions of MMICs include the secretion of immunosuppressive factors and contact-dependent immunoregulatory mechanisms requiring engagement of immune-inhibitory surface molecules (Schatton et al. 2010), which will be discussed in more detail below. A relative immune privilege of CSCs was also demonstrated in alternative malignancies, including glioblastoma (Di Tomaso et al. 2010; Wei et al. 2010a, b), bladder cancer (Chan et al. 2009), colorectal carcinoma (Todaro et al. 2009), and leukemias (Majeti et al. 2009), indicating that immunomodulation might represent a common feature of CSCs (Schatton and Frank 2009). Clearly, the possibility of a CSC-driven tumor immune escape has profound implications not only for the development of improved cancer immunotherapeutic protocols but also for the design of biologically relevant assays for the study of CSC behavior. Specifically, assessment of tumor-initiating ability in the absence of antitumor immunity now appears to represent an inadequate assay system for the study of MMICs, because host immune environments incapable of immunologic tumor clearance might enable melanoma bulk populations to also initiate and sustain experimental tumor growth. Because preferential inhibition of IL-2 production is one mechanism by which MMICs evade antitumor immunity (Schatton et al. 2010), assessment of melanoma initiation in a murine model, that is, IL-2 receptor null (i.e., NSG mice) (Quintana et al. 2008) is not an appropriate environment for the accurate enumeration of MMIC frequency, because

host immunity is abnormally impaired. In light of these considerations, it is not surprising that tumorigenicity experiments performed using more immunocompromised hosts with defective IL-2 receptors (i.e., NSG mice) (Quintana et al. 2008) yielded higher estimated MMIC frequencies compared to xenotransplantation assays utilizing more immunocompetent hosts (i.e., NOD/SCID mice) (Quintana et al. 2008; Schatton et al. 2008). In our view, tumor initiation in melanoma, one of the most immunogenic cancers, should be assessed in a setting that allows for host antitumor immune responses to occur. Ideally, model systems for the accurate assessment of MMIC biology might involve chimeric murine xenograft recipients that are orthotopically xenografted with human cancer cells into syngeneic human tissues of cancer origin in the presence of an adoptively transferred hematopoietic system originating from the same patient (Frank et al. 2010).

In addition to alterations of host antitumor immune response, co-injection of matrigel, a solution containing growth factors and ECM constituents (Kleinman and Martin 2005), accounted for a marked increase in tumorigenic capacity of unfractionated human melanoma cells and estimated MMIC frequencies in NSG mice (Quintana et al. 2008). In our view, these findings imply that the stage can be set for MMICs to appear more abundant by exposing them to microenvironmental stimulants such as ECM and growth factors. It is conceivable that in the absence of such growth-promoting signals, only MMICs might possess the ability to survive and as a consequence maintain the tumorigenic process. This possibility is indeed supported by findings in other CSC-driven cancers (Calabrese et al. 2007; Li et al. 2009b). The clinical cancer scenario is typically characterized by low nutrient and growth factor availability and deregulated ECM function (Hendrix et al. 2003b). It thus seems plausible that MMICs might preferentially produce both ECM and growth factors, thereby sustaining tumor maintenance. In support of this hypothesis, Duda and colleagues demonstrated that circulating cancer cells, which are coated with autologous stromal components, including ECM factors, have the preferential capacity to seed distant metastasis and promote initial tumor growth compared to those cancer cells that are not incorporated in stromal elements (Duda et al. 2010). Upon establishment of a growth-promoting environment through ECM-expressing tumor subsets at the early stage of metastatic foci, additional cancer populations can also home to secondary tumor sites and contribute to neoplastic growth and progression during later disease stages (Duda et al. 2010). Similarly, genetic lineage-tracing experiments revealed that melanoma bulk populations could furnish the growing tumor with cellular progeny in the presence of MMICs (Schatton et al. 2008). Thus, it is conceivable that the co-injection of growth-promoting factors to melanoma cell inocula may mask MMIC-specific functions and could allow most melanoma cells to initiate experimental tumor growth. Subpopulations of differentiated cancer cells that do not normally initiate tumors and do not display the ability for sustained self-renewal under clinical conditions may thus be able do so in the presence of the appropriate microenvironment. Similarly, melanoma cells can give rise to induced pluripotent stem cells (iPSCs) through exogenous reprogramming factors (Utikal et al. 2009). While these findings clearly establish the *potential* of melanoma cells to generate iPSCs, it seems, however, unlikely that such gains of cellular plasticity

occur naturally in human patients. In aggregate, these findings further highlight the importance of establishing translationally relevant assays for the study of MMICs, which accurately mimic the environmental factors found in clinical cancers (e.g., low-nutrient levels, necrosis, hypoxia, and relatively intact antitumor immunity) rather than further deviating from them. Assays that are very permissive to revealing the tumorigenic potential of melanoma cells might thus vastly overestimate MMIC frequencies. A major weakness of the hypothesis that the CSC model might not apply to melanoma Quintana et al. (2008, 2010) is that MMICs must be rare. The observation that tumorigenic melanoma cells may be more common when assay conditions are modified does not imply that MMICs might not exist. Indeed, rarity is clearly not a defining criterion of CSCs according to the consensus definition (Clarke et al. 2006).

While the above outlined differences in microenvironmental cues (i.e., immune selective pressures and growth and ECM factor availability) could account for variations in estimated MMIC frequencies in NOD/SCID vis-à-vis NSG xenotransplantation experiments (Quintana et al. 2008; Schatton et al. 2008), they cannot explain apparent discrepancies in tumor-initiating ability of unfractionated melanoma cells assayed in the presence of matrigel and using identical mouse models (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Quintana et al. 2008). Specifically, the marked frequency of up to 1 in 4 unfractionated human melanoma cells with tumor-seeding ability in NSG hosts described by Quintana and colleagues (Quintana et al. 2010; Quintana et al. 2008) has not been confirmed to date. Indeed, independent laboratories have found that a minimum of 100 unfractionated patient-derived melanoma cells were required to consistently initiate tumors in NSG mice under equivalent experimental conditions (i.e., in the presence of matrigel) (Boiko et al. 2010; Boonyaratanakornkit et al. 2010), paralleling findings in additional CSC-driven malignancies, including pancreatic and head and neck cancers (Ishizawa et al. 2010). These new studies (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Ishizawa et al. 2010) alert us to the fact that MMICs might generally not be as common as suggested by Quintana et al. (2008, 2010). Thus, even experimental models that are very permissive to experimental tumor growth support the view that only a minority of melanoma cells is capable of initiating and maintaining the disease.

How can the differences of the findings by Quintana and colleagues and other studies using apparently identical model systems (Boiko et al. 2010; Boonyaratanakornkit et al. 2010) be explained? For instance, variations in enzymatic tumor dissociation, isolation, and/or inoculation techniques between different laboratories could lead to different estimated MMIC frequencies (Shackleton 2010). Specifically, the aforementioned laboratory procedures could potentially account for differences in surface marker expression and/or viability among inoculated melanoma cell suspensions (Murphy et al. 2014). Also, the use of distinct matrigel batches with varying growth and/or ECM factor compositions could result in altered melanoma growth. Alternatively, the use of tumor specimens from patients with more advanced disease (Quintana et al. 2008), which have been demonstrated to contain elevated CSC numbers compared to primary tumors (Schatton et al. 2008), might also account for higher relative proportions of melanoma cells capable of

initiating experimental tumor growth. In addition, the assessment of CSC frequency, utilizing tumor cell isolates from melanoma xenografts that have been passaged in vivo for extended periods of time in lieu of directly patient-derived tumor samples, could further obscure the accurate assessment of CSC frequencies and biological functions (Boiko et al. 2010; Quintana et al. 2008). Indeed, Boiko and colleagues demonstrated that both in vitro and in vivo passaging of melanoma cells can result in the emergence of tumorigenic subclones independent of their immunophenotype (Boiko et al. 2010). Similarly, tumor cell lines may have lost the hierarchical structure of the primary tumor from which they originated (Zhou et al. 2009). A lesson to be learned from the apparently conflicting evidence regarding the frequency of tumorigenic melanoma cells in NSG mice (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Quintana et al. 2008) is that careful comparison of experimental methodologies may serve to explain differing results. Clearly, these discordant studies demonstrate that both the tumor environment and experimental procedures can govern tumor growth and underline the importance of establishing standardized assays for the study of MMIC biology that could enable the consolidation of experimental findings from different laboratories.

In summary, differences in MMIC frequency and function suggested by results obtained in alternative host immune milieus in the presence or absence of co-injected ECM and/or growth factors (Boiko et al. 2010; Quintana et al. 2008, 2010; Schatton et al. 2008, Murphy et al. 2014) strongly suggest critical interactions of MMIC with the tumor host environment. Thus, bioassays and experimental model systems for the detection of clinically relevant MMICs require further approximation of, rather than further abstraction from, the naturally occurring tumor–host interactions in human patients (Frank et al. 2010; Schatton et al. 2009; Murphy et al. 2014).

12.5 Melanoma Stem Biology: Therapeutic Opportunities

The ability to prospectively identify CSCs has permitted researchers to begin characterizing specific molecular and cellular mechanisms preferentially associated with CSCs that may contribute to tumor initiation and growth, in addition to those associated with their defining features of unlimited self-renewal and proliferative capacities (Frank et al. 2010). Among the recently uncovered CSC functions likely to influence tumor development are mechanisms of tumor immune evasion (Schatton and Frank 2009). Findings of increased tumor incidence in immunocompromised patients suggest that immunosurveillance might serve to eliminate malignant cells at early stages of tumorigenesis (Mapara and Sykes 2004). Relative immune privilege and/or selective immunomodulatory functions could thus enable CSCs to evade antitumor immune responses in favor of inexorable tumor growth (Schatton and Frank 2009). This possibility is especially relevant to melanoma development because melanoma is a particularly immunogenic cancer (Rosenberg et al. 2008). Indeed, several mechanisms by which MMICs evade antitumor immunity have recently been identified (Boiko et al. 2010; Schatton et al. 2010).

For example, MMICs can downregulate their expression of TAAs, such as MART-1, tyrosinase, ML-IAP, and the cancer testis antigens NY-ESO-1, and MAGE-A (Boiko et al. 2010; Schatton et al. 2010). Importantly, T cells reactive against TAAs are commonly observed in melanoma patients where they elicit antitumor immune responses directed at TAA-expressing melanoma cells (Lee et al. 1999; Stockert et al. 1998). Decreased expression of TAAs would thus enable MMICs to evade antitumor immune responses (Boiko et al. 2010; Schatton et al. 2010), providing for a potential explanation for the relative ineffectiveness of tumor-reactive T cells and autologous vaccination strategies in halting tumor growth (Schatton and Frank 2009). ABCB5$^+$ MMICs were also found to express decreased levels of MHC class I molecules (Schatton et al. 2010), which represent an established mechanism of tumor immune evasion and neoplastic progression (Aptsiauri et al. 2007; Khong et al. 2004), further suggesting preferential MMIC evasion from immunological clearance.

In addition to reduced expression of MHC class I molecules, ABCB5$^+$ MMICs selectively expressed the B7.2 (CD86) costimulatory ligand and the negative costimulatory receptor, PD-1 (Schatton et al. 2010). Of note, interactions of B7.2 with its receptor CTLA-4 and of PD-1 with its ligands PD-L1 and PD-L2 can down-modulate immune responses by inducing T cell anergy and/or by activating Tregs (Greenwald et al. 2005; Li et al. 2009a), suggesting that MMICs might regulate antitumor immune responses in favor of inexorable tumor growth via the involvement of negative costimulatory pathways. Indeed, selective blockade of MMIC-expressed B7.2 maintained CD4$^+$CD25$^+$FoxP3$^+$ Treg frequencies among cocultured lymphocytes and regulated their secretion of the immunosuppressive cytokine IL-10 (Schatton et al. 2010). Importantly, accumulating evidence in various cancers, including melanoma, suggests that Tregs might represent important mediators of clinical tumor immune evasion (Ahmadzadeh et al. 2008; Clark et al. 2008; Curiel et al. 2004), underlining the potential importance of MMIC-mediated Treg induction for melanoma progression.

In addition, MMICs were also found to inhibit human peripheral blood mononuclear cell proliferation more efficiently than tumor bulk populations (Schatton et al. 2010). Consistently, ABCB5$^+$ melanoma subpopulations preferentially inhibited production of the proliferative cytokine, IL-2, by both cocultured mitogen-activated lymphocytes and patient-identical peripheral blood mononuclear cells in the absence of a mitogenic stimulus (Schatton et al. 2010). Importantly, decreased IL-2 levels correlate with increased melanoma growth in animal models and human patients (Eklund and Kuzel 2004). Given its immune-activating effects, IL-2 is used as adjuvant therapy for the treatment of advanced stage melanoma (Eklund and Kuzel 2004). Thus, preferential inhibition of IL-2 signaling might not only represent an important mechanism underlying MMIC-driven tumor growth but could also provide for a novel explanation for the commonly observed inability of therapeutic regimens involving IL-2 in producing durable patient responses (Eklund and Kuzel 2004). The preferential inhibition of IL-2 production by ABCB5$^+$ MMICs might also explain observed differences in MMIC frequency in NSG (IL-2R$\gamma^{-/-}$) (Quintana et al. 2008) versus NOD/SCID (IL-2R^{WT}) hosts (Quintana et al. 2008;

Schatton et al. 2008). Specifically, assessment of tumorigenicity outcomes in the absence of functional IL-2 signaling might overestimate MMIC frequency because a host environment characterized by abnormally impaired antitumor immunity might enable melanoma bulk populations to also contribute to tumor growth.

Additional immunomodulatory functions of MMICs include the secretion of immunosuppressive factors, such as immune-inhibitory TGF-β pathway members (Schatton et al. 2010). Importantly, these soluble mediators can be produced by tumors to dampen the anticancer immune response (Gorelik and Flavell 2001; Inge et al. 1992).

In sum, numerous MMIC-specific immunological mechanisms have been unraveled that may enable them to evade and/or modulate the antitumor immune response to promote neoplastic growth and progression. Strikingly, a relative immune privilege of CSCs was also demonstrated in alternative malignancies, including glioblastoma (Di Tomaso et al. 2010; Wei et al. 2010a, b), bladder cancer (Chan et al. 2009), colorectal carcinoma (Todaro et al. 2009), and leukemias (Majeti et al. 2009), indicating that immunomodulation might represent a common feature of CSCs (Schatton and Frank 2009). Clearly, the possibility of CSC-driven tumor immune escape has profound implications for the design of biologically relevant assays for the study of CSC behavior.

Perhaps, most significant are the implications of an MMIC-driven tumor immune escape for the development of improved cancer immunotherapeutic protocols. In addition, MMIC biology could provide a highly relevant tool for the evaluation of patient responses to current immunotherapeutic regimens, including those aimed at modulating critical regulatory elements of patient immune cells to enhance their antitumor reactivity. Importantly, a number of such novel immunotherapeutics have recently entered clinical practice or ongoing trials (Kirkwood et al. 2008; Ugurel et al. 2016). These include inhibitors of costimulatory molecules or paracrine immunosuppressive cytokines, including monoclonal antibodies directed at CTLA-4, PD-1, 4-1BB, and TGF-β (Fong and Small 2008; Kirkwood et al. 2008; Lahn et al. 2005; Lynch, 2008). In contrast to current immunotherapeutic regimens, these novel agents exert inhibitory effects on both immune effector cells and Treg function (Kirkwood et al. 2008). They might thus prove more effective in inducing durable patient responses. Indeed, a phase III study demonstrated that treatment of stage III or IV melanoma patients with therapy-resistant disease with the anti-CTLA-4 antibody Ipilimumab resulted in improved overall survival compared to patients treated with a gp100 peptide vaccine (Hodi et al. 2010) and Ipilimumab was subsequently approved for clinical use. A potential additional explanation for this anti-melanoma effect of CTLA-4 inhibition arises from the fact that tumorigenic MMICs preferentially expressed the CTLA-4 ligand, B7.2, and induced Tregs in a B7.2-dependent fashion (Schatton et al. 2010). Inhibition of MMIC-specific immune escape mechanisms might thus contribute to the antitumor efficacy of Ipilimumab. Given the preferential expression of PD-1, 4-1BBL, and TGF-β pathway members by MMICs, it is further conceivable that responses observed in melanoma patients treated with clinically approved anti-PD-1 antibodies (Brahmer et al. 2010), or therapeutic agents directed at 4-1BB (Lynch 2008) or TGF-β (Kirkwood et al. 2008), might potentially

also relate to the ability of these immunotherapeutics to block MMIC-specific immunomodulatory functions. The observed failure of immunotherapies directed at the bulk of tumor cells (e.g., vaccination strategies targeting differentiation antigens such as MART-1 or tyrosinase) or those that elicit nonspecific immune activation (e.g., neoadjuvant IL-2 therapy) in mediating sustained patient responses (Rosenberg et al. 2004) might, on the other hand, relate to the inability of such regimens to effectively target the MMIC compartment (Schatton and Frank 2009), given its low expression levels of TAAs (Boiko et al. 2010; Schatton et al. 2010) and its preferential ability to inhibit IL-2 production (Schatton et al. 2010). Taken together, these findings highlight the possibility that immunotherapeutic strategies aimed at enhancing the endogenous immune responses to melanoma might prove most efficient if MMIC-specific immune escape mechanisms are concurrently impaired. In light of this intriguing possibility, it might be relevant to analyze the clinical effectiveness of novel immunotherapeutic agents, including Ipilimumab or PD-1 antibodies, not only with regard to the pattern and duration of immune responses (Reuben et al. 2006) but also in the context of their impact on the MMIC subset and its immunomodulatory properties (Schatton and Frank 2009).

In addition to their preferential refractoriness to current immunotherapeutic protocols, MMICs also demonstrate enhanced resistance to several structurally unrelated chemotherapeutic agents with distinct mechanisms of action (Schatton et al. 2009), a phenomenon also termed multidrug resistance (MDR) (Dean et al. 2005). MDR can result through several different means, including impairment of tumor apoptotic pathways, alterations in cell cycle checkpoints, and decreased drug accumulation (Gottesman et al. 2002). The latter mechanism is of particular interest to MMICs because one mechanism resulting in reduced intracellular drug levels is the excretion of cytotoxic agents by energy-dependent efflux pumps, known as ATP-binding cassette (ABC) transporters (Gottesman et al. 2002). Specifically, the MMIC determinant, ABCB5 (Schatton et al. 2008), mediates resistance to multiple chemotherapeutic agents in malignant melanoma, hepatocellular carcinoma, breast cancer, and leukemias, including doxorubicin (Cheung et al. 2011; Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Yang et al. 2010), camptothecin, mitoxantrone, and 5-fluorouracil (Huang et al. 2004; Lehne et al. 2009). A broader role for the ABCB5 transporter in chemotherapeutic resistance to additional agents is suggested by the observation that ABCB5 mRNA expression levels across a panel of human cancer cell lines used by the National Cancer Institute for drug screening correlated significantly with chemoresistance to 45 out of 119 anticancer agents (Frank et al. 2005). Evidence for a preferential resistance of CSCs to both chemotherapy and radiotherapy has also been generated in numerous additional CSC-driven malignancies, including leukemias, gliomas, breast carcinomas, and pancreatic cancer (reviewed in (Schatton et al. 2009)), underscoring the need to dissect further the molecular pathways responsible for CSC-specific therapy resistance. For example, Chartrain et al. showed that ABCB5-expressing melanoma cells selectively survive not only when exposed to dacarbazine, a long-standing reference treatment of metastatic melanoma, but also to vemurafenib, a recently approved inhibitor of the mutated kinase V600E BRAF and other various chemotherapeutic

drugs. These results showed that anti-melanoma chemotherapy might participate in the chemoresistance acquisition by selecting tumor cell subpopulations expressing ABCB5. This is of particular importance in understanding the relapses observed after anti-melanoma treatments and reinforces the interest of ABCB5 and ABCB5-expressing cells as potential therapeutic targets in melanoma (Chartrain et al. 2012). The therapeutic promise of MMIC-directed treatment strategies, which could enhance current treatment modalities for patients with advanced stage melanoma, is further highlighted by recent findings of a relationship of MMICs to neoplastic melanoma progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010; Gray et al. 2015; Reid et al. 2013) and adverse clinical outcome (Sharma et al. 2010; Reid et al. 2013). In light of the CSC concept and findings of MMIC-specific immunoregulatory properties, a number of novel therapeutic approaches can be envisioned that could potentially increase the efficacy of current forms of therapy (reviewed in Frank et al. 2010), if MMICs are indeed the major culprits of melanoma initiation and clinical virulence. For instance, MMIC ablation through prospective markers might prove useful in enhancing current anti-melanoma therapies. In support of this possibility, selective killing of MMICs via ADCC using an anti-ABCB5 monoclonal antibody halted experimental tumor growth (Schatton et al. 2008). Alternatively, targeting of MMICs via preferentially expressed surface molecules and/or MMIC-specific pathway interference might also yield improved melanoma patient responses. Such treatment strategies could include inhibition of costimulatory signaling events mediated by MMIC-expressed (Schatton et al. 2010) B7.2, PD-1, and/or 4-1BB. Disruption of surrogate MMIC-specific immune evasion pathways could also represent treatment modalities that might enhance responsiveness to current anti-melanoma regimens. Additionally, melanoma cell-intrinsic functions of PD-1 expressed on ABCB5^{+} MMIC could potentially be exploited for therapeutic benefit. Recently, we showed that melanoma cell-intrinsic PD-1 promotes tumorigenesis, even in mice lacking adaptive immunity. PD-1 inhibition on melanoma cells by RNAi, blocking antibodies, or mutagenesis of melanoma-PD-1 signaling motifs suppressed tumor growth in immunocompetent, immunocompromised, and PD-1-deficient tumor graft recipient mice. Conversely, melanoma-specific PD-1 overexpression enhanced tumorigenicity, as did engagement of melanoma-PD-1 by its ligand, PD-L1, whereas melanoma-PD-L1 inhibition or knockout of host-PD-L1 attenuated growth of PD-1-positive melanomas. Mechanistically, the melanoma-PD-1 receptor modulated downstream effectors of mTOR signaling. These results identified melanoma cell-intrinsic functions of the PD-1:PD-L1 axis in tumor growth and suggested that blocking melanoma-PD-1 might contribute to the striking clinical efficacy of anti-PD-1 therapy (Kleffel et al. 2015). In addition, driving MMICs into differentiation could lead to improved therapeutic outcomes. Consistent with this possibility, differentiation of glioblastoma CSCs via bone morphogenetic protein 4 (BMP4) exposure resulted in inhibition of experimental tumor growth concomitant with enhanced survival (Piccirillo et al. 2006). Interestingly, the BMP4 receptor, BMPR1a, is preferentially expressed by ABCB5^{+} MMICs (Schatton et al. 2008), suggesting that a similar strategy could

also be promising in this malignancy. Additionally, the ABCB5 marker of MMIC itself has been shown to provide a target for melanoma differentiation therapy: In MMIC, we showed that ABCB5 controls IL1beta secretion, which serves to maintain slow cycling, chemoresistant cells through an IL1beta/IL8/CXCR1 cytokine signaling circuit. This CSC maintenance circuit involved reciprocal paracrine interactions with ABCB5-negative cancer cell populations. ABCB5 blockade-induced cellular differentiation, reversed resistance to multiple chemotherapeutic agents, and impaired tumor growth in vivo. Together, these results defined a novel function for ABCB5 in CSC maintenance and tumor growth that could be therapeutically targeted (Wilson et al. 2014). An additional MMIC-directed targeting strategy could involve the use of antiangiogenic and/or antivasculogenic regimens. This possibility is indicated by findings of a preferential induction of neovascularization by CSCs in other cancers (Bao et al. 2006b). MMICs might likewise be involved in angiogenesis and/or vasculogenesis given their preferential expression of vasculogenic differentiation markers VE-cadherin and TIE-1 (Schatton et al. 2008). For example, we showed that VEGFR-1 expressed by MMIC is required for tumor growth and that melanoma-specific shRNA-mediated knockdown of VEGFR-1 blocked the development of $ABCB5^{+}$ vasculogenic mimicry (VM) morphology and inhibited $ABCB5^{+}$ VM-associated production of the secreted melanoma mitogen laminin. Moreover, melanoma-specific VEGFR-1 knockdown markedly inhibited tumor growth, identifying VEGFR-1 as a novel MMIC therapeutic target (Frank et al. 2011) Reversal of chemoresistance and/or radioresistance mechanisms operative in MMICs might also successfully increase anti-melanoma therapeutic efficacy (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004). This is further suggested by findings in glioblastoma CSCs (Bao et al. 2006a), among other cancers (Diehn et al. 2009; Vlashi et al. 2009). Lastly, given the importance of the tumor environment in governing CSC behavior (Scadden 2006), the disruption of protumorigenic MMIC-niche interactions could also optimize anticancer therapeutic protocols. It is important to recognize that therapeutic efficacy will often depend also on a significant reduction of melanoma bulk populations, which may cause excessive tumor burden. Therefore, combination therapies that involve both MMIC-directed agents as well as debulking regimens would be predicted to prove most effective in improving clinical treatment responses and patient outcomes.

In summary, several biological functions of MMICs have recently been identified, including their preferential ability to modulate and/or evade antitumor immune responses (Boiko et al. 2010; Schatton et al. 2010). In addition to their refractoriness to current immunotherapeutic protocols, MMICs also demonstrate increased resistance to a number of chemotherapy agents (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004; Chartrain et al. 2012). Taken together with the recently established relationship of MMICs to melanocytic tumor progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010; Gray et al. 2015; Reid et al. 2013), these findings underscore the potential clinical relevance of MMIC-directed targeting approaches. Importantly, proof-of-principle

for the potential therapeutic utility of targeting MMICs has been established, by demonstrating that selective eradication of ABCB5+ melanoma subpopulations can halt experimental tumorigenesis (Schatton et al. 2008). A number of additional targeting approaches might likewise prove useful in blocking MMIC-driven tumor development and progression, including disruption of immune-inhibitory pathways, differentiation therapy, and chemoresistance reversal. While more work is required to translate these research developments into the clinic, they point to a critical relevance of MMICs for successful melanoma therapy. Further molecular screens of purified MMIC populations employing microarrays, RNA-interference, or drug-screening libraries could allow identification of further targetable pathways leading to MMIC-tailored eradication strategies for melanoma therapy.

Conclusions

A growing body of literature supports the existence of MMICs (Boiko et al. 2010; Boonyaratanakornkit et al. 2010; Schatton et al. 2008; Civenni et al. 2011). Additionally, important links have been established between MMICs, neoplastic progression (Fukunaga-Kalabis et al. 2010; Gazzaniga et al. 2010; Ma et al. 2010; Schatton et al. 2008; Sharma et al. 2010; Vasquez-Moctezuma et al. 2010; Gray et al. 2015; Reid et al. 2013), chemoresistance (Elliott and Al-Hajj 2009; Frank et al. 2005; Fukunaga-Kalabis et al. 2010; Huang et al. 2004; Chartrain et al. 2012), and refractoriness to immunotherapy (Boiko et al. 2010; Schatton et al. 2010). Moreover, novel biological features of MMICs are currently being intensively explored. For instance, the ability of MMICs to modulate the antitumor immune response (Schatton and Frank 2009; Schatton et al. 2008) might be especially informative for the optimization and/or evaluation of targeted immunotherapies that were recently found to improve overall survival in patients with recurrent metastatic melanoma (Hodi et al. 2010; Ugurel et al. 2016). Together, these findings highlight the relevance and promise of novel MMIC-centered diagnostic and therapeutic approaches in human melanoma.

Acknowledgments This work was supported by National Cancer Institute, NIH, grants 1RO1CA113796, 1RO1CA138231, and 2P50CA093683. T. Schatton is supported by an Outrun the Sun, Inc. 2010 National Melanoma Research Scholar Award.

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The Birth of Malignancy: Embryogenesis and Melanoma

13

Alec Gramann, William Tyler Frantz, and Craig J. Ceol

13.1 Introduction

Embryonic development involves high levels of proliferation, migration, and differentiation to form all the tissues and organs of an organism. Under normal circumstances, cell–cell signaling in an embryo is coordinated both temporally and spatially to allow development to occur without error. Embryogenesis is a once-in-a-lifetime event, and many developmental signaling pathways and processes are not physiologically active in adult tissues, with notable exceptions in the maintenance of certain stem cell populations (Goldstein and Horsley 2012). However, in disease states such as cancer, malignant cells adopt similar characteristics to those seen in embryonic cells – proliferation, migration, and lack of differentiation. Over recent decades, investigation into various stages of melanocyte development has given great insight into specific characteristics of melanoma, leading to a deeper understanding of melanomagenesis. In this chapter, we review key literature describing connections between embryonic development and melanoma, including aspects of embryonic patterning and melanocyte lineage specification that are co-opted to promote melanoma progression.

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_13

13.2 Role of Embryonic Patterning Factors in Melanoma

During early embryonic development, a complex signaling program directs the fates of pluripotent cells and their descendants (Sadler and Langman 2010). This process of embryonic patterning involves a balance of signals. It directs apoptosis or proliferation, fate specification or maintenance of an undifferentiated state, and varying levels of migratory capacity. Each of these properties is important in the initiation and progression of many tumor types, including melanoma (Postovit et al. 2008). Of the many signaling molecules and pathways active in early embryonic development, Nodal and Notch signaling have crucial roles in patterning and have been linked to melanoma progression.

13.2.1 Nodal

Nodal, a member of the TGF-β superfamily, is a secreted protein that binds its receptor and, acting through SMAD signaling, activates transcription of target genes to direct embryonic development (Conlon et al. 1994). While Nodal is known to have multiple roles during embryogenesis, those of most interest in the context of cancer are maintenance of pluripotency and regulation of migration during gastrulation (James et al. 2005; Quail et al. 2013). Early embryonic development relies on maintaining cells in a self-renewing, pluripotent state before cell-fate specification and differentiation. Maintenance of pluripotency is critical in mesendoderm and neurectoderm, and in these tissues, Nodal signaling is essential. Loss of Nodal causes the loss of expression of stem cell factors and leads to expression of differentiation markers of neuroectoderm (James et al. 2005; Vallier et al. 2009). During gastrulation, Nodal is required to maintain both the primitive node and the primitive streak (Conlon et al. 1994). Loss of Nodal causes disruption of the formation of the streak and node, which causes defects in the migration of mesoderm between the hypoblast and epiblast layers of the embryo. In addition, markers of mesodermal migration, such as Twist-1 and Mox-1, are absent when Nodal is lost. Interestingly, Twist affects epithelial-to-mesenchymal transition (EMT, discussed in detail below), which is implicated in metastasis of many tumor types. Nodal signaling is also crucial in defining left–right asymmetry. Nodal secreted by the primitive node acts on the left side of the embryo and is prevented from acting on the right by active cilia. The cilia sweep Nodal to the left and allow distinct signaling on the right by Lefty1 and Cerberus (Levin et al. 1995; Zhu et al. 1999). Nodal signaling in embryogenesis directs many processes crucial to normal development, but when ectopically activated, these processes contribute to the malignant phenotypes observed in cancer.

The role of Nodal in melanoma was initially proposed by Hendrix and colleagues after a screen to investigate interactions between melanoma cells and embryonic stem cells (Topczewska et al. 2006). They injected fluorescently labeled human melanoma cells, which were previously characterized for gene expression,

invasiveness, and metastatic potential, into embryonic zebrafish and monitored changes in development. Those melanoma cells categorized as more aggressive induced changes in the structure of endogenous tissue, while less aggressive cell lines did not. Morphologic abnormalities observed included additional outgrowths and secondary body axes, which led to the hypothesis that melanoma cells were able to stimulate the same patterning pathways as embryonic cells. Given the nature of the morphologic abnormalities, Nodal was investigated as a candidate morphogen secreted by melanoma cells. Through evaluation of patient samples, they showed that Nodal expression is almost exclusively limited to those melanomas with more aggressive pathologic characteristics, such as an early vertical growth phase, local tissue invasion, earlier and more metastases, and lack of differentiation. Additional studies have shown that melanomas expressing Nodal have pathologic and clinical characteristics associated with a poorer prognosis than do other melanomas (Topczewska et al. 2006).

The characteristics of Nodal-expressing melanomas bear a relationship to Nodal-regulated processes in embryogenesis. For example, Nodal has been shown to activate EMT factors in melanoma, such as Snail family proteins, which, like Twist, promote embryonic mesodermal migration (Fang et al. 2013). Additionally, when melanoma cells were treated with exogenous Nodal in vitro, they became less adhesive and express increased matrix remodeling proteins. These characteristics are associated with EMT in both cancer and embryogenesis (Fang et al. 2013). Nodal has also been shown to drive dedifferentiation of melanoma cells toward a stem-cell-like state, resulting in expression of earlier melanocyte lineage markers as well as loss of pigmentation (Topczewska et al. 2006). Conversely, when Nodal is knocked down, the cells become more adherent and differentiated. Additionally, Nodal knockdown abrogates overall tumor growth in xenotransplant models (Hardy et al. 2010; Postovit et al. 2008).

The role of Nodal in aggressive melanomas has stimulated interest in it as a drug target. Nodal is expressed in few adult tissues (Hendrix et al. 2007), so an anti-Nodal therapy would be relatively tumor-specific, and off-target effects would be limited. Recently, anti-Nodal antibodies have been evaluated in vitro with dacarbazine for effects on melanoma cells (Hardy et al. 2015). Previous studies have shown Nodal-positive cell subpopulations in heterogeneous tumors are resistant to dacarbazine therapy. The combination treatment of anti-Nodal antibodies with dacarbazine decreased proliferation and increased apoptosis of melanoma cells when compared with treatments of each single agent alone. Follow-up evaluation of the combination therapy in xenografts showed a similar decrease in tumor growth rate. Anti-Nodal antibodies have been compared to BRAF^{V600E} inhibitor therapy in vitro and in vivo (Strizzi et al. 2015). Notably, a greater decrease in tumor volume in xenografts treated with anti-Nodal antibodies was observed when compared to controls treated with IgG antibodies or BRAF^{V600E} inhibitor. While direct targeting of Nodal for melanoma therapy shows promise in these initial evaluations, it is not yet known whether anti-Nodal therapies can improve patient clinical outcomes.

13.2.2 Notch

Notch is a highly conserved transmembrane protein that controls gene expression to regulate cell-fate determination, survival, development, and neurogenesis. There are four Notch receptors in humans, designated Notch 1–4. Five Notch ligands, designated Delta Like Ligand 1/3/4 and Jagged 1/2, activate Notch receptors. Notch ligand–receptor interactions stimulate juxtamembrane signaling, resulting in a series of proteolytic cleavage steps mediated by TNFa-converting enzyme and the γ-secretase protease complex. Following cleavage, the Notch intracellular domain (NICD) is released into the cytosol (Brou et al. 2000; Edbauer et al. 2003; Hartmann et al. 2002). NICD then translocates to the nucleus where it binds to mastermind, CBF1, and p300 and regulates transcription of genes, including the Hes and Hey gene families (Fryer et al. 2002; Nam et al. 2003; Wu et al. 2000). Notch signaling is an important part of normal embryogenesis, regulating asymmetric cell divisions that are crucial to neurogenesis, somite compartmentalization, and other developmental processes (Feller et al. 2008; Matsuzaki 2000). By regulating cell-fate specification and cell function, Notch activity contributes to a broad spectrum of biological processes, including stem cell maintenance, hematopoietic fate decisions, and intestinal epithelium renewal (Bedogni 2014; Montagne and Gonzalez-Gaitan 2014; Pinnix and Herlyn 2007). Notch targets CyclinD1, p21, and SKP2 suggesting a role in cell cycle regulation (Pinnix and Herlyn 2007). Taken together, these and many additional studies show Notch regulates cell function during embryogenesis and other contexts.

Notch signaling is required for the maintenance of melanoblasts and melanocyte stem cells (MSCs). Mice that are depleted of Notch activity in the melanocyte lineage exhibit severe coat color dilution caused by inappropriate apoptosis of melanoblasts during embryogenesis (Moriyama et al. 2006). Notch1 and Notch2 receptors are critical for melanoblast survival (Schouwey et al. 2007). Furthermore, Notch activity is required for MSC survival, as these cells are lost during successive hair cycles in Notch-depleted animals. Death within the melanocyte lineage may result from a failure to maintain melanoblasts and MSCs in a de-differentiated state. In support of this notion, gain-of-function studies by the Herlyn group found that expression of constitutively active NICD was sufficient to convert differentiated melanocytes into multipotent neural crest stem-like cells (Zabierowski et al. 2011). Notch signaling is indeed important for development of the neural crest, where it is involved in initial specification of neural crest cells and simultaneously prevents their premature differentiation (Cornell and Eisen 2002; Endo et al. 2002; Glavic et al. 2004). In cultured neural crest stem-like cells, Notch signaling is required for cell survival and is inhibited during ultraviolet-light-induced differentiation of these cells into melanocytes (Fukunaga-Kalabis et al. 2015). Overall, Notch signaling acts in cells of the melanocyte lineage, either neural crest, melanoblasts, or MSCs, to promote cell survival and prevent terminal differentiation.

Notch signaling is active in melanomas where it regulates key aspects of tumor progression (Hendrix et al. 2002). The microRNA miR-146a acts through Notch to promote melanoma cell survival and progression (Forloni et al. 2014). miR-146a is upregulated by oncogenic BRAF or NRAS signaling, and a key miR-146a target is

NUMB. Repression by miR-146a downregulates NUMB, relieving its inhibition of Notch signaling. Notch interacts with additional microRNAs to promote melanoma metastasis. Notch activation in melanoma cells through contact with neighboring keratinocytes leads to disruption of microphthalmia-associated transcription factor (MITF)/Recombination signal binding protein for immunoglobulin kappa J region (RBPJK)-mediated repression of miR-222/221 (Golan et al. 2015). This keratinocyte-induced Notch signaling results in miR-222/221 expression, which promotes melanoma invasion and metastasis. Lastly, Notch signaling is implicated in melanoma progression through an association with Nodal (Hardy et al. 2010). In aggressive melanoma cell lines, Nodal expression is correlated with and depends on Notch4 expression and activity, respectively. Inhibition of Notch4 blunts aggressive features of these cells including anchorage-independent growth. These activities of Notch in melanoma cells are consistent with its role in the melanocyte lineage of promoting a less differentiated and more proliferative state.

13.3 Adopting Melanocyte Lineage Characteristics in Promoting Melanoma

There are specific aspects of neoplasia that hold true for nearly every type of cancer – an ability to bypass cell cycle checkpoints, to suppress growth arrest and apoptotic signaling being a few commonalities. But between cancers arising from various cell types, and even between subtypes of the same cancer, there is a wide range of behaviors that are observed. Some develop more indolent characteristics, such as a slow growth rate or minimal invasion from the primary site. Others are known to be much more malignant, spreading and growing quickly, sometimes before a primary tumor is clinically apparent. While there is much variability within cancers that arise from same cell type, some malignancies are prognostically inherently worse or better than others. Some of these differences can be explained by the propensity for specific oncogenic mutations, unique environmental exposures to carcinogens, or particular viral infections. But there is also an underlying component of development that predisposes cancers to behave in specific ways.

Melanoma is often more aggressive and prone to metastasis than other cancers (Gupta et al. 2005a). While this propensity for aggressive behavior is likely related to the function of mutations in specific oncogenes and tumor suppressors, the underlying biology of melanocytes plays a role in the phenotypes observed in melanoma. In an experiment evaluating characteristics of different precursor cells with the same oncogenic mutation profile, multiple cell types were immortalized using simian virus-40-expressing LT and ST proteins and were then transformed with oncogenic Ras (Gupta et al. 2005a). The resulting transformed cells were evaluated for various malignant characteristics, such as invasive and metastatic capacity. The majority of transformed cells showed the ability to form tumors but were unable to invade or metastasize. However, transformed melanocytes were very invasive under the same transformation conditions. These results suggest that there is an inherent predisposition of melanocytes to metastasize when compared with

other cell types. This predisposition is due, in part, to epigenetic differences between melanocytes and other cell types (Gupta et al. 2005a). In a more recent study, Kaufman et al. showed that in zebrafish, a marker expressed only in the neural crest during embryogenesis, *crestin*, is re-expressed during the formation of melanomas (Kaufman et al. 2016). This further supports the notion that the embryonic lineage from which a cell is derived plays a role during malignant transformation. One of the major embryonic programs operating in these precursor cells is the epithelial-to-mesenchymal transition (EMT).

As previously discussed, the first major EMT event is the migration of mesoderm during gastrulation (Conlon et al. 1994). The second major EMT event involves the migration of neural crest cells from the neural crest to multiple locations around the developing embryo (Sadler and Langman 2010). In most cases involving EMT, there are key steps that allow cells to make the transition and to begin to migrate. These steps are delineated by the expression of markers known to be associated with EMT, such as Snail1, Snail2 (previously Slug), Twist1, Zeb1, and Zeb2 (Cano et al. 2000; Comijn et al. 2001; Grooteclaes and Frisch 2000; Yang et al. 2004). The first key step is alteration of adherence molecules on the cell surface. In pre-migratory melanocytes, the major adherence molecule involved in maintaining cell–cell and cell–matrix connections is E-cadherin (reviewed in Kerosuo and Bronner-Fraser 2012). During EMT, E-cadherin expression is replaced by expression of N-cadherin, driven by the neural crest transcription factor Snail2 (Cano et al. 2000). By downregulating E-cadherin, cells are able to free themselves from neighboring cells to begin migrating. Another key step is the upregulation of metalloproteinases and other matrix remodeling proteins. Key proteins such as ADAM10, ADAM13, and MMP-2 have increased expression that correlates with the timing of delamination of the neural crest cells from the neural tube (Kuriyama and Mayor 2008). By downregulating adhesion and upregulating remodeling proteins, neural crest cells are able to migrate to their final destinations. While promoting migratory characteristics of neural crest cells and melanocyte precursors, EMT also maintains a de-differentiated and minimally proliferative state in cells during migration (Vega et al. 2004).

Many aspects of the epithelial-to-mesenchymal transition during migration of the neural crest are similar to those that are characteristic of melanoma. In support of this similarity, many factors involved in physiologic EMT during development are also active in melanoma. In particular, Twist1, Zeb1, Zeb2, and Snail family proteins have been implicated in melanoma metastasis. These EMT factors are activated through MAP kinase signaling and promote a de-differentiated and migratory state in melanoma (Caramel et al. 2013). Additionally, the proliferation rate of cells with active EMT markers is significantly decreased, making them more resistant to many cytotoxic therapies that target rapidly proliferating tumor cells.

In the context of melanoma, EMT is associated with aggressive, therapy-resistant tumor types. Thus, better understanding the role of EMT during embryogenesis and tumorigenesis offers potential clinical value. EMT factors are expressed almost exclusively during embryogenesis and are not expressed in normal adult tissues (Gupta et al. 2005b). This affords the possibility of diagnostic techniques focused on these factors as biomarkers for the presence of malignancy. For example, Snail2

levels are highest during early transformation of melanocytes (Gupta et al. 2005a; Shirley et al. 2012). This knowledge could provide a means to more accurately identify lesions at risk for malignant transformation for further observation or removal. Additionally, in embryogenesis, these factors work in the context of various signaling pathways that keep them in check. Further exploration of how these EMT factors are regulated in embryonic development could inform potential means of controlling them in melanoma. For example, it has been shown that treatment which upregulates MITF, the master regulator of melanocyte differentiation, can drive differentiation of melanocytes expressing EMT markers, increasing susceptibility to chemotherapy and decreasing migratory capacity (Caramel et al. 2013). In addition to melanoma, EMT is present in the progression of other tumor types, and any discovery that informs the prognosis or treatment of melanoma based on these factors has the potential to be applied more broadly to other malignancies.

13.4 WNT Signaling

Wnt signaling plays a role in many aspects of embryonic development (Sadler and Langman 2010). Wnt secreted glycoproteins canonically signal through a series of intracellular steps to stabilize β-catenin so that it can translocate to the nucleus, form a complex with TCF/LEF transcription factors, and activate expression of target genes (Moon 2005). Wnt signaling regulates the melanocyte lineage at various stages of development (Lewis et al. 2004). Wnt signaling is important in neural crest specification, working in conjunction with BMP and FGF signals to prime pluripotent ectodermal cells to receive appropriate signaling to induce neural crest formation (Garnett et al. 2012; Jin et al. 2001; Sato et al. 2005). After neural crest formation, Wnt signaling plays a role in the expression of other neural crest markers and EMT factors (Garnett et al. 2012). Importantly, Wnt cooperates with Zic and Pax transcription factors to promote the expression of Snail family proteins in the neural crest, allowing appropriate neural crest migration (Sato et al. 2005). Additionally, Wnt signaling is involved in activation of Sox10, which is required for development of some nonmesenchymal neural crest derivatives, including melanocytes (Lewis et al. 2004). After neural crest migration, Wnt signaling is involved in the final specification, through multiple mechanisms, of melanocytes from precursors. Importantly, activated β-catenin itself promotes expression of MITF (Dorsky et al. 2000; Raible and Ragland 2005). Additionally, Wnt-activated expression of Sox10 increases Sox10 binding to the MITF promoter and reinforces expression of MITF. Through these mechanisms, canonical Wnt signaling promotes both neural crest and melanocyte identity.

13.4.1 Canonical Wnt Signaling in Melanoma

Canonical Wnt signaling has been implicated in the proliferation, invasion, and metastatic potential of melanoma. Multiple studies have shown Wnt ligands, ones that specifically activate the canonical pathway, are commonly overexpressed in

both melanocytic nevi as well as in melanoma (Pham et al. 2003; You et al. 2004). Examination of primary human melanoma samples has shown increased nuclear localization of β-catenin (Rimm et al. 1999). Melanoma cell lines with high levels of Wnt activity also show increases in transcription of known Wnt targets, such as MITF (Widlund et al. 2002). Despite high levels of nuclear β-catenin, mutations in β-catenin, adenomatous polyposis coli (APC), and other Wnt pathway components are infrequent in melanoma, indicating alternative means of Wnt activation (Reifenberger et al. 2002; Rimm et al. 1999). Taken together, these results show that the Wnt pathway is indeed active at various stages of melanoma progression.

Canonical Wnt signaling has complex and context-dependent roles through various stages of melanoma development. Experiments examining the role of Wnt on proliferation have shown that increased Wnt signaling decreases the proliferation of both human and mouse melanoma cell lines (Chien et al. 2009). Furthermore, cells overexpressing Wnt showed a decrease in tumor size in a xenotransplantation model. Similarly, overexpression of Wntless Wnt Ligand Secretion Mediator (WLS), a protein that supports Wnt secretion, causes a decrease in the proliferation of cells in vitro, while knockdown of WLS causes an increase in proliferation (Yang et al. 2012). These results suggest that signaling by canonical Wnt ligands acts to suppress proliferation of melanoma cells. In support of this idea, studies have shown that increased Wnt activity, as measured by nuclear staining for β-catenin, is associated with improved patient survival (Chien et al. 2009). These studies provide evidence for Wnt signaling acting as an inhibitor of proliferation. However, other studies support a role for the Wnt/β-catenin pathway in promoting proliferation, suggesting that Wnt actions are dependent on biological context. Damsky et al. expressed a stabilized version of β-catenin in a $BRAF^{V600E}/PTEN^{KO}$ mouse model and showed that it increased the proliferative index and overall growth of melanomas (Damsky et al. 2011). Similarly, Delmas et al. showed expression of stabilized β-catenin, in the context of activated NRAS, was able to immortalize primary skin melanocytes and induce melanoma formation in mice (Delmas et al. 2007). These results suggest that the role of Wnt signaling is highly dependent on the context in which it is active and potentially on how the Wnt pathway itself is activated.

Similar to its effect on proliferation, regulation of invasion and metastasis by Wnt signaling is likely dependent on biological context. Experiments involving Wnt overexpressing cell lines show a decrease in metastasis in a mouse xenograft model (Chien et al. 2009). In addition, WLS knockdown cells show an increase in metastasis when applied to a mouse xenograft model, suggesting that loss of Wnt signaling increases metastasis (Yang et al. 2012). In other contexts, Wnt signaling adopts alternative roles. In $BRAF^{V600E}/PTEN^{KO}$ mice expressing stabilized β-catenin, increased metastasis was observed when compared with controls (Damsky et al. 2011). Likewise, Gallagher et al. showed that, when combined with an activated NRAS oncogene, stabilized β-catenin was able to induce more metastases in mice (Gallagher et al. 2013). As with proliferation, the effects of Wnt signaling on invasion and metastasis of melanoma depend on how Wnt activation is achieved and in which genetic background experiments are performed. These studies, as well as those on proliferation described above, suggest the mode of Wnt activation is

important in its effect on tumorigenesis. Specifically, a difference exists between stabilized β-catenin and Wnt ligand-mediated activation: Wnt ligand overexpression shows a generally tumor suppressive role, while ligand-independent activation and stabilization of β-catenin promotes tumor progression.

Differential effects of Wnt signaling are also manifest in melanoma therapy. Suggesting that Wnt signaling facilitates therapeutic targeting, a Wnt3a-dependent increase in apoptosis was observed when $BRAF^{V600E}$-positive cell lines were subjected to BRAF inhibition (Biechele et al. 2012). In a later study, patients were retrospectively evaluated for response to BRAF inhibitors based on β-catenin staining prior to initiating therapy (Chien et al. 2014). Here, increased β-catenin staining prior to treatment correlated with a poorer response to BRAF inhibitors, arguing that Wnt signaling dampened the response to BRAF inhibitors. These results again suggest that the complexity of canonical Wnt signaling extends to melanoma therapy.

13.4.2 Noncanonical Wnt Signaling

Noncanonical Wnt signaling has two main sub-pathways: the planar cell polarity (PCP) pathway and the Wnt/calcium pathway (Liu et al. 2014; Veeman et al. 2003). Both pathways signal independently of β-catenin. The PCP pathway, activated by a subset of Wnt ligands binding to Wnt receptors, involves the activation of downstream factors that help rearrange cytoskeletal elements. The calcium pathway involves Wnt ligand and Frizzled receptor-induced activation of phospholipase C, leading to differential regulation of calcium within the cell. By regulating cell migration, cell polarity, and other fundamental processes, noncanonical Wnt signaling plays a role in several phases of embryonic development (Prasad et al. 2015).

The role of noncanonical Wnt signaling in promoting melanoma was first hypothesized after a screen of melanoma cell lines revealed overexpression of Wnt5a, a Wnt ligand that predominantly activates noncanonical pathways (Bittner et al. 2000). Further examination of tumor samples showed Wnt5a expression increased with melanoma progression (Bittner et al. 2000), and its expression correlated with poor patient outcomes (Forno et al. 2008). Mechanistic studies in vitro showed that exogenous Wnt5a added to melanoma cells augmented changes in polarity and migration, suggesting a role in promoting invasion and metastasis (Dissanayake et al. 2007). Supporting this result, targeting endogenous Wnt5a with an inhibitor decreased migration of melanoma cells (Jenei et al. 2009). Additional in vitro studies showed overexpression of Wnt5a caused redistribution and decreased expression of adhesion molecules (Witze et al. 2008), whereas knockdown increased adhesion molecule expression, leading to a decrease in invasion (Jenei et al. 2009). Extending these studies in vivo, Dissanayake et al. showed administration of Wnt5a in a xenograft model promoted metastasis (Dissanayake et al. 2008). Interaction of noncanonical Wnt signaling has also been implicated in resistance mechanisms to therapy. Anastas et al. have shown that elevated Wnt5a expression is correlated with a decrease in patient response to BRAF inhibitors (Anastas et al. 2014). This

resistance is proposed to be mediated by an increase in AKT/PI3K signaling activation. In this study, knockdown of Wnt5a was able to rescue sensitivity to BRAF inhibition. Additionally, Webster et al. have shown Wnt5a induces a senescent, yet still invasive, phenotype in melanoma cells, allowing continued progression of tumors while avoiding conventional chemotherapy or targeted therapies (Webster et al. 2015). While Wnt5a has been shown to affect signaling independent of β-catenin, it has also been shown that there may be interactions between canonical and noncanonical Wnt signaling, especially in later-stage melanomas. In vitro experiments showed Wnt5a signaling disrupted interaction between β-catenin and N-cadherin, allowing a larger pool of available β-catenin to translocate to the nucleus and induce transcription (Grossmann et al. 2013). This interaction between canonical and noncanonical signaling pathway creates another layer of complexity in interpreting the effects of Wnt signaling.

13.5 MITF

Microphthalmia-associated transcription factor (MITF) is the master regulator of the melanocyte lineage. It activates the transcription of melanin biosynthesis genes such as the receptor MC1R, the melanosome transport protein RAB27A, the melanin biosynthetic rate limiting enzyme Tyrosinase, and other genes such as PMEL17 and MLANA (Vachtenheim and Borovanský 2010). MITF is regulated by a combination of PAX3, SOX10, and LEF1, the latter of which directly links Wnt signaling to MITF regulation (Liu et al. 2014; Vachtenheim and Borovanský 2010). Several studies in mice, zebrafish, and other systems have shown that MITF is required for melanocyte development and function (Dorsky et al. 2000; Opdecamp et al. 1997; Patton and Nairn 2010).

The role of MITF in regulating melanocyte and melanoma cell proliferation and differentiation has been extensively studied. Early studies showed a complex relationship between MITF signaling and melanoma progression. In 2005, Carreira et al. found that MITF, when expressed at low levels, acts as an anti-proliferative factor, inducing G1 cell cycle arrest via $p21^{Cip1}$ and $p16^{INK4a}$ (Carreira et al. 2005). In a follow-up study, Carreira found short-term depletion of MITF leads to $p27^{Kip1}$-dependent cell cycle arrest while simultaneously increasing the invasiveness of melanoma cells (Carreira et al. 2006). By contrast, Garraway et al. found copy number amplification of the *MITF* gene in melanomas and showed that its elevated expression promoted melanocyte transformation, suggesting MITF can act as an oncogene (Garraway et al. 2005). The results of these studies indicate that the effects of MITF in melanoma depend largely on its expression level and corresponding transcriptional activity.

To account for the differential effects of MITF, Goding and colleagues proposed the “rheostat” model to describe the observed changes in cellular phenotype based on low, moderate, or high levels of MITF signaling (Carreira et al. 2006; Hoek and Goding 2010). Under this model, cells with low levels of MITF are expected to exhibit a stem-like state where they proliferate slowly and are more invasive. Cells expressing moderate levels MITF are expected to proliferate by expressing

MITF-target genes such as *CDK2* and *BCL2*, regulating cell cycle progression and survival. Melanocytes expressing MITF at high levels are nonproliferative and noninvasive, expressing genes such as *MART1* and *Tyrosinase* that promote terminal differentiation. In sum, MITF is proposed to modulate cell activity through variable expression just as a rheostat alters resistance in a circuit.

In concordance with this new model, Cheli et al. showed that low MITF levels are associated with a greater degree of “stemness” and tumor initiation (Cheli et al. 2011). Ablation of low MITF cells from a heterogeneous tumor cell population greatly reduced tumor initiation. Additionally, the subpopulation of cells with high MITF levels were poor at initiating tumors. However, inhibition of MITF in this subpopulation upregulated the stem cell markers Oct4 and Nanog and increased tumorigenic potential. MITF inhibition led to p27 upregulation, consistent with a model in which slow growing, MITF-low cells are more stem cell-like and have the greatest potential to initiate tumors.

As an extension of the rheostat model, manipulation of MITF levels or activity has been proposed as a means of melanoma therapy. The goal of altering MITF is to change cellular phenotype, causing a switch from a stem-like phenotype to a more proliferative or differentiated phenotype. To accomplish such “phenotype-switching,” methotrexate was used to increase MITF expression in melanoma cells, driving them toward a more proliferative and differentiated state (Sáez-Ayala et al. 2013). This higher rate of proliferation increased the susceptibility of the cells to treatment with the antimetabolite 3-O-(3,4,5-trimethoxybenzoyl)-(−)-epicatechin (TMECG), an inhibitor of folate metabolism. This result highlights the potential utility of using MITF-specific treatment as an adjuvant to standard of care in highly invasive and aggressive melanomas. MITF expression has also been investigated as a prognostic factor in melanoma. In 2014, Peeper and colleagues showed that a low MITF/AXL ratio was predictive of resistance to BRAF inhibitors (Müller et al. 2014). These are recent examples of how understanding MITF function in embryonic melanocyte development can further the development of clinically relevant tools and effective therapeutics.

Conclusion

Embryonic development requires many temporally and spatially regulated steps to develop mature melanocytes. From early embryonic patterning to final fate specification, multiple signaling pathways and intrinsic factors are required to complete these steps, perfectly coordinated to correctly direct development. Because embryogenesis is a unique biological scenario, many of these pathways and factors that drive proliferation and migration are only active during development, and not active in adult tissues. However, because these properties are crucial in tumor initiation and progression, the embryonic programs are often reawakened during melanoma initiation and progression. In particular, the melanocyte development program provides nascent melanomas with a toolset to promote proliferation, invasion, and metastasis. Many of the embryonic properties co-opted by melanomas are broadly shared across many forms of cancer. This common thread that connects different malignancies suggests that discoveries relating melanocyte development to melanoma could inform our understanding of biological processes underlying other cancers.

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14 Tumor Microenvironment for Melanoma Cells

Lawrence W. Wu, Meenhard Herlyn, and Gao Zhang

14.1 Introduction

Melanoma is the most deadly cancer in the skin with a 5-year survival rate of advanced, metastatic disease at 15 %. Fortunately, recent years have witnessed breakthroughs in melanoma therapies such as targeted therapy, vemurafenib, to block the MAPK signaling pathway by specifically targeting mutated oncogenic BRAF (Bollag et al. 2010; Flaherty et al. 2010). Additionally, combining a BRAF inhibitor (Dabrafenib) with an MEK inhibitor (Trametinib) increased efficacy of treatment by overcoming certain resistance mechanisms with BRAF inhibition alone (Johnson et al. 2014; Shi et al. 2014). This increased efficacy is also seen in the combination of vemurafenib and cobimetinib, another MEK inhibitor (Larkin et al. 2014). Immune checkpoint blockade therapies targeting immune inhibitory molecules have yielded tremendous clinical responses as well. Targeting cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) using Ipilimumab (anti-CTLA-4) paved the way for other immune checkpoint blockade therapies, such as nivolumab and pembrolizumab (anti-PD1), which target programmed cell death protein 1 (PD-1) (Hodi et al. 2010). Nivolumab was found to have greater response and efficacy than Ipilimumab as a single agent therapy (Robert et al. 2015). Additionally, the combination of nivolumab and Ipilimumab is more effective than single agent ipilumumab (Postow et al. 2015). Lastly, talimogene laherparepvec (T-VEC),

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_14

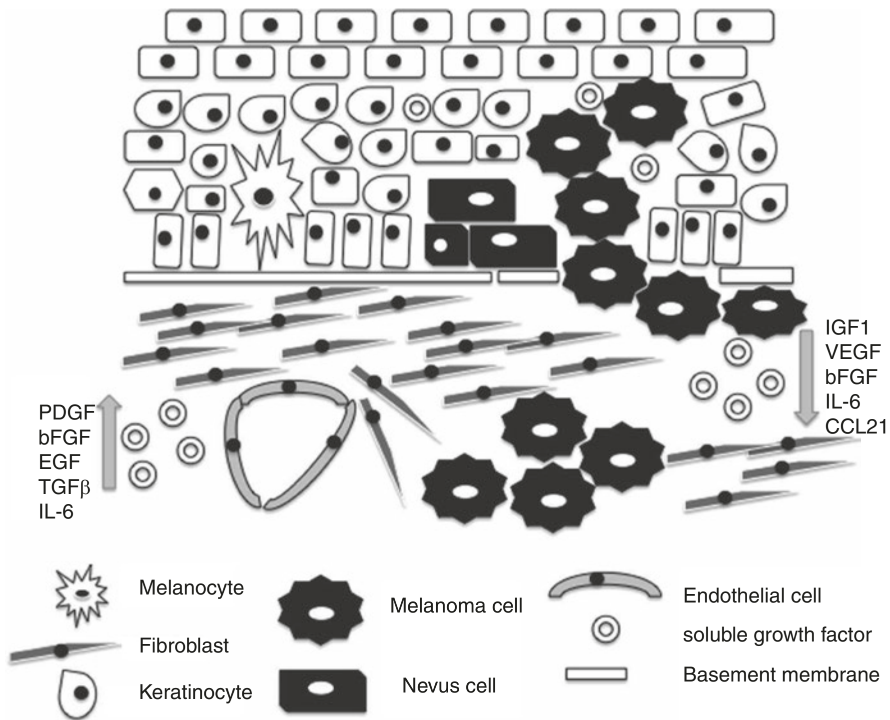

Fig. 14.1 Schematic representation of cross-talk between melanoma cells and tumor microenvironment. Normal melanocytes are localized at the basement membrane that separates epidermis from dermis and they are tightly regulated by surrounding keratinocytes through direct cell–cell contact. Upon transformation, the tight regulation mediated by E-cadherin is lost and replaced by N-cadherin between melanoma cells and adjacent cells. During melanoma development and progression, melanoma cells secrete soluble factors to recruit distant fibroblasts or immune cells in tumor microenvironment to its peritumoral zone. Stromal cells, including fibroblasts, endothelial cells, and immune cells, are activated and participate through paracrine signaling pathways to facilitate degradation of the basement membrane, invasion into the dermis, and metastatic dissemination. The dynamic communication between melanoma cells and the tumor microenvironment is mediated by direct cell–cell contact or soluble growth factors including bFGF, PDGF, VEGF, TGFβ, cytokines, and inflammatory factors

an oncolytic immunotherapy, was the first of its kind to demonstrate therapeutic benefit in a clinical trial (Andtbacka et al. 2015). The area of the immune microenvironment and mechanisms of immune-response to these checkpoint inhibitors is a very urgent topic among clinicians and scientists (see also Chaps. 16 and 17).

Melanoma cells activate, recruit, and continuously interact with other cells in the tumor microenvironment to facilitate their own growth, survival, migration, and invasion. As illustrated in Fig. 14.1, the tumor microenvironment of melanoma consists of neoplastic cells, normal cells, soluble growth factors, and extracellular matrix components that together cooperate to drive tumorigenesis. Perhaps the most striking evidence to support this notion came from our observation that metastatic

melanoma cells lose their tumorigenic properties if forced to attach to keratinocytes (Hsu et al. 2000). Similarly, melanoma cells can be reprogrammed by an embryonic microenvironment to a benign phenotype (Díez-Torre et al. 2009; Gerschenson et al. 1986; Topczewska et al. 2006), further underscoring the plasticity of malignant cells and the existence of inhibitory cues in the microenvironment.

14.2 Melanocyte, Melanoma, and the Microenvironment

Human melanocytes are specialized melanin-producing cells of neural crest cell origin. They are embedded at the conjunction of epidermis and dermis. All pigmented melanocytes are derived from embryonic neural crest cells that are highly migratory (White and Zon 2008). Multipotent neural crest stem cells (NCSC) can give rise to at least six differentiated cell lineages including melanocytes, glia, sensory neurons, adrenal cells, craniofacial cartilage and bone, and smooth muscle (Bronner-Fraser and Fraser 1988; Yu et al. 2006; Li et al. 2010; Cichorek et al. 2013). Several factors play a role in establishing multipotent neural crest stem cell, restricting neural crest stem cells toward a melanoblast fate, and driving differentiation of pigmented melanocytes (White and Zon 2008) (see also Chap. 13). Melanocytes make close contacts with keratinocytes, primarily through E-cadherin. On the basement membrane, the ratio between melanocytes and keratinocytes ranges from 1:5 to 1:8. Isolated melanocytes grow relatively rapidly in culture, and display bi- or tri-polar dendrites. By contrast, melanocytes rarely grow in the physiological setting of skin and display multiple (~35) dendrites to communicate with surrounding keratinocytes. It is the cell–cell contact through E-cadherins that closely regulates growth and proliferation of melanocytes and prevents early transformation of melanocytes to nevi (see also Chap. 2). With few exceptions, melanoma cells lose contact with keratinocytes and communicate with themselves and fibroblasts, which is due to the switch from E-cadherin to N-cadherin (Hsu et al. 2000; Li et al. 2001). The restoration of E-cadherin in melanoma cells of low levels of E-cadherin led to adhesion to keratinocytes and inhibited the invasion of melanoma cells into the dermis by downregulation of invasion-related molecules (Hsu et al. 2000). Oncogenic BRAFV600E can induce repression of E-cadherin to promote melanoma cell invasion through T-Box3 transcriptional repression (Boyd et al. 2013).

There are several mechanisms for malignant transformation. Hypoxia is one of the significant characteristics of the skin and tumor microenvironment and can accelerate transformation (Bedogni et al. 2005; Monsel et al. 2010). In particular, hypoxia contributes to heterogeneity in melanoma in a HIF1α-dependent manner (Widmer et al. 2013). These experiments have provided an important hint that the surrounding tissue contributes to transformation. The combination of bFGF and ultraviolet (UV) B could readily lead to pigmented lesions, some of which resemble low-grade melanoma (Berking et al. 2001). Interestingly, melanocytes display melanoma-like aggressive phenotypes when grown on melanoma cell-derived matrix (Seftor et al. 2005), suggesting that in addition to genetic approaches, the microenvironment can also reprogram normal melanocytes into melanoma-like

Table 14.1 Growth factors or inhibitors for melanocytes derived from human epidermal keratinocytes and dermal fibroblats

Melanocyte growth regulator	Keratinocytes	Fibroblasts
α-MSH	+++	NA
bFGF	+++	++
ET-1	+++	NA
ET-3	NA	+++
HGF	NA	+++
GM-CSF	+++	++
SCF	+++	+
DKK1	NA	+++

phenotypes. Conversely, malignant cells can be reprogrammed by human or chick embryonic stem cell matrix to a benign melanocyte-like phenotype (Kulesa et al. 2006; Postovit et al. 2006) (see also Chap. 13). Sustained stress in the microenvironment may lead to epigenetic reprogramming to a malignant transformation (Mologno ni et al. 2011). Together, these examples demonstrate how the surrounding microenvironment can play a major role in modulating of both normal and malignant melanocytes.

14.3 Growth Regulation of Human Melanocytes by Epidermal Keratinocytes and Dermal Fibroblasts

Human epidermal melanocytes isolated from fetal or adult skin do not propagate *in vitro* unless cultured in a defined growth medium, including phorbol ester and cholera toxin (Eisinger and Marko 1982) or growth factors that replace their activities. Conditioned medium derived from melanoma or astrocytoma cells (those selected for low TGF-ß production) support the growth of melanocytes in the absence of growth factors suggesting that malignant cells produce their own growth factors for autocrine stimulation (Table 14.1 and Eisinger et al. 1985).

Melanocytes are closely surrounded in skin by keratinocytes and survive and proliferate when co-cultured with keratinocytes or in the presence of keratinocyte-conditioned medium, suggesting that most melanocyte growth-stimulating factors are derived from keratinocytes (see also Chap. 2). Basic fibroblast growth factor (bFGF) is produced by keratinocytes to enhance melanocyte proliferation (Halaban et al. 1988). Not only does bFGF have an impact on melanocyte survival, growth, and proliferation, bFGF can also promote melanocyte migration via phosphorylation of focal adhesion kinase ($p125^{FAK}$) (Wu et al. 2006). bFGF can also be produced by dermal fibroblasts. α-MSH stimulates melanocyte growth by binding to its high affinity receptor and its activity requires bFGF and/or activation of protein kinase C (De Luca et al. 1993). Upon exposure of human keratinocytes to UVB, secreted interleukin 1-α and interleukin 1-β (IL1-α and IL1-β) from keratinocytes stimulate the secretion of endothelin (ET-1), which plays an important role in melanocyte proliferation (Imokawa et al. 1992; Jamal and Schneider 2002). Similarly, UVA/B--irradiated keratinocytes secrete granulocyte/macrophage colony-stimulating factor (GM-CSF), which also stimulates DNA synthesis and differentiation of melanocytes

in a dose-dependent manner (Hirobe et al. 2004; Imokawa et al. 1996). Interestingly, direct reprogramming of mouse and human fibroblasts into melanocytes can be achieved through expression of MITF, SOX10, and PAX3 (Yang et al. 2014).

There is no apparent direct contact between epidermal melanocytes and fibroblasts in the dermis of the skin. However, fibroblasts can produce growth factors that are important for melanocytes including bFGF and hepatocyte growth factor (HGF). IL1-α from UVB-irradiated keratinocytes can stimulate the production of HGF in fibroblasts (Mildner et al. 2007). Interestingly, HGF also plays a role in promoting melanocyte motility depending on CD44v6 expression (Damm et al. 2010), protecting melanocytes from apoptosis in an MITF-dependent manner (Beuret et al. 2007), and conferring resistance to RAF inhibitors (Straussman et al. 2012). Another paracrine factor, neuregulin-1, is derived from dermal fibroblasts. It can effectively increase pigmentation of melanocytes in monolayer and the reconstructed skin model (Choi et al. 2010) and also promotes melanocyte proliferation while inhibiting differentiation (Buac et al. 2009). On the other hand, dermal fibroblasts can have an inhibitory effect on density, proliferation, and differentiation of melanocytes via secreted DKK1 (Yamaguchi et al. 2007). DKK1 is highly expressed by fibroblasts and can suppress β-catenin and MITF in melanocytes, which are essential for melanocyte growth and proliferation. Interestingly, DKK1 expression can inhibit invasiveness of melanoma cells (Chen et al. 2012). Treatment of keratinocytes with DKK1 can increase their proliferation and decrease their uptake of melanin (Yamaguchi et al. 2008).

14.4 Inflammatory Mediators on Human Melanocyte Function

The epidermal and dermal microenvironment harbors inflammatory mediators, cytokines, hormones, and growth factors that can alter human melanocyte function in a profound way. Many of these factors are released into the microenvironment after UV exposure and subsequently affect melanocyte pigmentation, proliferation, differentiation, cytokine production, and motility.

Tumor necrosis factor-α (TNF-α) is among many cytokines responsible for an inflammatory response in skin. Upon UVB irradiation, keratinocytes release TNF-α, which is likely stimulated by IL-1α. IL-1α is the primary mediator that responds to inflammation and injury in skin. Secretion of IL-1α increases as keratinocytes and fibroblasts age (Okazaki et al. 2005). It has been reported that increased secretion of IL-1α can stimulate the production of HGF by dermal fibroblasts in a paracrine manner and the production of ET-1 by keratinocytes for autocrine stimulation, which together stimulate proliferation of melanocytes and induce their tyrosinase activity. Nuclear receptor retinoid X receptor-α (RXR-α) expressed in keratinocytes is one of the upstream regulators of these cytokines and can protect keratinocytes and melanocytes from UV-induced DNA damage and enhance proliferation via modulation of secretion of heparin-binding EGF-like growth factor, GM-CSF, IL-1α, and cyclooxygenase-2 and activation of mitogen-activated protein kinase pathways (Wang et al. 2011). The depletion of RXR-α in epidermal keratinocytes leads to alterations in expression of keratinocyte-derived secreted factors such as ET-1, SCF, HGF, FGF2, and α-MSH, which modulate proliferation and activation

of melanocytes after UV irradiation (Wang et al. 2011). Matrix metalloproteinases (MMP-2 and MMP-9) can also be co-up-regulated with inflammatory cytokines, serving as another important regulator in the epidermis (Decean et al. 2013).

Melanocytes express IL-8 mRNA when stimulated with TNF-α or IL-1α, and the supernatants from stimulated melanocyte cultures become positive for neutrophil and monocyte chemotactic activity, suggesting a possible role of melanocytes in participating in the initiation of an inflammatory response (Zachariae et al. 1991). The treatment of melanocytes with TNF-α, IL-1α, or IL-6 leads to the inhibition of tyrosinase activity and DNA synthesis (Swope et al. 1991), which suggests that they function as paracrine factors. Since melanocytes synthesize and transfer melanin to keratinocytes upon UV irradiation, it may implicate a negative feedback loop consisting of these factors to modulate melanocyte function. α-MSH can serve as a primary anti-inflammatory factor by opposing TNF-α-induced NF-kB activity in human melanocytes (Haycock et al. 1999). It is mainly produced by epidermal keratinocytes suggesting intricate interactions between keratinocytes, melanocytes, and inflammatory mediators.

14.5 Interplay Between Inflammation and Tumor Initiation, Promotion, and Progression

Ample evidence has pointed to a role of chronic inflammation in underlying tumor initiation and development (Grivennikov et al. 2010; Luo et al. 2004). IL-6/STAT3 and IKKβ-dependent NF-kB signaling pathways are key players linking inflammation and cancer by regulating an array of cytokines (He and Karin 2010; Yu et al. 2009). Inflammation contributes to the onset of malignancies by several potential mechanisms, including (1) stimulated cell proliferation, which can increase the likelihood of acquiring the transforming mutation, (2) production of metabolites such as reactive oxygen species (ROS) or endothelial nitric oxide synthase (eNOS), which can cause DNA damage, (3) suppression of cell-mediated immune response, which can create an environment that enhances tumor growth, and (4) inhibition of apoptosis (Kitasato et al. 2007; Rubin et al. 2004). An immunohistochemistry study demonstrated that chronic inflammation could contribute to malignant transformation in the human upper airways by the production of eNOS (Pacova et al. 2009).

UVB is regarded as an environmental carcinogen that is critical for melanoma development. UVB but not UVA can initiate melanoma using the HGF mouse genetic model (De Fabo et al. 2004). Exposure of keratinocytes to UV increases ROS production (Yoshihisa et al. 2010). In murine fibroblasts, UV irradiation enhanced ROS production via PKCdelta signaling (Bossi et al. 2008). Human epidermal keratinocytes have higher basal hydrogen peroxide (H_2O_2) levels than melanocytes and can transfer hydrogen peroxide to melanocytes (Pelle et al. 2005). UVB and UVC can differentially activate STAT3 in human keratinocytes and fibroblasts via ROS and DNA damage (Bito et al. 2010). Interestingly, IFN-γ promotes melanocytic cell survival and immune-evasion in UVB-induced melanocyte activation (Zaidi et al. 2011). Fibronectin-containing extracellular vesicles also protect against UVB radiation and associated cytotoxicity (Bin et al. 2016).

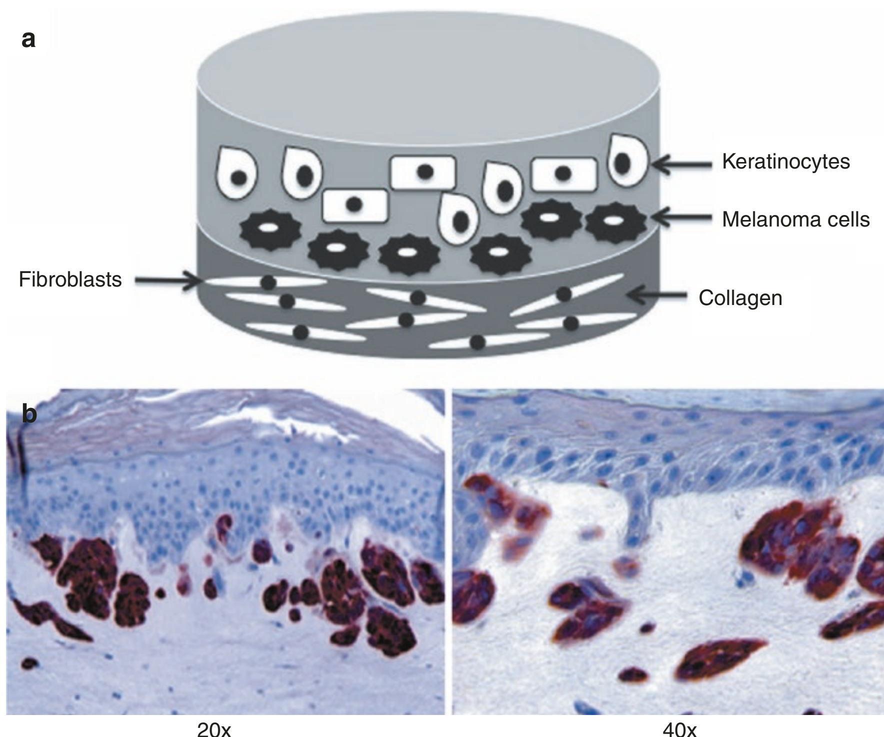

Fig. 14.2 Human skin reconstruct using the 3-D organotypic culture system. (**a**) A schematic representation of human skin reconstruct at which human melanocytes or melanoma cells are grown as 3-D with keratinocytes, fibroblasts, and collagen that mimic the microenvironment. (**b**) VGP human melanoma cells WM3248 are grown in 3-D skin reconstruct comprising of keratinocytes, fibroblasts, and collagen. Typical invasion into dermis of WM3248 cells is a characteristic of RGP melanoma line (Images are captured at 20× and 40× (Courtesy of Dr. Ling Li, the Wistar Institute))

Together, these results suggest that human epidermal keratinocytes and/or dermal fibroblasts represent major sources of ROS for epidermal melanocytes following UV irradiation and initiate inflammatory response via the STAT3 signaling pathway. The UVB radiation of primary cutaneous melanoma can promote metastatic progression (Bald et al. 2014). The release of high mobility group box 1 (HMGB1) from epidermal keratinocytes damaged by UVB and Toll-like receptor 4 (TLR4) recruit and activate neutrophils (Bald et al. 2014). This inflammatory response stimulates angiogenesis and melanoma migration toward endothelial cells. UVR targets TP53 to cooperate with oncogenic BRAFV600E to induce melanomagenesis (Viros et al. 2014). It has been noted that UVB, along with growth factor such as bFGF, can transform normal melanocytes (Berking et al. 2001, 2004). However, the underlying molecular basis of malignant transformation remains unclear. Given the evidence that ROS and DNA damage are implicated in UV irradiation-induced inflammatory response in skin keratinocytes and fibroblasts, an intriguing possibility is that UV-mediated inflammatory responses in the skin environment, including generation

of ROS and release of secreted cytokines, could further cause DNA damage in epidermal melanocytes, and contribute to genetic alterations (Fig. 14.2).

Conclusion

Transformation of normal melanocytes can be partially attributed to dysregulated tissue homeostasis executed by keratinocytes. Furthermore, transformed melanocytic cells send signals throughout the microenvironment to recruit other cells types. Those cells become activated, communicate with the neoplastic cells, and elicit soluble pro-survival and anti-apoptosis signals through autocrine or paracrine signaling patterns or direct cell–cell contacts in order to create a suitable tissue microenvironment that supports melanoma progression. Over the decades, experimental studies have identified key intrinsic signaling pathways that mediate transformation of melanocytes into melanoma cells. In the skin, melanocytes are mainly surrounded by epidermal keratinocytes and they also communicate with dermal fibroblasts. How the microenvironment of melanocytes plays a role in transformation remains to be clarified. We propose to use human melanocytes as a paradigm to study and understand cell–cell communication and growth regulation between melanoma and the microenvironment. An understudied area is the role of inflammation in melanoma development and progression. The immune microenvironment is another area where we plan to explore in melanoma. We are isolating skin fibroblasts (or peripheral blood mononuclear cells) from melanoma patients or healthy individuals who are predisposed or susceptible to melanoma, reprogram them into iPS cells and then differentiate them into melanocytes. These melanocytes are being used to model how the tumor microenvironment and UV radiation trigger oncogenic transformation. These results can provide the foundation for future studies in prevention and development of new targets for therapy.

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Melanoma Model Systems

15

Birgit Schittek, Miriam de Jel, and Thomas Tüting

15.1 Complex In Vitro Cell Culture Model Systems

15.1.1 Introduction

Although there is experimental evidence that human tumor cell lines grown in culture can be representative of the original tumor lesion (Masters 2000; Smalley et al. 2006b; Meier et al. 2000), it is obvious that tumor cells in vivo grow in an environment consisting of extracellular matrix components, stromal cells, inflammatory cells, and endothelial cells (Bissell and Radisky 2001). The contribution of the tumor microenvironment to tumor progression has already been recognized in 1889 by the "seed and soil" theory of Paget (Paget 1889) and later extended and confirmed by Meenhard Herlyns group (Li et al. 2003). This theory claims that not only genetic changes in the tumor cells determine an aggressive phenotype, but that microenvironmental factors have an impact on tumor cell behavior. Factors secreted by stromal cells as fibroblasts or direct cell–cell contacts between the tumor cells and the surrounding stromal cells may either inhibit malignant transformation or promote tumor progression (Li et al. 2003). Cells grown in monolayer can differ considerably in their morphology, cell–cell and cell–matrix interactions, and differentiation from those growing in more physiological three-dimensional (3-D) environments (Yamada and Cukierman 2007). Furthermore, 3-D culture gene expression profiles have been

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_15

shown to more accurately reflect clinical expression profiles than those observed in 2-D cultures. Finally, preclinical drug screening in 3-D culture models more reliably predict clinical efficacies, and monolayer culture of tumor cells has remained a poor predictor of a patient's response toward therapeutic agents (Johnson et al. 2001; Voskoglou-Nomikos et al. 2003; Burdett et al. 2010) (Table 15.1).

Animal models are not suitable for high throughput screening but often provide definitive tests of the importance of specific molecules or drug targets. However, one has to keep in mind that animal models may not adequately reproduce features of human tumors or therapeutic responses (Kung 2007; Teicher 2006; Burdett et al. 2010). On the other hand, most 3-D in vitro models lack the complex vascular systems that perfuse tissues in vivo. Since "Life isn't flat" (Smalley et al. 2006b), in vitro 3-D tissue models provide an approach to bridge the gap between traditional cell culture and animal models (Griffith and Swartz 2006; Rangarajan et al. 2004; Yamada and Cukierman 2007).

15.1.2 Culture of Melanoma Cells in Extracellular Matrix Scaffolds

The specific extracellular matrix microenvironment provided to cells can substantially influence experimental outcome. Both the composition and stiffness of the extracellular matrix surrounding the cells have major effects on signaling and behavior (Yamada and Cukierman 2007). Therefore, a widely used strategy is to propagate cells in tissue culture and then implant them in a 3-D extracellular matrix scaffold as either single cells or as tissue-like aggregates. Three-dimensional scaffolds have been generated from purified molecules such as collagen I, Matrigel, or from native extracellular matrices secreted and isolated from fibroblasts or keratinocytes.

Because of its ubiquitous nature and relative ease of isolation, collagen was one of the earliest biomaterials to be widely used for 3-D cell culture. Collagen gel embedding involved the encapsulation of small tumor explants (1–2 mm) or of dissociated tumor cells within a collagen matrix that allowed the explants to maintain their viability and cellular architecture ex vivo (Burdett et al. 2010). Cells suspended in extracellular matrix components are easily visualized by phase contrast or immunofluorescence microscopy. In addition, the biological response of cells suspended in this 3-D system can be compared to that of cells grown in monolayer. Viable cells may also be removed from the 3-D cell culture system for further experimentation, including biological analysis and flow cytometry. Collagen gels can mimic loose or dense connective tissue depending on the concentration of collagen; such gels have been used widely in studies of fibroblast and tumor cell migration and signaling (Grinnell 2003). Since each tissue in vivo has a characteristic matrix microenvironment, for a given study, it is crucial to select an appropriately matched 3-D in vitro matrix (Yamada and Cukierman 2007). Besides collagen, the basal membrane components fibronectin, laminin, and collagen IV as well as Matrigel (BD Bioscience, San Jose, CA) have been used for melanoma cell culture. Matrigel is a basement membrane extract derived from the Engelbreth–Holm–Swarm mouse sarcoma that contains a diverse array of components, including collagen type IV, laminin, and

Table 15.1 Complex in vitro cell culture model systems

Melanoma model system	Application	Advantages	Disadvantages
Culture in extracellular matrix scaffolds	Influence of extracellular matrix components on melanoma cell growth, invasion, and drug sensitivity	Easy to perform; different matrix components can be tested	Extracellular matrix components absorb drugs or growth factors; mostly higher drug concentrations have to be used
Microcarrier bead culture	High cell density culturing in matrix components possible	Several carrier materials can be used; ideal for large-scale culturing in matrix components	Expensive and more complicated
Transwell chambers	Analysis of chemotaxis, migration, and invasion	Easy to perform when precoated transwells are used, fast assay	Expensive, high variations possible if individual coating of the inserts is performed
Organotypic explant culture	Invasion, migration, and drug response	Culturing of melanoma cells in a physiological environment	Limited culture time; patient material necessary
De-epidermized dermis (DED)	Influence of extracellular matrix components and basal membrane on melanoma cell invasion	Native extracellular matrix and basal membrane present	Limited culture time; patient material necessary
2-D coculture systems	Influence of growth factors or cell–cell contact on melanoma cell growth, survival, invasion, gene expression	Easy to perform, secreted factors as well as cell contact can be analyzed separately	Patient material necessary for primary cells; sometimes separation of the cells necessary before analysis
Mono- or multi-cellular spheroids	Influence of melanoma cells grown in aggregates on drug sensitivity, secreted factors or cell–cell contact with melanoma cells itself or other cell types	Easy to perform, fast assay, high throughput screening possible, variable platforms and conditions possible	Suboptimal physiological conditions; limited informative value about melanoma invasiveness, not all melanoma cells can be used
Organotypic skin equivalent culture	Growth, survival, migration, and invasion of melanoma cells in a physiological environment	Most sophisticated melanoma model system, physiological environment, dermal and epidermal culture systems available	Laborious, expertise needed, not all melanoma cells can be used

other ECM molecules, as well as various soluble factors, such as cytokines and growth factors (Burdett et al. 2010). However, because Matrigel is a largely undefined and variable mixture of proteins, Matrigel culture has not been widely used for drug screening purposes.

More sophisticated models use other biomaterial scaffolds for culturing melanoma cells on other natural substrates (Fischbach et al. 2007; Zhang et al. 2010). Prototypical scaffolds were developed, in which freeze-dried and then cross-linked solutions of collagen and glycosaminoglycans create biodegradable, sponge-like structures (Griffith and Swartz 2006). Hyaluronic acid can also be used as a scaffold for bioengineered skin (Scuderi et al. 2008). Furthermore, native extracellular matrix components secreted by fibroblasts can be isolated by a protocol described by Beacham et al. (2007). The advantage of using native extracellular matrix components is that the natural composition and 3-D structure of the matrix molecules remain intact. Extracellular matrix components can serve as a reservoir for growth factors secreted by neighboring cells or melanoma cells and by this can influence morphology and growth of neighboring cells. Together with fibroblasts, immune cells, and blood vessels, the extracellular matrix builds the tumor stroma and influences tumor progression, invasion, and metastasis (Mueller and Fusenig 2004).

15.1.3 Microcarrier Bead Culture

Microcarrier beads are also widely used (Smit et al. 1995). A microcarrier is a support matrix allowing for the growth of adherent cells in bioreactors. Carrier materials for cells may be composed by gelatin, porous glass, collagen, or cellulose, with dimensions of 170–6,000 μm. In microcarrier culture, cells grow as monolayers on the surface of small spheres or as multilayers in the pores of macroporous structures that are usually suspended in culture medium by gentle stirring. By using microcarriers in simple suspension culture, fluidized or packed bed systems, yields of up to 200 million cells per milliliter are possible. Therefore, microcarriers provide convenient surfaces for growing animal cells or increasing the yield of cells from standard monolayer culture vessels and perfusion chambers. The high cell density confers more stability and improves the longevity of the culture, making macroporous microcarriers suitable for long-term culture. It has been described that normal human melanocytes or melanoma cells can be coated on microcarrier beads and polymerized within fibrin or fibrin–collagen hydrogels. A layer of dermal fibroblasts was added to more accurately simulate the microenvironment. Invasion can be monitored over a time period of 7 days. This model shows that the melanoma cell lines recapitulate their in situ growth patterns in this environment (Ghajar et al. 2007).

Microcarrier beads can also be rolled on a lymph node endothelial surface, which was created by growing endothelial cells on a differentiating extract of lymph node biomatrix, and testing the ability of tumor cells to invade across Matrigel-coated

filters. Interestingly, compared to the contact with plastic, Lewis lung carcinoma and B16 melanoma cell invasiveness were increased after exposure to "lung endothelial surface." This indicates that a lymph node environment may modulate the metastatic potential of tumor cells (Whalen et al. 1994).

15.1.4 Transwell Chambers

Transwell chambers are often used to study migration or invasion of tumor cells. This system is also called Boyden chamber assay, originally introduced by Boyden for the analysis of leukocyte chemotaxis (Boyden 1962). It is based on a cylindrical cell culture insert nested inside the well of a cell culture plate. It consists of chambers of two medium-filled compartments separated by a microporous membrane. The insert contains a polycarbonate membrane at the bottom with a defined pore size. Depending on the composition of the porous membrane, either migration or invasion of tumor cells can be studied. Usually, cells are seeded in the upper compartment in serum-free media, while serum or similar chemoattractants are placed in the well below. Migratory cells move through the pores toward the chemoattractant below. The number of cells that have migrated to the lower side of the membrane is determined after staining the membrane and quantified in a plate reader. Therefore, the Boyden chamber-based cell migration assay has also been called filter membrane migration assay, transwell migration assay, or chemotaxis assay. A number of different Boyden chamber devices are commercially available (Chen 2005). Such a system can be used to obtain an objective numerical readout to assess the effects of drugs or the modulation of target gene expression on cell migration. Furthermore, by the addition of a Matrigel layer on top of the membrane, this assay can be modified to measure invasion, such that cells have to invade through the matrix to reach the underside of the filter.

15.1.5 Organotypic Explant Culture: Ex Vivo Cultures

Organ explant slices can be cultured on a semiporous membrane or are embedded in a 3-D collagen gel (Pampaloni et al. 2007). Tumor tissue slices can be cultivated for up to 7 days and have been mainly used to determine drug responses and to predict tumor responses (Hickman et al. 2014; Gerlach et al. 2014). Organotypic slice cultures preserve the cytoarchitecture and cellular differentiation of the original tissue. Although this approach benefits, in that epithelial/endothelial cells are cultured in a relatively physiologically normal microenvironment, the culture period during which the organ remains viable is limited. A problem is also how to obtain the starting material, especially if it is of human origin (Hegerfeldt et al. 2002; Berry et al. 1975; Friedl et al. 2004). With this method one can also perform 3-D invasion assays, which support the invasion of tumor cell clusters from cancer explants.

15.1.6 De-epidermized Dermis (DED)

The skin model based on de-epidermized human dermis populated with keratinocytes and fibroblasts was originally developed for resurfacing burned patients (Ghosh et al. 1997; Chakrabarty et al. 1999; Sahota et al. 2003; Harrison et al. 2006). On de-epidermized dermis (DED) stromal cells, keratinocytes, or melanoma cells can be seeded and cultured for several days and the invasive capacity of melanoma cells in this environment can be analyzed (Dekker et al. 2000). Using this model one can evaluate the contribution of the extracellular matrix, the basal membrane or normal skin cells on melanoma cell invasion. The advantage of this system is that the de-epidermized dermis retains a native extracellular matrix and basal membrane and the contribution of the basal membrane in the invasive properties of melanoma cells can be analyzed easily. This in vitro system showed reliable invasion of highly invasive cells and was used to investigate the proteolytic mechanisms involved in melanoma cell invasion into dermal connective tissue (Dennhofer et al. 2003). By culturing melanoma cells either on the dermal side of the DED or on the basal membrane side, one can study the influence of the basement membrane on the invasive behavior of melanoma cells (Van Kilsdonk et al. 2010). It is also possible to study the role of matrix metalloproteases in this model on melanoma cell invasion (Dennhofer et al. 2003). The basement membrane components can also be removed by treatment of the DED by incubation with dispase (Harrison et al. 2006). Using this model, it has been shown that the dermoepidermal basement membrane can prevent invasion of metastatic melanoma cell lines in the absence of a stratified epidermis (Van Kilsdonk et al. 2010).

15.1.7 2-D Coculture Systems

The 2-D coculture system is ideally suited to analyze the effect of defined factors or genes especially on melanocyte transformation in an environment in which at least a quasi in vivo like cellular communication can take place. It is known that melanocytes cultured in vitro display different phenotypic characteristics than melanocytes in vivo. This suggests a role of microenvironmental signals in controlling the melanocytic phenotype. Indeed, it was shown that upon coculture with undifferentiated keratinocytes, melanocytes regain their normal phenotype resembling those in situ, indicating that keratinocytes regulate cell growth, dendricity, and antigen expression of melanocytes in vitro (Hsu et al. 2002; Shih et al. 1994). Following malignant transformation, the dominance of keratinocytes over the melanocytic phenotype is lost and melanoma cells become more and more autonomous (Valyi-Nagy et al. 1993; Shih et al. 1994; Hsu et al. 2000, 2002). To analyze independently the effects of soluble factors, the coculture can be performed in Transwell systems where melanocytes and keratinocytes are seeded in two compartments of a tissue culture well, which is separated by a semiporous membrane. By this, it was shown that E-cadherin-mediated cell adhesion is required for keratinocyte-mediated control of melanocytic cells (Li et al. 2004).

The cultivation of melanoma cells with normal dermal fibroblasts indicates that melanoma cells can direct gene expression in fibroblasts (Gallagher et al. 2005; Loffek et al. 2005; Ntayi et al. 2003). Interestingly, highly aggressive melanoma cells cannot only direct the activation and functional differentiation of stromal fibroblasts and endothelial cells but also can transdifferentiate by masquerading as endothelial cells to take over all or part of stromal functions, a phenomenon termed as vasculogenic mimicry (Maniotis et al. 1999; Hendrix et al. 2003, 2007).

15.1.8 Mono- or Multicellular Spheroids

Spheroids, or tumor cell aggregates, have been used since the 1970s, mostly for investigations into the mechanisms of action of radiotherapy and chemotherapeutic drugs as well as drug resistance (Hirschhaeuser et al. 2010; Mueller-Klieser 1997; Smalley et al. 2006b). The principle is that melanoma cells are grown under non-adherent conditions, which permits the formation of 3-D aggregations or spheroids. Once formed, the spheroids are implanted into a matrix of collagen I, which mimics the microenvironment of human skin.

Cellular spheroids take advantage of many cell types to aggregate. The cellular aggregates can range in size from 20 to 1 μm in diameter, depending on the cell type and growth conditions. However, fewer than 100 human tumor cell lines have been shown to have the capacity to grow in spheroid cultures (Friedrich et al. 2007, 2009). Melanoma cells are more suitable for spheroid formation since it was shown that 18 of the 26 analyzed melanoma cell lines can form spheroids (Smalley et al. 2006a, b). Spheroids more closely resemble the in vivo situation than monolayer conditions due to the architecture and the extensive cell–cell contacts provided by the spheroids. Spheroids exhibit many of the biological properties of solid tumors, including cell morphology, growth kinetics, gene expression, and drug response (Friedrich et al. 2007, 2009; Hirschhaeuser et al. 2010; Kunz-Schughart et al. 2004; Mueller-Klieser 1997). Human tumor spheroids are widely used for drug screening since cancer cells grown in these spheroids show greater resistance toward several anticancer drugs compared to cells grown in monolayer (Smalley et al. 2006b). This may be explained in part by increased cell–cell contact, 3-D cellular architecture, enhanced deposition of tumor-derived ECM within the spheroid, a lower overall cell proliferation rate, or a combination of these factors (Bates et al. 2000; Hamilton 1998; Burdett et al. 2010). Interestingly, similar to the situation in a tumor, a diffusion gradient exists within spheroids for oxygen and nutrients limiting the availability of these compounds to the innermost cells (Lin and Chang 2008).

In spheroids, melanoma cells form concentric, spherical structures that contain large proliferating cells in the periphery and smaller quiescent cells on the interior of the sphere (Fig. 15.1a, b). These spheroids can be implanted into a collagen-based matrix where they exhibit an invasive phenotype that is indicative of the progression stage of melanoma (Smalley et al. 2006b). This system can be further

explored by interspersing fibroblasts into the collagen before imbedding into a spheroid. These fibroblasts infiltrate the spheroid and produce ECM proteins (Smalley et al. 2005) (Fig. 15.1a, b). Spheroids can be established from a single cell type or can be multicellular mixtures of tumor, stromal, and immune cells (Hirschhaeuser et al. 2010). Several cell types such as fibroblasts and immune cells have been described, which can be successfully mixed with tumor spheroids under

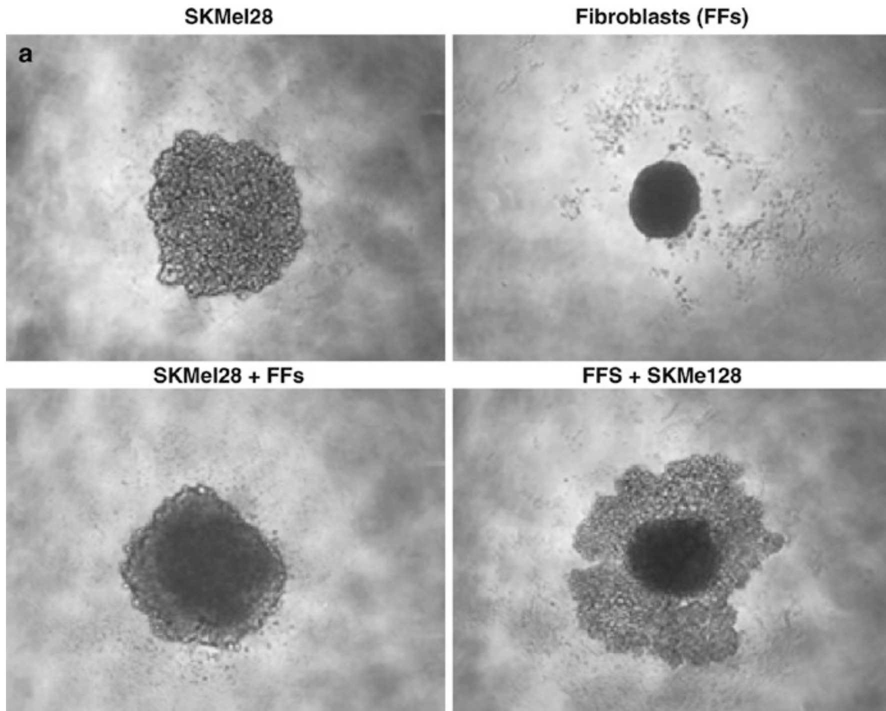

Fig. 15.1 Spheroid and organotypic skin equivalent models. (**a**) Phase contrast pictures of spheroids of SKMel28 melanoma cells or primary dermal fibroblasts (FFs) 48 h after aggregation. *Lower left* picture: Spheroids of SKMel28 melanoma cells were formed for 24 h and afterward cocultivated for 24 h with dissociated fibroblasts for further 24 h. *Lower right* picture: Spheroids of primary fibroblasts were formed for 24 h and afterward cocultivated for 24 h with SKMel28 melanoma cells for further 24 h. (**b**) Melanoma spheroids either untreated (control) or treated with a beta-catenin inhibitor (Sinnberg et al. 2010). Spheroids were stained with the vitality stains calcein-AM and ethidium bromide to visualize live and dead cells, respectively. As a measure of cell death induction ethidium bromide fluorescence indicates dead cells. (**c**) Organotypic epidermal skin equivalents (SE). Shown are hematoxylin/eosin stainings of organotypic epidermal skin reconstructs with either integrated primary melanocytes (marked with an *arrow*), a radial growth phase (RGP), vertical growth phase (VGP), or metastatic melanoma cell line. We have observed that skin reconstructs consisting of human fibroblasts and keratinocytes simulate human skin in vivo and that human melanocytes or melanoma cells from different stages of melanoma development and progression recapitulate in skin reconstructs the biological behavior in vivo. In the skin reconstructs, only an irregular basement membrane is formed as seen by the collagen IV staining (pictures taken with permission from (Meier et al. 2000)

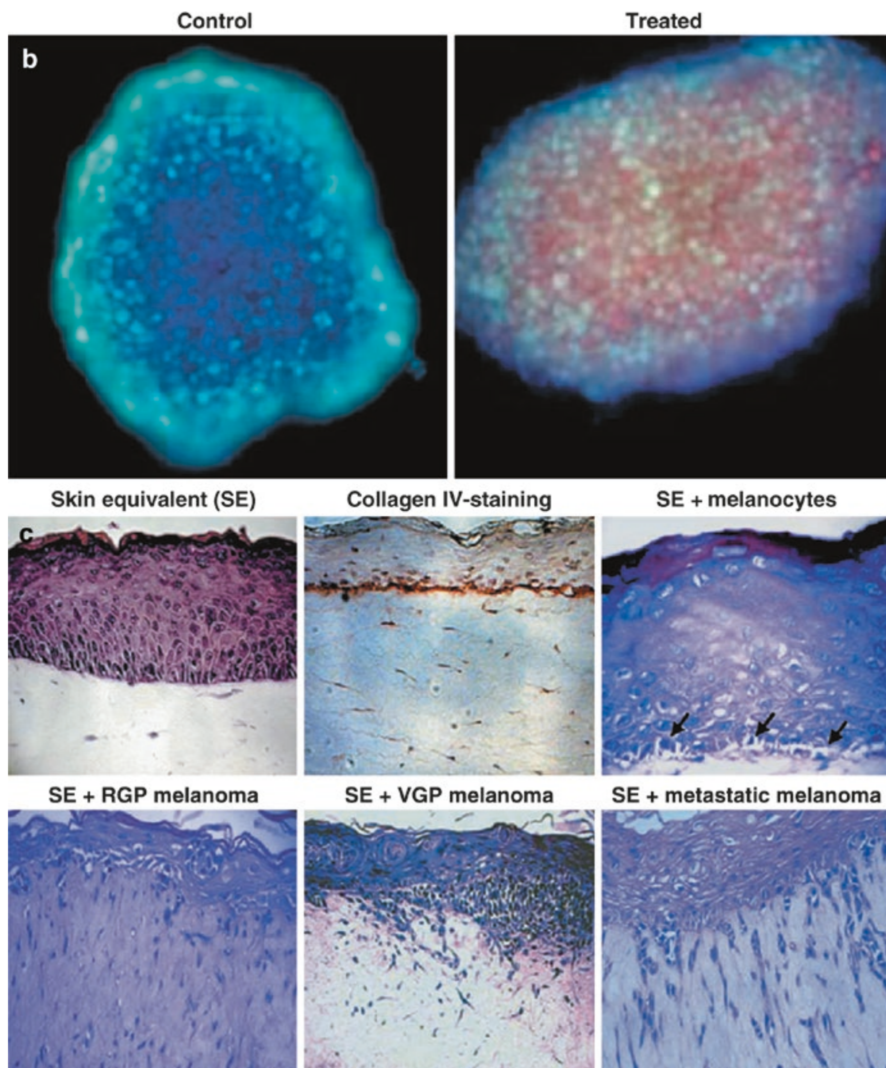

Fig. 15.1 (continued)

coculture conditions (Smalley et al. 2006b). Using this model system it was also shown that zebrafish embryo extracts promote sphere-forming ability of human melanoma cell lines (Na et al. 2009). Preformed multicellular tumor spheroids can be incubated with immune cells that migrate into the spheroid. As a result, tumor-associated migration and differentiation processes as well as cytotoxic and cytostatic activity of migrated immune cell populations can be examined (Konur et al. 1996; Gottfried et al. 2006; Pampaloni et al. 2007).

Spheroid formation can be induced by a variety of different techniques. In the spinner flask culture, fluid turbulence prevents attachment and promotes cellular

aggregation (Sutherland et al. 1971). In the rotary wall vessel, reactor cells are placed between rotating cylindrical walls to mimic microgravity (Lin and Chang 2008; Friedrich et al. 2007). A simpler method, which does not need specialized equipment or additional processing, is the liquid overlay method. This involves the liquid overlay of a cell suspension over a non-adherent surface, such as agar-coated plates (Yuhas et al. 1977; Burdett et al. 2010). The absence of fluid flow using this method results in a more pronounced diffusional gradient within the spheroids. Additionally, the spheroids formed through liquid overlay are more varied in size and number. To optimize this and to get more consistent spheroid size and composition, recent adaptations involve spheroid formation within hanging drops (Kelm et al. 2003; Timmins and Nielsen 2007) and microfluidic chips (Wu et al. 2008; Burdett et al. 2010). However, these methods need again specialized equipment and additional handling steps.

By contrast, the collagen-implanted spheroid model is easier and faster to perform and is suited to high throughput drug screening studies. This assay takes account of both cell–cell contact between adjacent tumor cells and the need for 3-D supporting matrix. It is based on the liquid overlay method in which a uniform cell suspension is plated on top of hard (1.5 %) agar, which prevents the tumor cells from adhering to the underlying tissue culture plastic. After 48–72 h, the tumor cells form small aggregates, or spheroids. These spheroids can be used directly for drug toxicity studies. The protocol developed by Smalley et al. (2006a) differs in that the spheroids are harvested and mixed with a suspension of bovine collagen type I. These are then plated on a 24-well plate, which is already layered with collagen to prevent the spheroids from settling onto the underlying plastic. These collagen-implanted spheroids can be used for the analysis of the invasive capability of melanoma cells. It was shown that the extent of collagen invasion correlated with the tumor stage and the cells from the early stages were poorly invasive, whereas the cells from the metastasis colonized the entire collagen gel (Smalley et al. 2006a, b). The spheroids are scored for cell survival by removing the cell culture media, washing in PBS, and then staining using calcein-AM and ethidium bromide (Fig. 15.1b).

15.1.9 Organotypic Skin Equivalent Culture Model

The organotypic skin equivalent culture model is the most advanced and complex model currently available. The Fusenig lab was the first to use the organotypic skin model to study invasion of squamous cell carcinoma cells (Borchers et al. 1997). In simplest term, an organotypic culture of skin can be engineered using its four major components, collagen, dermal fibroblasts, melanocytes, and epidermal keratinocytes (Parenteau et al. 1992). This model is ideally suited to study the effect of inhibitors or gene expression or UV on toxic effects or on invasive capability of tumor cells or melanocytes. These organotypic cultures have been extended to different tumor types, including breast, prostate, and ovarian cancer (Chioni and Grose 2008).

The experimental protocols for creating human skin reconstructs have been described elsewhere (Meier et al. 2000, 2003; Berking et al. 2001). In brief, for the creation of an epidermal skin reconstruct, a mixture of collagen and human skin fibroblasts are seeded out into 6- or 24-well plates. After the fibroblasts have constricted the collagen, a mixture of keratinocytes and melanocytes are overlaid on top of the stromal layer. After the culture is established, the tray is lifted and the top exposed to the air, which induces keratinocyte differentiation. At maturity, the human skin reconstruct has near identical histology to normal human skin and a basal membrane like structure is built (Meier et al. 2000, 2003) (Fig. 15.1c). For the generation of a dermal skin reconstruct, one processes only until the fibroblasts have been seeded on the collagen layer (Sinnberg et al. 2010). When melanoma cells from the different defined tumor stages are introduced into the epidermal reconstruct, they exhibit progression-specific behavior. Cells derived from the radial growth phase melanoma stay within the epidermal keratinocyte layer and do not breach the basement membrane (Meier et al. 2000, 2003). Cells from the vertical growth phase of melanoma invade through the basement membrane into the dermal fibroblast layer, and cells from metastatic lesions invade rapidly throughout the dermis (Fig. 15.1c). It is possible to include melanocytes or melanoma cells in which gene expression was modulated, and it was shown that this can have dramatic effects on aggressiveness or invasive capability (Meier et al. 2003; Berking et al. 2001). The human reconstruct is a very useful model for modeling not only the growth of melanoma cells in a 3-D microenvironment but also the interaction of melanoma cells with the surrounding keratinocytes and fibroblasts. This approach can also be applied to other tissues as human breast, colon, and esophagus (Smalley et al. 2006b). Thus, the composite culture model enables functional studies of individual genes and interactions between specific gene products in various skin cell types in a biologically relevant milieu (Hsu et al. 2002; Meier et al. 2000; Sinnberg et al. 2010).

Skin reconstructs have a maximal life span of approximately 1 month in vitro, which can be extended to several months once grafted to living hosts (Javaherian et al. 1998; Satyamoorthy et al. 1999; Berking et al. 2001; Hsu et al. 2002). In these models, melanocytes regain their physiological localization at the level of the basal layer in the epidermis (Fig. 15.1c). They preserve all their functionality since in response to UV rays they proliferate, synthesize, and secrete melanin (Auxenfans et al. 2009). Further advancements are the endothelialized skin equivalents in which human endothelial cells from umbilical vessels are seeded together with fibroblasts, which organize themselves into tubular structures with a well-defined lumen resulting in an endothelialized skin equivalent (Hudon et al. 2003; Auxenfans et al. 2009). Seeding of keratinocytes on this endothelialized dermis results in the formation of capillary structures (Velazquez et al. 2002; Smalley et al. 2006b). Finally, the immunocompetent skin equivalent model is available. The reconstruct is a novel 3-D culture system in which the migration of leukocytes toward tumor cells and the factors that influence leukocyte migration can be studied under in vivo-like conditions (Berencsi et al. 2007; Zhang et al. 2006).

15.2 Experimental Animal Models

15.2.1 Introduction

In melanoma research, a great variety of experimental animal models is available. Each model has distinct advantages for the investigation of particular scientific questions regarding melanoma formation, genetics, and therapy as reviewed recently (van der Weyden et al. 2016). Melanomas naturally occur in horses, dogs, minipigs, and fish. Already at the end of the 1920s melanoma development was documented in interspecies hybrids of the fish genus *Xiphophorus* (Gordon 1927; Häussler 1928; Kosswig 1928). Since then the genetics of melanoma susceptibility was intensively studied in this model with successful identification of relevant oncogenes like *Xmrk*. Zebrafish and medaka are other well-studied melanoma fish models which have helped to unravel the underlying mechanisms of spontaneous as well as carcinogen and ultraviolet (UV) induced melanoma formation. Mouse models are also widely used in melanoma research, as mice exhibit short breeding cycles with a relatively large number of offspring, are easily manipulated genetically, and can be treated with UV and chemical carcinogens. A major benefit of modeling melanoma in mice is its ability to investigate the role of different genetic abnormalities observed in patients on disease progression due to the high degree of homology with the human genome. Mice have been generated that develop melanoma in the skin which metastasize early and are highly resistant to chemo- and radiotherapy, a fundamental characteristic of malignant melanoma. Experimental models in the laboratory mouse have been of critical importance to understand the biologic mechanisms how melanoma cells migrate in and out of blood and lymph vessels, evade immune defense, and colonize distant organs. In addition to the study of gene function on an organismal level, mouse models have also been widely used to evaluate the efficacy of novel treatment strategies in controlling metastatic disease progression. Experimental mouse models for cancer research can principally be subdivided into three different categories:

- Transplantation of human tumor cells into immunodeficient mice
- Transplantation of mouse tumor cells into syngeneic immunocompetent mice
- Primary autochthonous (“genetically engineered”) mouse models of cancer

We will briefly sketch the historical development of each category and provide a few selected examples how technical advances have enabled new insights in different areas of melanoma research over the years. We do not intend to provide a comprehensive catalog of all possible model systems but hope to give the reader a broad and stimulating overview (Table 15.2).

Table 15.2 Experimental mouse models

Melanoma model systems	Application	Advantages	Disadvantages
Transplantation of human melanoma cells into immunodeficient mice	Analysis of gene functions in human melanoma cells for invasive and metastatic tumor growth in vivo; preclinical evaluation of new drug candidates	Fast, reliable assay that portrays important aspects of human melanoma biology	Excludes the role of immune cells in the tumor microenvironment, does not portray the early steps of malignant transformation
Transplantation of mouse melanoma cells into syngeneic immunocompetent mice	Analysis of gene functions in mouse melanoma cells for invasive and metastatic tumor growth in vivo; preclinical evaluation of drug efficacy	Fast, reliable assay that includes the role of immune cells in the tumor microenvironment	Does not portray the early steps of malignant transformation, may not adequately portray all aspects of human melanoma biology
Primary autochthonous (genetically engineered) mouse melanoma models	Analysis of gene functions in the pathogenesis of melanoma on an organismal level; preclinical confirmation of drug efficacy	Allows for the investigation of early steps in malignant transformation in a fully immunocompetent tumor microenvironment	Time consuming and costly experimental setting, may not adequately portray all aspects of human melanoma biology

15.2.2 Transplantation of Human Melanoma Cells into Immunodeficient Mice

The successful engraftment of human tumors onto immunodeficient mice was first reported in 1969 using the athymic nude mouse (Rygaard and Povlsen 1969). This approach was widely used in subsequent years to study the morphological and biochemical characteristics of different tumor cells in vivo and the response of such xenografts to antineoplastic agents (Giovanella et al. 1973; Seaman et al. 1975). Several groups investigated the ability of human melanoma cells to grow metastatically in nude mice either following intravenous injection ("experimental pulmonary metastasis assay" or "lung colonization assay") or following subcutaneous injection ("spontaneous metastasis assay"). In each case, the development of lung metastases

was assessed over time. It was noted that melanomas, like most malignant neoplasms, were composed of heterogeneous tumor cell populations with different capacities for invasion and metastasis. Intrinsic features of a given tumor line appeared to be major determinants in regulating metastatic spread (Kozlowski et al. 1984). These features could be selected for by serial in vivo passaging. In addition, it was noted that growth of melanoma metastases was limited by residual immune reactivity of nude mice against xenogeneic human tumor cells, which was mediated particularly by NK cells.

Over the years, a number of melanoma cell lines were established (e.g., A375, MeWo, SKMel28, 1205Lu, and many others), which could be transplanted in nude mice as well as in mice with severe combined immunodeficiency (SCID), which were described a few years later. With the emerging ability to genetically modify tumor cells in vitro, these model systems have been successfully used to functionally assess the role of individual gene products on invasive growth and metastatic spread of melanoma cells in vivo until today. As an example, Clark et al. serially transplanted A375-M cells in nude mice and used cDNA microarray analyses to identify RhoC as a protein associated with melanoma metastases. Retroviral overexpression confirmed that RhoC indeed promotes the ability of melanoma cells to metastasize in mice in vivo (Clark et al. 2000). The ability to stably knock down the expression of a target gene using RNA interference has provided an additional valuable tool to study gene function not only on the cellular but also on the organismal level in mice. This technology was used to show that the CYLD protein regulates the ability of a human melanoma cell line to grow invasively and metastasize in vivo (Massoumi et al. 2009).

Several technical advances have expanded the range of experimental possibilities for the study of human tumor cells in immunodeficient mice. To more adequately model the tumor microenvironment, the group of Meenhard Herlyn transplanted human skin onto SCID mice and injected melanoma cells into the xenografts. This "orthotopic" tumor transplantation model circumvented the significant anatomic and physiologic differences between mouse and human skin and recapitulated the close interaction between melanocytes and keratinocytes (Juhasz Albelda et al. 1993). Crossing SCID mice onto the nonobese diabetic (NOD) genetic background improved tumor engraftment rates because NOD/SCID mice show reduced macrophage and NK function, as well as an absence of complement-dependent hemolytic activity. These mice were used by the group of Weinberg to study the in vivo growth properties of human melanocytes, which were transformed by defined genetic modifications in vitro (Gupta et al. 2005). Using this experimental approach, they provided evidence that the developmental origins of melanocytes in the neural crest might be relevant to their metastatic propensity. By combining both approaches it has today become possible to transplant complex skin reconstructs generated in vitro onto immunodeficient mice to study their behavior in vivo as mentioned in Sect. 15.1 above.

Another technical improvement for studying human tumor cells in immunodeficient mice has been developed by hematologists investigating the development and function of hematopoietic stem cells in immunodeficient mice. To further facilitate the permanent engraftment of bone marrow precursors, they tested

various mice with additional genetic changes affecting the immune system. It was found that NOD/SCID mice, which lack the IL2 receptor common γ chain (NOD/SCID/γc mice), show extremely high engraftment rates using human hematopoietic cells (Ito et al. 2002). The reason for the high engraftment rates was attributed to multiple immunological functional defects that affect dendritic cells in addition to the absence of T, B, and NK cells. Using these highly immunocompromised NOD/SCID/γc mice, Carreno et al. demonstrated that the residual immunity in NOD as well as NOD/SCID mice affected the metastatic growth of A375 melanoma cells (Carreno et al. 2009). They used tumor cells stably expressing a luciferase gene and monitored tumor growth in the lungs in real time by measuring in vivo bioluminescence with a highly sensitive CCD camera. In addition, they demonstrated human melanoma recognition by residual NOD/SCID NK cells, which correlates with MICA/B expression and could be blocked by anti-mouse NKG2D antibodies.

The importance of using different immunodeficient mouse strains became prominently evident when analyzing the ability of different subpopulations of malignant cells derived from primary melanomas to generate tumor xenografts. The group of Markus Frank showed that only very few human melanoma cells can form tumors when transplanted into NOD/SCID mice, and that melanoma cells expressing the chemoresistance mediator ABCB5 were highly enriched for such "melanoma-initiating cells." Importantly, they demonstrated that this minor tumorigenic cell population could be therapeutically targeted and destroyed in vivo with a specific monoclonal antibody (Schatton et al. 2008). Sean Morrison's group showed that a slight modification of the xenotransplantation assay conditions and the use of more highly immunocompromised NOD/SCID/γc mice greatly increased the detection of tumorigenic melanoma cells (Quintana et al. 2008). The mechanisms underlying melanoma cell heterogeneity (which had already been observed in the early days of melanoma xenografting) can now be studied using genetically modified transplantable melanoma cell lines in NOD/SCID/γc mice. The group of Meenhard Herlyn reported that a dynamically regulated JARID1B-expressing subpopulation of melanoma cells is essential for continuous tumor growth (Roesch et al. 2010). These results suggest a new understanding of melanoma heterogeneity with tumor maintenance as a dynamic process mediated by spatially and temporarily distinct subpopulations. Highly sophisticated imaging techniques are currently being employed to monitor in vivo in real time the proliferation and migration of transplanted melanoma cells expressing various fluorescent and bioluminescent reporter genes placed under the control of constitutive or dynamically regulated promoters.

Recent trials aim at facilitating a personalized cancer therapy generating individualized tumor models by means of melanoma patient-derived xenografts (PDX). These PDXs serve as preclinical models to test drug response, unravel resistance mechanisms, and offer the possibility for new drug and biomarker discovery. Especially therapeutic strategies for patients with mutations of the BRAF and NRAS oncogene and accompanying resistance to targeted therapies by inhibitors like vemurafenib are frequently investigated (Das Thakur et al. 2013;

Guerreschi et al. 2013). In addition, as some melanomas are not easily accessible or difficult to biopsy an alternative method was established by using circulating tumor cell-derived xenografts (CDX), which is suitable for studying late-stage melanoma (Girotti et al. 2016). The high similarity between PDXs and the corresponding biopsies of the patient with regard to mutation and expression patterns as well as histopathologic and immunohistochemical properties was proven in many studies. Moreover, PDXs show similar resistance mechanisms as the donor tumor (Kemper et al. 2015) allowing for detailed analysis of the underlying processes. However, one has to keep in mind that PDXs may not display the whole genetic heterogeneity of the patient's tumor (Kemper et al. 2015) and therefore provide only a partial prediction of the patient's disease progression and treatment response. Although the clinical feasibility and translatability of PDXs could be demonstrated (Einarsdottir et al. 2014; Gao et al. 2015), their use is technically challenging with some cases of engraftment failure and a quite time-consuming and costly procedure.

Taken together, the transplantation of human melanoma cells into immunodeficient mice can be generally considered as a rather rapid, reliable, and reproducible experimental approach to functionally study the role of genes in the process of malignant progression to metastatic disease. Importantly, this experimental system most closely reflects the intrinsic biology of human melanomas and partly recapitulates the tumor microenvironment in vivo. Furthermore, it is of critical importance for preclinical testing of novel therapeutics to develop personalized treatment strategies and understand drug resistance. However, work with human tumors in immunodeficient mice has two major inherent drawbacks. Firstly, it excludes the interaction of tumor cells with the immune system, which is of critical importance in shaping the tumor microenvironment and the process of tumor progression. Secondly, transplanted tumors grow rapidly and do not portray the early events during tumor initiation.

15.2.3 Transplantation of Mouse Melanoma Cells into Syngeneic Immunocompetent Mice

To experimentally include the role of the immune system in tumor progression requires the use of immunocompetent animals. However, in mice, melanoma develops only very rarely. One of these rare events occurred at the base of the ear in a C57BL/6 mouse at the Jackson Laboratories in 1954. This tumor was serially transplanted and subsequently established in vitro as the B16 melanoma cell line by Isaiah Fidler in the early 1970s. B16 melanoma cells readily form solid tumors when injected s.c. or i.v. into syngeneic C57BL/6 mice and were widely used from the early 1970s on as a model for metastases research to investigate the steps involved in tumor dissemination. Following the concept of "seed and soil," originally proposed by Stephen Paget in the late nineteenth century (Paget 1889), Fidler and colleagues showed that both host factors and properties of the tumor cells contribute to the success or failure of the metastatic process. Already in 1977, it

was reported that different B16 melanoma cell subclones derived in vitro varied greatly in their ability to form lung metastases upon intravenous inoculation into syngeneic mice. This observation suggested that B16 melanoma cells were heterogeneous and that highly metastatic tumor cell variants preexisted in the parental population (Fidler and Kripke 1977). The origins of tumor cell heterogeneity were also analyzed using K1735 melanoma cells, which were established from a UV-irradiated C3H mouse (Fidler and Hart 1982). These transplantable mouse melanoma cell lines are still used today to understand the cellular and molecular mechanisms underlying the process of metastatic progression utilizing modern tools of molecular genetics to overexpress or silence individual genes and of in vivo imaging to follow fluorescent and bioluminescent reporter genes as described in the previous section.

The most important application of tumor transplantation models in immunocompetent mice, however, was to understand the role of innate and adaptive immune responses in tumor progression. Because B16 melanoma cells are poorly immunogenic and do not efficiently stimulate antigen-specific tumor immunity, they were widely used as a model to study tumor vaccine adjuvants, including various microbial extracts. Molecular insights into the mechanisms how immune responses against tumor cells develop was gained from studies with B16 melanoma cells genetically modified to secrete immunostimulatory cytokines. Unexpected at the time, the expression of GM-CSF effectively promoted the induction of tumor immunity (Dranoff et al. 1993). It later became clear that this cytokine acted as a potent growth factor for antigen-presenting dendritic cells, which are critically required to initiate adaptive immunity. The experimental strategy to overexpress (or knock down) immune-related genes in B16 melanoma cells continues to reveal important insights into the interaction between tumor and immune cells until today. This is best illustrated by a recent report showing that the expression of the chemokine CCL21 by B16 melanoma cells induces a lymphoid-like reticular stromal network and recruits regulatory leukocyte populations to promote an immunotolerant microenvironment in mice (Shields et al. 2010).

The transplantable B16 melanoma model has also been used to study antigen-specific vaccination strategies, including the use of synthetic peptides as well as recombinant proteins and genes. Initially, investigators targeted model foreign antigens such as chicken ovalbumin, which were stably transfected into B16 cells (Mayordomo et al. 1995). In our own work, we found that efficient induction of cellular immune responses against clinically relevant melanocytic self-antigens such as TRP2 or gp100, which are naturally expressed by B16 melanoma cells, required strong activation of dendritic antigen-presenting cells either by culture from bone marrow precursors in vitro or by recombinant viral vaccines in vivo (Tüting et al. 1999; Steitz et al. 2000). Importantly, by combining antigen-specific vaccination with genetic modification of B16 melanoma cells, we could show that the expression of type I IFNs promoted T cell effector functions in the tumor microenvironment (Steitz et al. 2001). Type I IFNs could also be efficiently stimulated in tumor tissue by adjuvant peritumoral injections of immunostimulatory nucleic acids such as polyI:C and CpG-rich DNA, which imitate viral RNA and DNA and activate

the innate immune system through the Toll-like receptors (TLR) 3 and 9 (Tormo et al. 2006a; Bald et al. 2014a).

The B16 model is also very suitable to study biological processes and therapeutic agents, which simultaneously affect both tumor and host cells, particularly immune cells. In addition to using genetically modified tumor cells, researchers have also employed genetically engineered hosts and injected specific monoclonal antibodies to dissect the underlying molecular and cellular mechanisms. Our own work with therapeutic oligonucleotides exemplifies this strategy. We explored the use of small immunostimulatory siRNA molecules, which simultaneously can silence a target gene in tumor cells, in our case the antiapoptotic gene Bcl2, and activate retinoic acid-inducible gene-I (RIG-I), a cytosolic sensor of viral RNA, in immune cells (Poeck et al. 2008). Silencing Bcl2 promoted tumor cell apoptosis and activation of RIG-I stimulated cytotoxic immune cells to destroy tumor cells. Surprisingly, we found that activation of RIG-I also contributed to apoptosis induction directly in tumor cells. Experiments in mice, genetically lacking the interferon alpha receptor 1 chain, demonstrated that this response was critically dependent on a functional type I IFN system. The injection of cytotoxic antibodies against NK cells showed that this cell type largely mediated the antitumor response.

The interaction between melanoma cells and T cells in the tumor microenvironment in vivo can be studied in greater detail using adoptive lymphocyte transfer approaches. This experimental strategy has been facilitated by the development of T cell receptor transgenic mice, which carry large numbers of genetically marked T cells that specifically recognize melanocytic antigens (Overwijk et al. 2003; Xie et al. 2010). The adoptive transfer and in vivo activation of T cells in mice bearing macroscopically detectable B16 melanomas enables investigations of the mechanisms that determine the balance between tumor regression and progressive disease, including the various possibilities of tumor immune escape. To more closely imitate the clinical situation, mice have been genetically engineered to express the human HLA-A2 molecule and T cells have been genetically engineered to express human T cell receptors (Frankel et al. 2010). These highly sophisticated experimental models have been of considerable help to translate this therapeutic approach into clinical reality for melanoma patients.

In summary, the transplantation of mouse melanoma cells into syngeneic immunocompetent mice also represents a rapid, reliable, and reproducible experimental approach to functionally study the role of genes in the process of malignant progression to metastatic disease. This experimental system may not fully reflect the biology of human melanomas, but it much more adequately portrays the role of the immune system in shaping the tumor microenvironment in vivo. It has been of critical importance for preclinical development and testing of novel therapeutics, including melanoma vaccines with dendritic cells and immunomodulatory agents such as the anti-CTLA4 mAb, which is currently evaluated in clinical phase III studies. However, transplanted mouse melanomas also progress very rapidly following tumor inoculation and do not recapitulate the gradual series of cellular changes from premalignant to malignant pathologies.

15.2.4 Primary Autochthonous (Genetically Engineered) Mouse Melanoma Models

Advances in molecular genetics and stem cell biology have made it possible to study the functional role of oncogenes and tumor suppressor genes in the pathogenesis of melanoma on an organismal level. Work over the last decade has shown that genetic alterations observed in human melanomas also promote the malignant transformation of melanocytes in mice. This most likely reflects the fact that the genetic control of melanocyte proliferation, migration, and differentiation in embryonic development is highly conserved during the evolution of vertebrates. Genetically engineered mouse tumor models initially involved the transgenic expression of viral or cellular oncogenes under the control of tissue-specific promoters. The transgenic expression of certain growth factors or growth factor receptors also promoted tumor development. Subsequently, it became possible to delete tumor suppressor genes by gene targeting. More recently, the conditional deletion of tumor suppressor genes or activation of oncogenes has been achieved via Cre-lox technology. Because genetically engineered mouse models for melanoma have been extensively reviewed (Chin et al. 2006; Larue and Beerman 2008; Zaidi et al. 2008), we will only briefly sketch their historical development and highlight some of the more important recent advances in the field.

The transformation of melanocytes in mice was first achieved by Beatrice Mintz's group who placed the oncogenic SV40 large T antigen under the control of a tyrosinase promoter construct specifically in melanocytes. Founder lines expressing high transgene levels developed eye melanomas very early while lines expressing low transgene levels developed eye melanomas much later. Grafting experiments of skin derived from mice with high melanoma susceptibility onto mice with low melanoma susceptibility revealed proliferating pigment cells close to areas of greatest wound healing, which subsequently evolved into invasively growing melanomas (Mintz and Silvers 1993). These observations strongly suggested that pro-inflammatory growth factors and cytokines known to be produced in wound repair can promote growth and malignant conversion of genetically susceptible melanocytes in vivo.

Lynda Chin and colleagues generated mice that expressed the activated Hras^{G12V} oncogene under a tyrosinase promoter in mice, which carried a deletion of the p16^{INK4a}/p19^{ARF} tumor suppressor gene locus (Chin et al. 1997). These mice spontaneously developed cutaneous melanomas after a short latency and with high penetrance. Her results indicated that activation of Ras and loss of p16^{INK4a}/p19^{ARF} can cooperate to accelerate the development of melanoma and provided the first in vivo experimental evidence for a role of p16^{INK4a}/p19^{ARF} deficiency in the pathogenesis of melanoma. In our own work, we could demonstrate the critical role of the p16^{Ink4a}–Cdk4 interaction in suppressing the transformation of melanocytes following oncogene activation (Tormo et al. 2006b). The transgenic overexpression of the hepatocyte growth factor (Hgf), which drives Ras signaling via its receptor tyrosine kinase c-Met, cooperates with a mutated oncogenic cyclin-dependant kinase 4 (Cdk4^{R24C}) knocked into the germline, which abrogates p16 binding. Hgf-Cdk4^{R24C}

mice spontaneously develop multiple small (benign) nevi and subsequently single progressively growing (malignant) melanomas, indicating that impairment of the p16^{Ink4a}-Cdk4 axis affects the complete spectrum of stepwise malignant transformation of melanocytes (Landsberg et al. 2010).

With the recent development of techniques allowing for tamoxifen-inducible conditional activation of oncogenes specifically in melanocytes, it became possible to show that the mutated oncogenic Braf^{V600E}, which is frequently found in human melanomas, also strongly drives melanocyte proliferation in the skin of mice leading to the development of multiple melanocytic nevi (Dhomen et al. 2009; Dankort et al. 2009). The simultaneous deletion of the tumor suppressor genes PTEN or p16^{INK4a} led to a decreased latency and increased penetrance of melanoma. These novel models will undoubtedly help to dissect the molecular mechanisms regulating melanocyte proliferation, oncogene-induced senescence and tumor progression in the microenvironment of nevi and melanoma in vivo. The introduction of fluorescent or bioluminescent marker genes that are specifically expressed by melanocytes will enable the in vivo imaging of early proliferative events during melanocyte transformation, particularly following neonatal UV irradiation which has been shown to significantly promote melanomagenesis in several models, including Hgf-transgenic mice (Noonan et al. 2001).

Another transgenic mouse model showing spontaneous melanoma development was generated by the insertion of a *Dct* promoter-Grm1 construct leading to an aberrant expression of the metabotropic glutamate receptor 1 (Grm1) in melanocytes (Pollock et al. 2003). With complete penetrance Tg(*Grm1*) mice develop pigmented lesions at hairless skin regions, including ear, tail, and anus within 4–6 months. Furthermore, not only local metastasis but also metastatic spread in distant organs like lung, liver, lymph node, and spleen occurs (Schiffner et al. 2012). Interestingly, the majority of these disseminated cells display phenotypic changes during metastasis in form of de-pigmentation as is also found frequently in human melanoma. During the last years, a variety of human melanoma cell lines and tissue samples were screened with respect to their Grm1 expression showing overall high levels in contrast to normal melanocytes or benign nevi emphasizing the importance of ectopic Grm1 expression in melanoma development. Indeed, the malignant transformation of murine melanocytes could be induced by solely stable Grm1 expression (Shin et al. 2008). The activation of the Grm1 receptor results in a constitutive activation of the MAPK and PI3K/AKT signaling pathway. Both can be suppressed by treatment with the glutamate release inhibitor riluzole with the effect of a reduced colony formation, migration and invasion ability of human melanoma cell lines (Le et al. 2010). Hence, GRM1 seems to be a promising therapeutic target for future clinical trials. In addition, GRM1 expression was detected recently in human uveal melanoma samples and in accordance Tg(*Grm1*) mice were shown to develop melanocytic hyperproliferation within the uveal tract resulting in some cases of choroideal melanoma (Schiffner et al. 2014). Therefore, Tg(*Grm1*) mice open up the new opportunity to analyze spontaneous uveal melanoma development and metastasis in a murine model system.

Genetically engineered mouse melanoma models also provide novel opportunities to experimentally investigate the regulation of immune cell functions in the tumor microenvironment and understand the role of “cancer immunosurveillance” and “cancer-associated inflammation” in the pathogenesis of primary and metastatic melanoma. This is nicely illustrated by a recent report that primary melanomas developing in mice expressing the Ret oncogene as a transgene disseminate early but remain dormant for varying periods of time (Eyles et al. 2010). The control of tumor growth is mediated at least in part by cytotoxic T cells, since antibody-mediated depletion of these cells resulted in faster outgrowth of visceral metastases. These findings suggest that immune responses can be essential for prolonging the survival of early stage melanomas and that therapeutic strategies designed to reinforce such immune responses may produce marked survival benefits.

However, the immune system can be a double-edged sword because an inflammatory response can also promote tumor growth as has been first observed by Beatrice Mintz almost 20 years ago. Pro-inflammatory mediators capable of activating NF-kB signaling may not only support tumor cell survival, regenerative proliferation, and migration but can also enhance neoangiogenesis. An important role for NF-kB signaling in melanomagenesis has indeed been obtained by the group of Ann Richmond (Yang et al. 2010a). They generated mice, which allowed the inducible genetic ablation of Ikkβ (a kinase which phosphorylates IkB leading to NF-kB nuclear translocation and activation), specifically in melanocytes expressing the oncogenic $HRas^{G12V}$ mutation on the $p16^{INK4a}/p19^{ARF}$-deficient background. The deletion of Ikkβ significantly inhibited the development of melanomas in these mice in vivo and promoted p53-dependent apoptosis and cell cycle arrest in cultured melanocytes in vitro. These results support a role for tumor cell-intrinsic activation of NF-kB signaling in the pathogenesis of melanoma in agreement with reports in genetically engineered mouse models for other tumor types (Grivennikov et al. 2010). NF-kB-driven inflammatory responses may also participate in the development of melanoma following burning doses of UV irradiation. This interesting question can now be experimentally addressed using UV-sensitive mouse models.

The impact of tumor-associated inflammatory responses on T cell-mediated immunosurveillance has been addressed by Anne-Marie Schmitt-Verhulst’s group in mice where activation of oncogenic $HRas^{G12V}$ and deletion of $p16^{INK4a}/p19^{ARF}$ can be induced simultaneously by tamoxifen treatment (Soudja et al. 2010). These “TiRP” mice develop two different types of melanoma: slowly growing, pigmented melanomas and more rapidly growing, nonpigmented melanomas that are infiltrated by $Gr1^+/CD11b^+$ inflammatory immune cells. TiRP mouse melanomas also express the T cell-defined tumor antigen P1A in melanocytes, which allows investigations with adoptively transferred T cells specifically recognizing the P1A antigen on melanoma cells. Using this experimental system, it could be shown that an initially protective adaptive immune response is subsequently suppressed by chronic tumor-associated inflammation associated with infiltrating bone marrow-derived immature myeloid cells and a systemic Th2/Th17-oriented cytokine production profile.

In our own work, we found that primary $Hgf\text{-}Cdk4^{R24C}$ melanomas can effectively escape recognition and destruction by cytotoxic effector T cells. To

experimentally introduce T cell-mediated immunosurveillance, we adoptively transferred melanoma-specific TCR-transgenic cytotoxic T lymphocytes in the context of a combination treatment protocol consisting of host preconditioning, viral vaccination, and adjuvant peritumoral injections with immunostimulatory nucleic acids, which trigger innate immune responses in the tumor microenvironment (Kohlmeyer et al. 2009). This frequently resulted in complete and long-term regression of primary cutaneous melanomas. However, primary tumors eventually recurred indicating that some melanoma cells survived. Importantly, we observed large areas of poorly melanotic, proliferating melanoma cells in recurring melanomas suggesting that cytotoxic inflammation caused a phenotypic switch in subpopulations of tumor cells reminiscent of inflammatory melanomas in the TiRP mice (Landsberg et al. 2010). This phenomenon might represent a conversion of tumor cells toward a mesenchymal, invasive phenotype, which has been shown to result in the suppression of adaptive immune responses (Kudo-Saito et al. 2009). Similar phenomena were observed in response to adoptive T cell therapy, which allowed tumor cells to escape immune destruction through reversible inflammation-induced dedifferentiation (Landsberg et al. 2012). Moreover, an inflammatory environment following UV irradiation of the skin promoted melanoma cell invasion and metastasis (Bald et al. 2014b).

Taken together, genetically engineered mouse melanoma models imitate the multistep pathogenesis of melanoma in man where primary tumor cells establish in a unique microenvironment, and naturally progress toward metastatic disease. These models are ideally suited to adequately address many of the key issues in melanoma biology, including the regulation of proliferation, differentiation, and senescence of melanocytes following oncogene activation; the origin of tumor cell heterogeneity and the mechanisms of phenotype switching; the relationship of melanoma cells with neural crest-derived precursor cells; the initiation of innate and adaptive immunity leading to immunosurveillance and inflammation; the role of pro-inflammatory mediators for tumor cell survival, proliferation, and migration as well as neoangiogenesis; and the dynamics of tumor cell migration, dormancy, and metastatic colonization.

15.2.5 Experimental Mouse Models: Conclusions

A number of observations in the clinic suggest a close relationship between the activation of oncogenic signaling pathways driving the malignant transformation of melanocytes and the activation of immune responses in the tumor microenvironment. Future work in different experimental mouse models will help to dissect the role of innate immune signaling pathways in tumor and immune cells, which determine the balance between tumor regression and tumor progression. Even more importantly, work in these model systems will inform us how targeted inhibition of signaling pathways will affect the survival of tumor cells in vivo in the tumor

microenvironment, including the response of immune cells. While genetically engineered mouse models adequately recapitulate many aspects of the early genetic events of melanocyte transformation, including the important role of the immune system in melanomagenesis, it must be kept in mind that they only partly reflect the features of human melanoma. Some of the more obvious shortcomings such as the low number of melanocytes in the interfollicular melanocytes may be overcome experimentally. Nevertheless, considerable differences will always remain in the molecular hardwiring of mouse tumor and immune cells that will have to be appreciated in each experimental setting. Furthermore, work with these model systems requires a long-term commitment since they are extremely time consuming, labor intensive, and costly. This may be alleviated in part by the generation of transplantable cell lines from primary melanomas with defined genetic alterations.

Conclusion

In this chapter, we have presented many of the commonly used complex in vitro cell culture model systems and the in vivo experimental mouse models that are employed by research groups today to study the various aspects of melanoma biology and to evaluate novel therapeutic strategies. The relative importance of each experimental approach depends largely on two important aspects: Firstly, how well does the model recapitulate the process it attempts to emulate? Secondly, how well does the model itself offer the flexibility to expand our knowledge relating to the pathologic process being evaluated? Today, researchers are more and more combining the strengths of new in vitro cell culture models, new "humanized" mouse strains for the transplantation of human melanomas, and new "genetically engineered" mouse models for primary melanoma to comprehensively analyze their particular aspect of melanoma biology.

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16 New Approaches to Signaling

Ryan J. Sullivan and Keith T. Flaherty

16.1 New Therapeutic Approaches in Signaling

16.1.1 Introduction

The emergence of selective BRAF inhibitors for the treatment of metastatic melanoma changed the therapeutic landscape of this disease. For decades, the melanoma field lagged others in achieving significant alteration in the natural history of metastatic disease with conventional cytotoxic chemotherapy. However, with the emergence of immune checkpoint inhibitor therapy, in particular anti-programmed death 1 (PD1) and anti-PD ligand 1 (PD-L1), monoclonal antibodies alone or in combination with the anti-cytotoxic T-lymphocyte antigen 4 (CTLA4), and the development of combined BRAF/MEK inhibitor therapy, the treatment landscape has dramatically changed. Still, the majority of patients will still die from this disease, and optimizing treatment of metastatic melanoma remains a critical need. It is hoped that the discovery of somatic genetic alterations in signal transduction pathways that regulate proliferation, cell cycle, differentiation, and survival will provide further opportunities for expanding the reach of targeted therapy (Table 16.1).

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_16

Table 16.1 Oncogenes and tumor suppressor genes in melanoma and therapeutic strategies

Oncogene	Pathway	Drug targeting strategy
BRAF	MAP kinase	Direct, ATP competitive kinase inhibitors Downstream, allosteric MEK inhibitors
NRAS	RAS	Combinations targeting RAS effecter pathways
C-KIT	RAS	Direct, ATP competitive kinase inhibitors
AKT3	PI3 kinase	Direct, allosteric AKT inhibitors
Cyclin D	P16/Rb	Indirect, ATP competitive CDK4 inhibitors
CDK4	P16/Rb	Direct, ATP competitive kinase inhibitors
GNAQ/GNA11	G-protein coupled receptors	Indirect, PKC, MAP kinase, PI3 kinase pathway inhibitors

Tumor suppressor gene	Pathway	Drug targeting strategy
NF1	RAS	Combinations targeting RAS effecter pathways
PTEN	PI3 kinase	PI3K, AKT, mTOR inhibitors
P16	P16/Rb	Indirect, ATP competitive CDK4 inhibitors
BAP1	Polycomb repressive complex 1	Indirect, histone deacetylase inhibitors

16.2 Oncogenes Amenable to Direct Targeting

16.2.1 TCGA and the MAP Kinase Pathway

The Cancer Genome Atlas (TCGA) in melanoma recently reported its first comprehensive profiling using whole exome sequencing (WES), RNA sequencing (RNA Seq), and proteomics from 331 patients with cutaneous melanoma (Cancer Genome Atlas 2015). The most important conclusions from this work were that four subsets of melanoma patients were defined based on the driving mutation (BRAF, NRAS, NF1, and triple wild-type) and that the great majority of tumors activate the mitogen-activated protein kinase (MAPK) pathway through oncogene (BRAF, NRAS, MEK1/2) or tumor suppressor gene mutation (NF1) mutations and/or at the gene expression level. These findings confirm that the most important targets of small molecule inhibitor therapy in melanoma are the mediators of the MAPK pathway. However, a deeper look at the data suggests that other key regulators of cancer biology are also potential targets, including the phosphoinositol-3-kinase (PI3K) pathway, the cell cycle regulation mediators cyclin D (CCND1) and cyclin-dependent kinase 4 (CDK4), regulators of apoptosis such as mouse double minute 2 homolog (MDM2) and B-cell lymphoma 2 (BCL2) family members, and even epigenetic regulators.

16.2.2 The Development of BRAF Inhibitors in Melanoma

The identification of activating mutations in the searing threonine kinase, BRAF, in 50–60 % of melanomas in 2002 was the watershed event that opened the door for investigations of molecularly targeted therapy in melanoma (Davies et al. 2002). BRAF is a constituent of the long-studied MAP kinase pathway, a known mediator of growth factor signals in cancer (also refer to Chap. 7 (Fisher)). However, prior to the identification of BRAF mutations, only RAS mutations were known to constrictively activate the pathway in a subset of cancers, including melanoma (Albino et al. 1984). Therefore, when BRAF mutations were screened for in a large series of diverse tumor types, and found, it was immediately apparent that these mutations might underlie the biologic importance of this pathway in 7–8 % of all cancers (Davies et al. 2002). The fundamental challenges that confronted the field were (1) understanding the potential vulnerability of BRAF inhibition despite the presence of numerous additional genetic alterations in the same tumors and (2) the development of potent and selective inhibitors.

In the immediate years following the identification of BRAF mutations, sorafenib was the only targeted therapy in clinical development known to have some capacity for inhibiting RAF kinases including BRAF (Wilhelm et al. 2006). Laboratory evidence suggested that sorafenib was able to have a cytotoxic effect on melanoma cell lines; however, this effect did not seem to depend on the presence or absence of BRAF mutations (Whittaker et al. 2010). This lack of cellular selectivity suggested the possibility that BRAF might be the important point of intervention even in tumors that lacked a RAF mutation. However, it was also known that non-pharmacologic methods for limiting the production of BRAF protein only impacted the growth and survival of melanoma cell lines with a BRAF mutation (Hingorani et al. 2003; Wellbrock et al. 2004). Clinical trials were rapidly designed and executed even before significant preclinical testing had occurred. Early in clinical testing, it was revealed that sorafenib was not associated with tumor regression when administered as a single-agent and only modestly inhibited the MAP kinase pathway in tumors analyzed during the first few weeks of therapy (Flaherty et al. 2005). Still, two large Phase III trials were opened evaluating the combination of chemotherapy (carboplatin and paclitaxel) with or without sorafenib, both in the front-line and second-line setting. These trials both showed no benefit of adding sorafenib to chemotherapy and successfully ended the era of non-specific inhibitors of RAF in patients with metastatic melanoma (Hauschild et al. 2009; Flaherty et al. 2013). This did, however, leave open the possibility that more potent and selective BRAF inhibitors might yield superior molecular effects as well as evidence of clinical efficacy.

Several BRAF inhibitors subsequently entered clinical trials that were developed and optimized in preclinical tumor models that harbor BRAF mutations. The most successful of these were agents are highly selective for BRAF. In fact, three selective BRAF inhibitors (vemurafenib, dabrafenib, and encorafenib) have emerged from

Phase I clinical trials and demonstrated clear antitumor activity (Flaherty et al. 2010; Kefford et al. 2010; Dummer et al. 2013). Further, both vemurafenib and dabrafenib have been shown, in randomized Phase III trials, to be superior to chemotherapy in patients with advanced (unresectable Stage III or Stage IV) melanoma harboring $BRAF^{V600E}$ or $BRAF^{V600K}$ mutations. Specifically, in the BRIM3 study, 675 patients with $BRAF^{V600E/K}$ mutations were randomized 1:1 to receive vemurafenib or dacarbazine (DTIC) (Chapman et al. 2011). The initial primary endpoint was overall survival; however, at the first interim analysis, the trial design was changed to use a composite endpoint of OS and progression-free survival (PFS) and allow for crossover from DTIC to vemurafenib at the time of progression. The initial analysis demonstrated the superiority of vemurafenib versus DTIC with respect to response rate (48 % vs 5 %), PFS (hazard ratio [HR] 0.26; 95 % confidence interval [CI] 0.20–0.33, p value < 0.001), and OS (HR 0.37, 95 % CI 0.26–0.55, $p < 0.001$). Similarly, in the BREAK3 study, 250 patients with advanced BRAFV600E/K mutant melanoma were randomized to receive either dabrafenib or DTIC; though as opposed to the BRIM3 study, patients were randomized at 3:1 to receive dabrafenib, patients were allowed to cross over from DTIC to dabrafenib from the beginning, and the primary endpoint was PFS (Hauschild et al. 2012). Not surprisingly, patients randomized to dabrafenib had superior outcomes to those randomized to DTIC and specifically had improved PFS (HR 0.30, 95 % CI 0.18–0.51, $p < 0.0001$) and RR (50 % vs 4 %). Based on the data from BRIM3 and BREAK3, the United States Food and Drug Administration (FDA) approved vemurafenib in 2011 and dabrafenib in 2013.

The efficacy of the BRAF inhibitors vemurafenib and dabrafenib were unprecedented and balanced by a favorable toxicity profile (Chapman et al. 2011; Flaherty et al. 2010; Hauschild et al. 2012; Sosman et al. 2012; Falchook et al. 2012). With that said, a number of toxicities are seen with therapy including those commonly seen with small molecule inhibitors such as nausea, diarrhea, and fatigue to those quite specific for BRAF inhibitors including hyperkeratosis, palmar–plantar erythema, arthralgia, and squamous cell carcinomas of the skin (cuSCC). These latter adverse effects have since been determined to be related to paradoxical upregulation of the pathway in tissues that are dependent on MAPK pathway signaling (Oberholzer et al. 2012; Su et al. 2012; Heidorn et al. 2010; Poulikakos et al. 2010). This occurs through the interaction of the BRAF inhibitor with wild-type BRAF in these cells that triggers a conformational change in BRAF that leads to robust homo- and hetero-dimerization with RAF isoforms and subsequent pathway activation (Poulikakos et al. 2010; Heidorn et al. 2010). This is best understood in the setting of cuSCC, where an upstream mutation in HRAS has been seen in the majority of these cases (Oberholzer et al. 2012; Su et al. 2012).

16.2.3 Building upon BRAF Inhibition and the Development of MEK Inhibitor Therapy in BRAF Mutant Melanoma

In tumor biopsy samples initially collected from patients treated with vemurafenib and dabrafenib, some degree of residual MAP kinase pathway activity persists even

when patients are administered maximum tolerated doses. Similarly, residual ERK activity can be detected in vitro when BRAF mutant cell lines are treated with high concentrations of the tool compound, PLX4720 (Paraiso et al. 2010). So, even before turning to combination regimens, it was felt that that there may be value in finding agents that inhibit the MAPK pathway at distinct points. MEK inhibitors were developed in BRAF mutant melanoma for this express purpose. The first such inhibitor to be evaluated was trametinib, which quickly was developed from Phase I trials to a randomized Phase III trial in patients with advanced $BRAF^{V600E/K}$ mutant melanoma. In the METRIC study, 322 patients were randomized on a 2:1 ratio to receive either trametinib or chemotherapy (single agent DTIC or paclitaxel) (Flaherty et al. 2012b). The trial met its primary endpoint of improved overall survival (HR 0.54, 95 % CI 0.32–0.92, $p = 0.01$), as well as secondary endpoints of improved PFS (HR 0.45, 95 % CI 0.33–0.63, $p < 0.001$) and RR (22 % compared to 9 %). Additionally, trametinib was well tolerated with most common toxicities, which were related to inhibition of the MAPK pathway in normal tissues, such as acneiform rash, diarrhea, fatigue, peripheral edema, nausea, and hypertension with rare toxicities including reduced ejection fraction and ophthalmologic effects such as central serous retinopathy and, less commonly, retinal vein thrombosis. Based on these data, the FDA approved trametinib for the treatment of advanced $BRAF^{V600E/K}$ melanoma in 2013.

With the FDA approval of BRAF inhibitors (vemurafenib and dabrafenib) and a MEK inhibitor (trametinib), an important issue that arose was how to use these types of drugs in relation to the other. For example, would sequencing or combination of BRAF and MEK inhibitors be more effective and if combination were effective, would it be tolerable. To address this issue, it is important to understand the status of the MAPK pathway at the time of resistance to BRAF inhibitors. For example, if the MAPK pathway were active at the time of BRAF inhibitor resistance, than perhaps targeting the pathway downstream might be a useful endeavor, whereas if the pathway were not reactivated, targeting alternative pathways rather than the MAPK pathway would likely be more fruitful. Over the years, following the availability of BRAF inhibitors on clinical trials and commercially, a number of mechanisms of resistance have been identified including adaptive changes in receptor tyrosine kinase activity (AXL, IGFR1, PDGFR, etc.) and genetic aberrations that lead to MAPK pathway reactivation and/or upregulation of other pro-survival signaling pathways including the phosphoinositide-3-kinase (PI3K) pathway (Montagut et al. 2008; Smalley et al. 2008; Emery et al. 2009; Johannessen et al. 2010; Nazarian et al. 2010; Paraiso et al. 2010, 2011; Villanueva et al. 2010; Poulikakos et al. 2011; Shi et al. 2012; Wilson et al. 2012). Importantly, reactivation of the MAPK pathway, through a multitude of mechanisms (including upstream RAS mutation, BRAF amplification, alternative splicing of BRAF, and downstream MEK1/2 mutations), occurs in up to 70 % of cases, though nearly 20 % of the time both MAPK and PI3K activation is seen (Shi et al. 2014; Van Allen et al. 2014). These data predict that effective, downstream inhibition of the MAPK pathway after BRAF inhibitor therapy would be an effective therapy in most patients with BRAF inhibitor-resistant, BRAF mutant melanoma.

To test the hypothesis that downstream MAPK pathway inhibition would be effective in the setting of BRAF inhibitor resistance, a Phase II trial of trametinib following BRAF inhibitor therapy was performed in patients with BRAF mutant melanoma who had either received prior BRAF inhibitor therapy (Cohort A) or had not received prior BRAF inhibitor therapy but had been treated with chemotherapy or immune therapy (Cohort B) (Kim et al. 2013). In Cohort A, no responses were seen in any of the 40 patients treated, and the median PFS was 1.8 months. In Cohort B, responses were seen in 25 % of the 57 patients enrolled and the median PFS was 4 months, which was similar to the data for patients treated in the METRIC study. The conclusion from this trial is that MEK inhibitor therapy, in this case trametinib, is not effective in patients with BRAF mutant melanoma who have previously received BRAF inhibitor therapy.

The next issue to be addressed was whether combined BRAF and MEK inhibitor therapy would be tolerable and effective. It was hoped that such a combination would suppress MAPK signaling to even greater degrees than are achievable with either agent alone. Additionally, it was hypothesized that the co-administration of a MEK inhibitor with a selective BRAF inhibitor would counter the paradoxical upregulation of the MAPK pathway that occurs in normal tissues following selective BRAF inhibitor therapy. Thus, it was thought that the consequence of dual BRAF and MEK blockade may be less toxicity, whereas the prediction is for most combination regimens to engender greater toxicity. The first such trial was the combination of dabrafenib and trametinib, which in a Phase I/II trial, was shown to be tolerable at the maximum tolerable dose (MTD) levels of each individual drug and was associated with high response rates (~70 %) and median PFS in excess of 9 months (Flaherty et al. 2012a). Two additional BRAF/MEK combinations have shown to be tolerable and effective, vemurafenib and combimetinib (MEK inhibitor), and encorafenib and binimetinib (MEK inhibitor) (Ribas et al. 2014; Sullivan et al. 2015b). In all three combinations, the rate of symptoms thought to be related to paradoxical activation of MAPK pathway in non-BRAF mutant cells by the BRAF inhibitors were abrogated by the MEK inhibitor. Specifically, the rate of hyperkeratosis, palmar–plantar erythema, and cuSCC are greatly reduced in the combination compared to single-agent BRAF inhibitors.

More recently, three Phase III trials have been completed showing the superiority of the combination of BRAF/MEK inhibitor therapy to single-agent BRAF inhibitors. In COMBI-d, 423 patients with previously untreated and advanced $BRAF^{V600E/K}$ mutant melanoma were randomized to the combination of dabrafenib and trametinib versus dabrafenib and placebo (Long et al. 2015). Patients treated with the combination had improved RR (69 % vs 53 %), PFS (median 11 months vs. 8.8 months; HR 0.67, 95 % CI 0.53–0.84, $p = 0.0004$), and OS (median 25.1 months vs. 18.7 months; HR 0.71, 95 % CI 0.55–0.92, $p = 0.0107$). In COMBI-v, 704 patients were randomized to either dabrafenib plus trametinib versus vemurafenib and placebo (Robert et al. 2015). Similar to COMBI-d, patients treated with the combination had superior RR (64 % vs. 51 %), PFS (median 11.4 months vs. 7.3 months; HR 0.56, 95 % CI 0.46–0.69, $p < 0.001$), and OS (1 year rate 72 % vs. 65 %; HR 0.69, 95 % CI 0.53–0.89, $p = 0.005$) compared to those treated with vemurafenib and placebo. The

third such trial, coBRIM, compared the combination of vemurafenib plus cobimetinib versus vemurafenib plus placebo and, like the COMBI-d and COMBI-v trials, demonstrated superiority compared to vemurafenib plus placebo in RR (65 % vs. 59 %), PFS (median 12.5 months vs. 6.2 months; HR 0.58, 95 % CI 0.46–0.72), and OS (median 22.3 months vs. 17.4 months; HR 0.70, 95 % CI 0.55–0.90) (Larkin et al. 2014). A fourth trial, COLUMBUS, is ongoing and randomizing patients to encorafenib plus binimetinib, encorafenib plus placebo, and vemurafenib plus placebo (NCT01909453).

16.2.4 Adding to the BRAF/MEK Inhibitor Backbone in BRAF Mutant Melanoma

Given the myriad mechanisms of resistance to BRAF-targeted therapy in melanoma, which are often seen in the same tumor or in multiple tumors in a patient, it is hard to imagine that targeting one specific signaling pathway mediator (ERK, PI3K, AKT, etc.) will be associated with dramatic clinical benefit. Notwithstanding, targeting ERK with the ERK1/2 inhibitor BVD523 has been associated with transient responses in some patients with BRAF mutant melanoma who were previously treated with BRAF/MEK inhibitor therapy (Infante et al. 2005). While this is predictable given that the majority of patients will have reactivation of the MAPK pathway, it is also not surprising that the benefit was transient, given that multiple mechanisms are often involved. It is this pervasive heterogeneity in the mechanism of resistance to BRAF-targeted therapy that forces researchers and clinical investigators to think about alternative targets (immune system, apoptosis, oncogene chaperones, early adaptive changes to therapy) as opposed to the traditional targets, namely mediators of signal transduction pathways (e.g., PI3K pathway or MAPK pathway).

16.2.4.1 Dual BRAF and Immune Targeting

Combination of BRAF-targeted therapy with immunotherapies is an attractive approach for several reasons. First, BRAF does not appear to be an important constituent of the MAP kinase pathway in mature immune cells, including T cells (Tsukamoto et al. 2008). Second, emerging evidence suggests that inhibition of the MAPK pathway in patients with melanoma, through inhibition of BRAF and/or MEK, leads to increased melanoma antigen expression, decreased immunosuppressive cytokines, increased trafficking of CD8+ T-lymphocytes, and increased expression of programmed death 1 (PD1) and its ligand (PDL1) (Donia et al. 2012; Cooper et al. 2013; Frederick et al. 2013; Wilmott et al. 2012). Also, it has recently been shown that patients with a higher proportion of preexisting tumor-infiltrating lymphocyte clones, determined by T-cell receptor (TCR) rearrangement sequencing (so-called ImmunoSeq), have better outcomes with BRAF/MEKi therapy, although nearly all patients have an increase in TCR clonality in the setting of BRAF/MEKi therapy (Cooper et al. 2013). The major conclusion from these data is that MAPK pathway targeting enhances antitumor immunity within the tumor microenvironment, providing a strong rationale for combination of MAPK-targeted therapy with immunotherapy.

The first wave of combined BRAF-targeted therapy plus ipilimumab studies have been performed and limited by tolerability. Specifically, the vemurafenib plus ipilimumab study was closed in dose escalation when it was observed that six of the ten evaluable patients had developed grade 3 or 4 elevation of transaminases (Ribas et al. 2013). Interestingly, when dabrafenib was combined with ipilimumab, there was less hepatic toxicity, though when the triple combination of dabrafenib, trametinib, and ipilimumab was tested, two of nine patients developed bowel perforation (Puzanov et al. 2014). Based on the results of these trials, the development of ipilimumab-based targeted therapy–immunotherapy trials has been halted.

The next wave of combined targeted-immunotherapy trials has been evaluating various combinations of BRAF plus or minus MEK inhibitors with anti-PDL1 antibodies. The first such trial explored the safety and efficacy of vemurafenib plus the anti-PDL1 antibody atezolizumab. In the initial cohort, concomitant initiation of therapy was associated with excess toxicity (elevated liver enzymes, fatigue, rash); however, with employing a lead-in of 28 days to perform the combination phase of the trial, the combination has been shown to be well tolerated and associated with responses in the majority (76 %) of the 17 patients evaluable for response, including three complete responders (Hamid et al. 2015). This trial is now evaluating the triple combination of vemurafenib, cobimetinib, and atezolizumab (NCT01656642). The second trial to explore the utility of anti-PDL1 antibodies with targeted therapy is the Phase I/II trial of durvalumab with either trametinib (in BRAF wild-type patients) or dabrafenib/trametinib (in BRAF mutant patients). In the cohort of BRAF mutant patients, the response rate was 69 % (18/26 patients), with all patients having control of disease (response or stable disease) at first imaging (Ribas et al. 2015). Whether these combinations are associated with a more durable benefit rate than BRAF/MEK inhibitor therapy is unknown, but the initial results are promising that combined BRAF/MEK plus anti-PDL1 therapy is tolerable and associated with response in the great majority of patients. As durability of response will be the next clinical endpoint by which these combinations will be judged, substantially greater sample sizes and follow-up will be needed to glean the impact of these regimens.

16.2.4.2 Targeting Apoptosis

Constitutive activation of the MAPK pathway in the context of a BRAFV600 mutation is associated with a number of pro-survival consequences including inhibition of proapoptotic BCL-2 family members, inactivation of BAD, and downregulation of BIM to enhance cell survival (Boisvert-Adamo and Aplin 2008; Cartlidge et al. 2008). Preclinically, inhibition of BRAF or MEK leads to apoptosis, at least in part, through the upregulation of BIM and its inhibition of the antiapoptotic proteins BCL-2 and MCL-1 (VanBrocklin et al. 2009; Haq et al. 2013; Wroblewski et al. 2013; Frederick et al. 2014). Further, gene expression analyses of patient tumor biopsies demonstrate that treatment with either vemurafenib or dabrafenib and trametinib reduces MCL-1 mRNA levels and increases BIM mRNA and protein levels in most patients (Frederick et al. 2014). Nonetheless, the degree of apoptosis in the setting of BRAF inhibitor therapy is variable and likely attenuated by the antiapoptotic BCL-2 family members, BCL-x_L and BCL-w, both of which also increase with BRAF-directed therapy (Trunzer et al. 2013; Long et al. 2013; Frederick et al. 2014). These

findings support the addition of an agent that can augment BRAF and MEK inhibitor-induced apoptosis and serves as the preliminary evidence for a Phase I/II trial of dabrafenib, trametinib, and navitoclax, a small molecule inhibitor of BCL-2, BCL-x_L, and BCL-w (NCT01989585).

Another important mechanism of promoting cell survival in melanoma is the inactivation of the tumor suppressor gene/protein, p53. While p53 is mutated in a minority of patients (~15 %), it is functionally inactivated by MDM2 or MDM4 in approximately half of patients with melanoma (Cancer Genome Atlas 2015; Gembarska et al. 2012; Ji et al. 2012; Muthusamy et al. 2006; Polsky et al. 2001). Thus, small molecule targeting of MDM2 in combination with BRAF and/or MEK inhibitors is rational and supported by preclinical evidence showing synergy of the combination in both in vitro and in vivo studies (Ji et al. 2012; Saiki et al. 2014). Based on the rationale and preclinical data, clinical trials are being developed to evaluate this combination, and one such trial is actively enrolling patients with either trametinib plus or minus dabrafenib in combination with tMDM2 inhibitor AMG232 (NCT02110355).

16.2.4.3 Targeting an Oncogenic Chaperone (Heat Shock Protein 90)

Heat shock proteins act as cellular chaperones for client proteins and facilitate cellular signaling. Heat shock protein 90 (HSP90) serves as a molecular chaperone to a number of mediators of growth factor pathways and is a potential therapeutic target in the setting of BRAF inhibitor resistance. Included among its client proteins are insulin-like growth factor 1 receptor (IGF1R), BRAF, CRAF, AKT, cyclin D, and cyclin-dependent kinase 4 (CDK4), all of which have been implicated in BRAF inhibitor resistance. Supporting this is data showing that the HSP inhibitor XL888 blocks growth and survival, in vitro and in vivo, in BRAF-mutant melanoma cell lines that harbor acquired resistance mechanisms to BRAF inhibitor including COT overexpression, platelet-derived growth factor receptor beta (PDGFR-b) overexpression, concomitant BRAF and NRAS mutations, and cyclin D amplifications. Additionally, a reduction in levels of a number of both client proteins and growth pathway mediators was seen, as was a favorable change in the pro- and antiapoptotic molecules, respectively, BIM and MCL1. Similar data also have been generated with the HSP90 inhibitor AT13387, showing activity in cell lines with BRAF inhibitor acquired-resistance mechanisms and suppression of acquired resistance in BRAF mutant melanoma cell lines in xenograft models when mice are treated with both a BRAF inhibitor and AT13387 (Smyth et al. 2014). Based on these data, clinical trials have been launched evaluating single-agent BRAF inhibition (vemurafenib) with XL888 (NCT01657591), as well as the combination of dabrafenib, trametinib, and AT13387 (NCT02097225).

16.2.5 PI3 Kinase Pathway

The PI3 kinase pathway is another RAS effecter pathway that has been implicated in the pathophysiology of numerous cancers, including melanoma. Melanomas commonly feature loss of PTEN function, a negative regulator of AKT activation by

PI3 kinase (Guldberg et al. 1997). Hemizygous and homozygous deletion appear to be the most common type of genetic alteration in PTEN in melanoma. Additionally, numerous point mutations, thought to inactivate PTEN, and thereby permitting higher PI3 kinase pathway activity, have also been described in some melanomas (Celebi et al. 2000). A point of continued controversy is whether silencing of PTEN expression by promoter hypermethylation might also be a mechanism of gene silencing in melanoma (Mirmohammadsadegh et al. 2006; Furuta et al. 2004). In any case, it is clear that a substantial subset of melanomas have genetic alterations in PTEN, and the preponderance of evidence suggests that it is the consequence of overactivity in the PI3 kinase pathway that is of pathophysiologic significance. It should be noted that numerous other cytoplasmic and nuclear sites of PTEN activity have been described, but it is unclear if any of those are important to melanoma biology (Zhang and Yu 2010). Some evidence has been produced from array comparative genome hybridization experiments to support the presence of AKT3 amplification in a distinct subset of melanomas from those that have PTEN loss, and rare activating mutations in AKT3 have been reported (Stahl et al. 2004; Davies et al. 2008). This is further genetic evidence that the PI3 kinase pathway plays a critical role in a subset of melanomas. Notably, it appears that PTEN loss is frequently seen concomitant with BRAF mutation (Tsao et al. 2004). This suggests that both of these RAS effecter pathways are needed to contribute to melanoma formation.

Experimental evidence supports a role for the PI3 kinase pathway in melanoma invasion into the dermis, and potentially in metastatic spread. In particular, the introduction of activated AKT results in the conversion from a radial growth to vertical growth phase phenotype in primary melanoma in animal models (Govindarajan et al. 2007). Conversely, restoration of PTEN expression or blockade of PI3K/AKT signaling in melanoma cells blocks invasion and induces apoptosis (Stewart et al. 2002; Ruth et al. 2006). Introduction of AKT3 into melanoma cell lines promotes proliferation and survival, whereas knockdown of AKT3 mRNA, but not AKT1 or AKT2, inhibits both phenotypes (Stahl et al. 2004). Introduction of myristylated AKT prevented the expression of proapoptotic mediators such as Bim and Bmf and knockdown of AKT3 with siRNA greatly enhances the degree of apoptosis seen in BRAF mutant cell lines treated with a selective BRAF inhibitor (Shao and Aplin 2010).

The development of therapeutic agents targeting the PI3 kinase pathway, and particularly the components within it that are critical to melanoma pathophysiology, is at a very early stage. Due to the overall prevalence of PTEN loss, activating mutations in PI3 kinase itself, and activating mutations or amplification in AKT, significant efforts have been made in the pharmaceutical industry to develop antagonists of this pathway. Three classes of PI3 kinase inhibitors have emerged in clinical development: (1) inhibitors of specific PI3 kinase isoforms, (2) agents that inhibit with near equal potency all PI3 kinase isoforms, and (3) dual inhibitors of PI3 kinase and mTOR. When considering which of these agents might be most relevant in melanoma, the deficiency in our knowledge regarding which isoform or isoforms of PI3 kinase to target becomes apparent. In lymphocytes, it appears that the catalytic subunit p110delta (the delta isoform) is the one whose activity is most markedly upregulated in the setting of PTEN deletion (Janas et al. 2008). However, in

breast cancer models, it appears that PI3 beta is the most relevant isoform in mediating cell invasion in response to RAS activation (De Laurentiis et al. 2011). These observations remain to be confirmed, specifically in melanomas.

The therapeutic strategy at the level of AKT may be somewhat more straightforward based on available genetic and experimental evidence. Given that the most common activating mutations in AKT across cancers are in AKT1, the focus of pharmaceutical development has been on agents with preferential inhibitory effects on this isoform (Carpten et al. 2007). AKT2 appears to be the most critical isoform in glucose homeostasis, and therefore would be the one isoform to avoid targeting (Altomare et al. 1998). The first generation of AKT inhibitors in clinical development have fairly uniform potency against the three AKT isoforms. This may be problematic, as dose-limiting toxicity may be rendered by AKT2 inhibition, or possibly co-inhibition of all isoforms. The ideal AKT inhibitor for melanoma might be one that is relatively AKT3-specific, and such an agent has not yet been developed. In fact, the AKT inhibitor that is furthest into clinical development, MK-2206, is equally potent against AKT1 and AKT2, but fivefold less potent against AKT3 (Yap 2009). Thus, the melanoma field awaits the development of additional AKT inhibitors with more optimal properties relevant to the biology of AKT signaling in this disease.

16.2.6 Beyond BRAF: Targeting Other Molecular Subsets

16.2.6.1 NRAS Mutant Melanoma

Activating mutations in NRAS continue to be an elusive target in melanoma, more than 30 years after their initial identification (Albino et al. 1984). Approximately 25 % of all advanced melanoma cases harbor an activating mutation in and RAS, with Q61R mutation being the most common and mutations at position 12 in the amino acid sequence being the next most commonly affected (Curtin et al. 2006; Cancer Genome Atlas 2015). NRAS is not amenable to targeting with drugs of the sort that can compete with ATP binding as RAS does not consume ATP as an energy source. Rather, RAS is a GTPase, and the common mutation found in cancer impairs GTPase activity. Thus, an effective pharmacologic inhibitor of mutated ras would need to restore the lost GTPase activity, and such compounds have not been readily discovered to date. Experimental evidence demonstrating the biologic significance of RAS in melanoma stems from at siRNA knockdown experiments as well as genetically engineered models demonstrating its contribution to melanoma pathophysiology (Eskandarpour et al. 2009; Nogueira et al. 2010). More indirect evidence supporting the therapeutic potential of antagonizing RAS comes from experiments using farnesyltransferase inhibitors, which block one of the key post-translational modifications required for RAS to localize to the plasma membrane (Smalley and Eisen 2003). The introduction of mutated NRAS into melanocytes induces senescence, or transformation in the appropriate genetic background (Whitwam et al. 2007). In transgenic models, introduction of activating NRAS mutations combined with either p53 or p16 loss of function through genetic inactivation results in proliferative and invasive melanocytic lesions (Nogueira et al. 2010). In cell lines,

knockdown of NRAS mRNA results in growth arrest and apoptosis selectively in melanoma cell lines that harbor activating NRAS mutations (Eskandarpour et al. 2009). At exposures that inhibit the farnesylation of many signaling molecules, farnesyltransferase inhibitors will arrest the cell cycle and induce cell death in melanoma cells that harbor NRAS mutations. Additionally, this intervention sensitizes such cells to the cytotoxic effects of chemotherapy. Unfortunately, chemical trials with farnesyltransferase inhibitors have been largely disappointing, owing to the lack of evidence of single-agent efficacy in patients with RAS-mutated cancers when these agents are administered at maximum tolerated doses (Gajewski et al. 2006). While there is some evidence that partial inhibition of RAS signaling can be achieved with these agents, it appears that the degree of innovation is insufficient to significantly perturb these tumors (Sebti and Hamilton 2000). Thus, agents with more selectivity for RAS are anxiously awaited but for the reasons noted above continue to be a major technical challenge.

An alternative strategy would be to block the downstream signal transduction pathways that are activated in the setting of RAS mutation. There are numerous RAS effecter pathways, the MAP kinase and PI3K pathways being the best described, but the relative importance of each has not been elucidated. As drug targeting of RAS effecter pathways has been a major priority in pharmaceutical development, there are numerous agents emerging targeting the MAP kinase and PI3K pathways, which might be useful for NRAS mutant melanoma. In particular, potent and selective MEK inhibitors have demonstrated single agent activity in NRAS mutant melanoma. Specifically, binimetinib was associated with responses in over 20 % of patients with metastatic, NRAS mutant melanoma, and with a median PFS of 3.7 months (Ascierto et al. 2013). While these data are not dramatic, this was a notable benefit in a previously hard-to-treat subgroup of patients and provided the justification for a randomized, Phase III trial of binimetinib versus DTIC (so-called NEMO trial). The initial findings of this trial were recently publicized as part of a press release stating that treatment with binimetinib was associated with improved PFS (median 2.8 months vs. 1.5 months; HR 0.62, 95 % CI 0.47–0.80, $p < 0.0001$) compared to DTIC treatment.

The data with single-agent binimetinib is an encouraging first step, but better regimens are still needed for this patient population. One such regimen is the combination of MEK inhibitors with CDK4 inhibitors. There is ample preclinical data suggesting that cell cycle progression is a key mechanism of resistance to single-agent MEK inhibition in NRAS mutant melanoma. In patients, two trials of MEK and CDK4 inhibitors have been performed to date. The first is the combination of binimetinib with the CDK4/6 inhibitor ribociclib. In this Phase I/II trial exclusively enrolling patients with advanced NRAS mutant melanoma, the majority of patients across all dose cohorts experienced tumor regression, and responses were seen in 7 of the 22 patients enrolled, with another 11 experiencing stable disease (Van Herpen et al. 2015). The second trial evaluated the combination of trametinib plus the CDK4/6 inhibitor palbociclib. The difference with this study was that it allowed the enrollment of patients with any advanced solid tumor. In this dose escalation study, only two responses were seen (one in an NRAS mutant colon cancer patient and the

other in a NRAS/BRAF/NF1 wild-type melanoma patient) among the 26 patients treated (Sullivan et al. 2015a). In eight NRAS mutant patients, one responded (the aforementioned colon cancer patient), four had stable disease, and three had progression of disease as a best response.

16.2.6.2 CDK/Cyclin/p16

Germline mutation in CDKN2A, the gene that encodes both p16 and p14, have long been known to confer susceptibility to melanoma and is the highest penetrance allele yet discovered in familial melanoma (Hussussian et al. 1994). Mutations in CDKN2A, as well as hemizygous and homozygous deletions, are also observed in sporadic cases of melanoma (Wagner et al. 1998; Fujimoto et al. 1999). The best characterized point mutations identified in familial melanoma result in a disruption of the p16/CDK4 inhibitory interaction, suggesting that this may be the critical interaction related to melanoma formation (Haferkamp et al. 2008). The net consequence of p16 loss or mutation is that CDK4 is dysinhibited and pushes cells through the cell cycle without the usual checkpoint regulation. In cases where CDKN2A is wild-type, a distinct subset of melanomas harbor cyclin D amplification (Curtin et al. 2005). Cyclin D is another binding partner for CDK4 and is a critical co-activator of CDK4 activity. Thus, cyclin D application results in overactivity of CDK4, analogous to loss of p16 function. Lastly, CDK4 itself has been found to be amplified in some cases of sporadic melanoma and to contain activating mutations in the kinase domain in another subset (Muthusamy et al. 2006). This genetic evidence would support that the CDK4 cell cycle checkpoint is a critical suppressor of melanoma formation and, conversely, its overactivity appears to contribute to melanoma pathophysiology. In vitro as well as mouse transgenic models support cooperative the between activating BRAF mutations and genetic aberrations in p16/cyclin D/CDK4 (Dhomen et al. 2009; Robinson et al. 2010). What remains to be determined is whether dual targeting of this axis combined with an inhibitor of the MAP kinase pathway, such as BRAF, will have greater therapeutic value then inhibiting either target alone.

Restoring p16 function in the setting of mutation or deletion is beyond the scope of what current pharmacologic development can achieve. Additionally, cyclin D lacks an enzymatic function against which drugs could be readily generated. That leaves CDK4, with its kinase activity, as a potentially relevant point of intervention in tumors with p16/cyclin D/CDK4 aberrations. Potent and selective CDK4 inhibitors have been developed and completed or are in the process of completing Phase I clinical trials. An example of this class of drugs is PD032991, which is an orally available CDK4/6 inhibitor (Fry et al. 2004). As one might project for a cell cycle targeted therapy, this agent produced mild to moderate–mild suppression that was reversible during planned treatment interruptions in Phase I (Schwartz et al. 2007). This agent and other CDK4 inhibitors are actively being tested clinically in patients with melanoma. It is of particular interest to evaluate the efficacy of single-agent CDK4 inhibitor therapy in patients with p16/cyclin D/CDK4 alterations, though there are no such data that have been presented or published to date. As noted above for NRAS mutant melanoma, the genetic evidence supporting CDK4 dysregulation

as being critical to melanoma formation supports further clinical investigation in combination with other approaches. Now that BRAF and MEK inhibitors have established their own evidence of efficacy as single agents and in combination in BRAF mutant and NRAS mutant subtypes, the most feasible approach would be to test a combination of a selective CDK4 inhibitor with a BRAF with or without a MEK inhibitor in BRAF mutant patients (or with MEK inhibitor in NRAS mutant patients as described above). This is occurring as part of an additional dose escalation and expansion cohort in the Phase I/II trial of encorafenib plus binimetinib that was described above as well as in other trials as well (Sullivan et al. 2015b).

16.2.6.3 KIT

By the time CKIT mutations were reported in melanoma in 2006, KIT had already been validated as a therapeutic target in gastrointestinal stromal tumor (GIST) (Demetri et al. 2002). And subsequently, several second-generation KIT inhibitors established therapeutic benefit in this population as well (Fig. 16.1) (Demetri et al. 2006). However, it was not known whether the somatic genetic changes that might accompany KIT mutation in melanoma, perhaps differently than GIST, might render melanoma more or less susceptible to single-agent KIT-targeted therapy.

Imatinib was the first KIT inhibitor to demonstrate single-agent activity in GIST, and was the first agent to be evaluated clinically in melanoma (Hodi et al. 2008). In the years prior to the discovery of KIT and mutations in melanoma, three Phase II clinical trials were conducted with imatinib as a single agent in metastatic

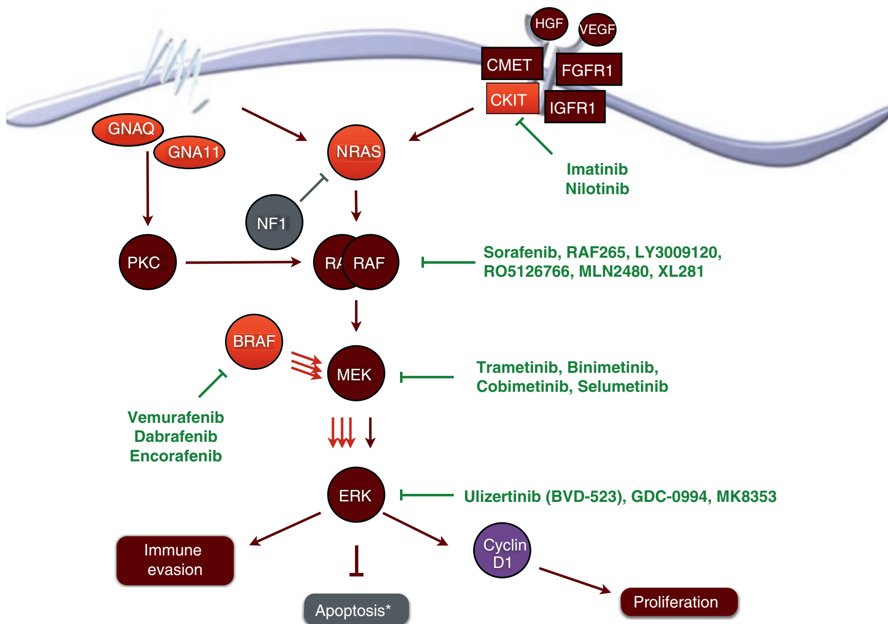

Fig. 16.1 c-KIT, RAF, and MEK inhibitors in clinical development for melanoma

melanoma (Ugurel et al. 2005; Wyman et al. 2006; Kim et al. 2008). However, nearly all of the patients enrolled on trials did not have melanoma that arose from the clinical subtypes in which KIT mutations can be found, mucosal, acral/lentiginous, and those associated with chronic sun damage. So, although KIT mutation analysis was not performed in the context of these trials, one would estimate that very few, if any, of these patients had KIT mutations. Only one patient experienced an objective response in those trials and this patient had a prior history of a mucosal melanoma primary. In addition to activating mutations in KIT, amplification has also been described, and can occur in the presence or absence of mutation. So, the hypotheses addressed in clinical trials with KIT inhibitors in melanoma are whether mutation and/or amplification might serve as predictive markers for response to these therapies. It should be noted that many of the mutations in KIT described in melanoma overlap with those previously seen in GIST. Some of these mutations have been described as imatinib-sensitive in the context of GIST, while others have not. And novel mutations in KIT have been described in some cases of melanoma as well. Thus, new questions remain to be answered in the context of KIT inhibitor trials in melanoma to understand not only whether mutations previously described as sensitive to existing drugs would be so in melanoma but also to determine if amplification or novel mutations are similarly predictive of response to therapy.

A single patient with mucosal melanoma whose tumor was found to harbor an internal tandem duplication in the juxtamembrane domain of KIT was treated with imatinib and an objective response lasting for at least several months was observed (Hodi et al. 2008). This first report established that KIT targeted therapy could be effective in melanoma.

There are now three Phase II trials with imatinib in KIT mutant or amplified melanoma that have been completed. In one of those trials, Carvajal and colleagues screened 295 patients with metastatic melanoma whose primary tumors were mucosal, acral/lentiginous, or arose from chronic sun-damaged skin (Carvajal et al. 2011). Archival tumor specimens were screened for the presence of amplification in KIT or mutations in exons 9, 11, 13, 17, and 18. A total of 51 of 295 (17 %) of these patients were found to have aberrations in KIT and 28 were treated with imatinib. Two patients experienced complete responses and two had partial responses (16 % response rate) among the 25 evaluable patients. The median time to progression was 12 weeks and median overall survival was 46.3 weeks. All of the responders had either an exon 11 or 13 mutation. In another Phase II trial, Guo and colleagues enrolled 43 patients with KIT mutation in exons 9, 11, 13, 17, and 18 or amplification only (Guo et al. 2011). The starting dose of imatinib was 400 mg daily, with escalation of the dose to 600–800 mg daily upon disease progression. Forty-three patients were enrolled, all of whom had a response evaluation by the time of publication. Ten patients (23 %) had an objective response, and nine had either an exon 11 or 13 mutation. In patients with exon 11 or 13 mutations, the response rate was 35 % (6 of 17) and 33 % (3 of 9), respectively. A third Phase II trial of imatinib in patients with CKIT aberrations treated patients with 400 mg daily to be escalated to 400 mg twice daily in the setting of no initial response (Hodi et al. 2013). Twenty-five patients were enrolled, and all but one was evaluable for response. Seven patients had disease

response; all but one had either an exon 11 or 13 mutation (there was one responder with an exon 17 point mutation).

Based on these two trials, several preliminary conclusions can be made: (1) the previously described imatinib-sensitive mutations in GIST also predict responsiveness to imatinib in melanoma, (2) several patients with predicted imatinib sensitivity did not respond, and (3) on the basis of response rates in the 20–25 % range, patients with CKIT exon 11 and 13 mutations are a subgroup worthy of further investigation.

Additionally, Phase II trials of nilotinib in patients with CKIT mutant melanoma who have progressed or who are intolerant of first-line KIT inhibitor therapy has been completed (Carvajal et al. 2015). In this trial, 20 patients were enrolled to one of two cohorts, Cohort A prior KIT inhibitor therapy or Cohort B patient with brain metastases. The primary endpoint was 4 months of disease control, and three of eleven patients treated in cohort A and one of eight in Cohort B met this endpoint. The conclusion of this trial was that nilotinib may be associated with clinical benefit in patients with CKIT mutant melanoma but is associated with limited benefit in patients with brain metastases.

Clearly, the proof of concept has been established that KIT mutant melanoma can be responsive to imatinib. However, much work remains to be done to understand the response rate for particular mutations or in particular exons. KIT amplification alone remains of uncertain significance with regard to connoting responsiveness to therapy. And the durability of response or disease stabilization and its impact on overall survival remains to be determined. The pace with which these questions can be answered is limited by the rarity of genetic alterations in KIT in the overall melanoma population, and their presence in a minority of patients with the relevant clinical subtypes in which they can be found.

16.2.7 Oncogenes Not Readily Amenable to Direct Targeting

16.2.7.1 MITF

The microopthalmia transcription factor, MITF, is the master regulator of melanocyte differentiation and expression of melanocyte-specific antigens (see also Chap. 9 (Abdel-Malek)). Its potential role as an oncogene in melanoma has recently been suggested based on the discovery of high-level amplification of the MITF locus in approximately 15 % of melanomas (Garraway et al. 2005). In such tumors, and some others without amplification, knockdown of MITF with siRNA impairs proliferation. Being a member of the transcription factor superfamily that includes MYC, a contribution to cancer pathophysiology would not be unprecedented. However, targeting MITF with pharmacologic inhibitors is not currently possible. This molecule lacks an enzymatic domain against which small molecules could readily be developed. Rather, agents that block the association of MITF with cofactors, or key promoter regions in MITF target genes, would be needed. In order to avoid pleiotropic effects on the expression of a vast array of genes, likely resulting in unacceptable toxicity, a deeper understanding of which MITF target genes contribute most significantly to

melanoma biology is required. Such a detailed understanding would potentially generate alternative targets that are amenable to pharmacologic therapy. In the meantime, there is evidence that epigenetic regulation of MITF may be apparent in the cancer setting and could be ameliorated to some degree with the use of the histone deacetylase inhibitor (Yokoyama et al. 2008). A clinical trial is currently ongoing in metastatic melanoma patients using a class to specific histone deacetylase inhibitor (LBH589).

16.2.7.2 GNAQ/GNA11

In 2008, activating mutations in the G protein-coupled receptor signaling molecule, GNAQ, were first described in a large subset of uveal melanomas (Van Raamsdonk et al. 2009). The same mutations are not found in other melanoma subtypes. The following year, analogous mutations in the highly homologous GNA11 were described in initial in an additional portion of uveal melanoma cases. In total, either of these mutations appears to be present in approximately 80 % of uveal melanomas. It had previously been shown that BRAF and NRAS mutations are not found in uveal melanoma, so the discovery of these activating mutations filled a significant void in the understanding of oncogenic drivers in this clinical subtype of melanoma. Like RAS, these proteins are GTPases, in which the GTPase activity is disabled by the mutation that had been described. Therefore, pharmacologic agents would need to restore the GTPase activity in order to antagonize the signaling capacity of these molecules. As has been noted, such an approach appears to be extremely technically challenging, and such candidate therapies do not currently exist.

Outside of the context of cancer, these G protein alpha subunits (GNAQ and GNA11) are known to activate several downstream signal transduction pathways, including the MAP kinase and PI3 kinase pathways (Van Raamsdonk et al. 2004). Early experimental evidence in melanoma cell lines harboring a GNAQ mutation suggested that the MAP kinase pathway may be a point of vulnerability for therapeutic purposes (Van Raamsdonk et al. 2009). Given the availability of potent and selective MEK inhibitors, clinical trials employing single agent MEK inhibitor therapy were performed. The first of these studies randomized 101 patients with metastatic uveal melanoma to either the MEK1/2/inhibitor selumetinib or chemotherapy (both temozolomide and DTIC were allowable) (Carvajal et al. 2014). The primary endpoint, PFS was superior in the selumetinib arm (HR 0.46, 95 % CI 0.30–0.91; $p<0.001$) compared to the chemotherapy arm, though the OS was not statistically significantly different (HR 0.66; 95 % CI 0.41–1.06; $p=0.09$). The follow-up trial randomized patients to chemotherapy or chemotherapy plus selumetinib and failed to meet its primary endpoint of improved PFS. Obviously, further preclinical work is needed to understand more about the hierarchy of signal transduction pathways downstream of these G proteins so that rational combination drug strategies can be formulated.

16.2.7.3 BAP1

The most recent genetic discovery in melanoma also comes in the uveal melanoma subpopulation. It has long been known that the short arm of chromosome 3 is frequently deleted in uveal melanomas, and particularly those that metastasize and

result in fatality (van Gils et al. 2008). However, a potential candidate tumor suppressor gene on this chromosome had not been described. Recently, massively parallel exome sequencing has uncovered mutations in BRCA1-associated protein 1 (BAP1) in more than 80 % of metastatic uveal melanomas (Harbour et al. 2010). The distribution of these mutations in regions of the gene/protein that interacts with two distinct deubiquitinases, UCH and ULD, suggests that that BAP1 is indeed a tumor suppressor gene. As with any other tumor suppressor gene in cancer, we currently lack the ability to restore the function that is lost through inactivating mutations or deletion. However, a greater mechanistic understanding of the activities of BAP1 binding partners may provide opportunities for therapeutic intervention. The two deubiquitinases that the genetic evidence suggests to be important in BAP1 function are likely not ideal therapeutic targets as ubiquination and deubiquination are fundamental levels of regulating protein stability that pharmacologic inhibitors of such molecules may have far-reaching and toxic effects. One observation made in previous mechanistic studies regarding BAP1 function suggests that BAP1 regulates the activity of the polycomb repressive complex 1 (PRC1), which regulates histone acetylation and acetylation (Scheuermann et al. 2010). Experimental evidence suggests that in BAP1-mutated uveal melanoma cell lines, histone deacetylase inhibitors have a growth-inhibitory effect (Harbour et al. 2010). This approach is certainly possible to investigate in clinical trials, as numerous histone deacetylase inhibitors have been developed.

Conclusion

The discovery of somatic genetic changes in oncogenes and tumor suppressor genes, and the subsequent biological validation of their significance, has opened the door for the development of molecularly targeted therapies aiming to antagonize the very genetic underpinnings of melanoma. The proof of principle has been established with BRAF-targeted therapy, that melanoma can be vulnerable to such an approach, despite the vast array of genetic alterations present within any one tumor. However, it is equally clear that no single point of intervention will eradicate metastatic melanoma, and that understanding the hierarchy of oncogenes and signal transduction pathways becomes critical to develop rational combination therapies, which might make an even more significant impact. Numerous challenges and hurdles remain, and the development of more effective molecularly targeted therapies for each of the recently described molecular subsets of melanoma remains a major focus of investigation. Clearly, combination targeted therapy strategies will require that each component within the combination be highly selective for its intended target, or that the target is uniquely relevant to melanoma biology and not normal physiology. Given our increasingly detailed understanding of the network of genetic changes and aberrant signal transduction and the downstream phenotypic consequences on various cellular processes, there is no shortage of essential points of intervention or types of combinations. It will be critical for the melanoma field to remain mindful of the ultimate goal, which is to tailor the therapeutic approach to the individual patient based on the unique constellation of alterations within their tumor.

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Immune Checkpoint Inhibitors in Melanoma Define a New Era in Immunotherapy Aiming for Cure

17

Alexander M.M. Eggermont, Dirk Schadendorf, and Caroline Robert

17.1 The New Paradigm: Breaking Tolerance Is the Prerequisite

Advances in melanoma therapies are at present mainly in the field of immunotherapy and mutation-driven drug development (Eggermont et al. 2014). Breaking tolerance represents a major paradigm shift and the impact of the first checkpoint inhibitors, i.e. anti-CTLA-4 (cytotoxic T lymphocyte antigen-4) and anti-PD1/anti-PDL1 (programmed death-1 receptor and its ligand PD-L1) is unprecedented (Pardoll 2012). In only 5 years, advanced melanoma has been transformed from an incurable disease into a curable disease (Eggermont et al. 2013; Robert et al. 2013). Breaking tolerance has a transversal impact throughout solid tumor oncology.

17.2 Anti-CTLA4

17.2.1 Ipilimumab in the Therapeutic Setting of Advanced Melanoma

Monoclonal antibody blocking of cytotoxic T lymphocyte antigen 4 (CTLA-4) leads to breaking immune tolerance and can induce tumor regressions. In 2011, the fully humanized monoclonal anti-CTLA4 antibody ipilimumab was approved in the USA in first- and second- line for patients with advanced melanoma and in second

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© Springer International Publishing AG 2017
A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8_17

line in Europe at a dose of 3 mg/kg. The approval was based on randomized controlled trial (RCT) results that showed that ipilimumab alone or combined with a peptide vaccination provided a significant survival benefit of about 33 % compared to vaccination alone (Hodi et al. 2010). In another RCT, but in first-line, ipilimumab at 10 mg/kg combined with dacarbazine provided only a small, albeit statistically significant, benefit over treatment with dacarbazine alone, but there seems no reason to advocate the use of this combination (Robert et al. 2011). Mature data in thousands of patients indicate that about 20 % of patients treated with ipilimumab have the potential to survive for at least 3 years and up to 10 years from treatment initiation (Schadendorf et al. 2015). Also the efficacy in patients with brain metastases has been established and reported (Margolin et al. 2012). Ipilimumab responses can occur after the initial tumor progression or the appearance of new lesions. For this reason, immune-related response criteria (irRC) have been developed to avoid premature treatment cessation (Wolchok et al. 2009; Hoos et al. 2010).

Adverse events (AE) occur in about 40 % of patients and are mostly immune-related (irAE), such as skin rashes, colitis, hepatitis, and hypophysitis. Grades 3–4 adverse events occur in about 20 % of patients and can, in rare cases, be fatal. Usually, they resolve spontaneously or after steroid therapy. Endocrinopathies behave differently and pituitary–adrenal axis failure usually requires permanent hormonal substitution. High-dose steroids are indicated for severe irAEs, but other immunosuppressive agents, like anti-TNF-alpha antibodies may also be needed, especially in the context of severe colitis (Weber et al. 2012).

Good biomarkers for response to ipilimumab therapy still remain to be established. Immune-related adverse events, an increase in lymphocyte counts, an increase in eosinophil counts, the presence of NY-ESO-1 antigen, and the resistance in vitro to T-regulatory cell functions seem to be associated with higher response rates (Attia et al. 2005; Ku et al. 2010; Delyon et al. 2013; Ménard et al. 2008). Recently, the high levels of soluble CD25 in the serum, especially in combination with high levels of LDH, were demonstrated to be a very strong prognostic factor for poor outcome (Hannani et al. 2015).

Even the optimal dose and schedule for ipilimumab remain to be established. A randomized phase II trial comparing 0.3 mg/kg, 3 mg/kg, and 10 mg/kg suggested 10 mg/kg to be the more effective dose, but associated it with more toxicity (Wolchok et al. 2010). The results of the RCT comparing 3 mg/kg versus 10 mg/kg are not yet mature. The value of four thrice-weekly administrations (induction) compared to induction followed by further administrations (maintenance) has not been established.

17.2.2 Ipilimumab in the Adjuvant Setting of Resected Stage III Melanoma

The results of a double-blind placebo-controlled adjuvant trial EORTC18071 in stage III patients at high risk for relapse were recently published (Eggermont et al. 2015). In 951 patients with high-risk stage III disease (palpable nodal disease or sentinel node positive disease with metastases >1 mm in diameter according to the

Rotterdam Criteria (van Akkooi et al. 2008; van der Ploeg et al. 2011, 2014)), ipilimumab was dosed at 10 mg/kg and administered every 3 weeks over the first 12 weeks (induction) and thereafter every 12 weeks for up to 3 years or relapse. A significant impact on RFS (HR 0.75, $p = 0.0013$) for the ITT population was reported. Most patients came off treatment after four to five administrations of ipilimumab. The potential value of maintenance therapy will therefore remain unanswered. irAEs were consistent with what has been observed in advanced melanoma trials, but at a higher frequency, especially regarding endocrinopathies. Post hoc analyses demonstrated a significant impact both in patients with sentinel node-positive disease and palpable node-positive disease. Similar to EORTC adjuvant trials 18952 and 18991 with IFN and pegylated-IFN, patients with sentinel-positive disease derived a greater benefit (Eggermont et al. 2005, 2008, 2012a). Patients with an ulcerated primary derived the greatest benefit like in the meta-analysis of the IFN trials 18952 and 18991, indicating that ulcerated melanoma is a separate biologic entity (Eggermont et al. 2012b, c). In contrast, however, to the experience in the adjuvant IFN trials EORTC 18952 and 18991, patients with non-ulcerated melanomas also derived a benefit in the adjuvant ipilimumab setting (van Akkooi et al. 2008). This is in contrast to the total lack of benefit in IFN trials, which has also recently been confirmed in the individual patient data (IPD) meta-analysis of all adjuvant IFN versus observation trials (Suciu et al. 2014).

17.2.3 Combination Therapies with Ipilimumab

Various combinations of ipilimumab with other immune-modulating, anti-angiogenic or chemotherapeutic, or targeted agents have been reported or are ongoing. Guiding principles for combination treatment designs could be to use drugs that lead to immunogenic cell death (Kroemer et al. 2013; Vacchelli et al. 2014a; Galluzzi et al. 2012; Zitvogel et al. 2013). Since radiotherapy can also induce immunogenic cell death, the reported observation of abscopal antitumor effects after radiotherapy and ipilimumab has led to a number of clinical studies to further investigate this phenomenon (Postow et al. 2012).

17.2.3.1 Chemotherapy

Three studies regarding the combination of chemotherapy with ipilimumab in melanoma patients have been published thus far.

1. *Dacarbazine*: A phase III trial comparing DTIC versus DTIC plus ipilimumab at 10 mg/kg in first-line in patients with advanced melanoma showed a survival benefit for patients treated with the combination (Robert et al. 2011). The median benefit of only 2.1 months was, however, disappointing and the combination is not believed to bring a benefit over ipilimumab alone.
2. *Fotemustine*: In an open-label, single-arm phase II trial, 86 patients with advanced melanoma, 20 of them with asymptomatic brain metastases, received induction treatment of 10 mg/kg intravenous ipilimumab every 3 weeks for a total of four

doses, and 100 mg/m^2 intravenous fotemustine weekly for 3 weeks and then every 3 weeks from week 9 to week 24 (Di Giacomo et al. 2012). Patients with a confirmed clinical response were eligible for maintenance treatment from week 24, with ipilimumab every 12 weeks and fotemustine every 3 weeks. Forty patients (46.5 %) in the study population achieved disease control, as did 10 patients with brain metastases (50 %). Toxicity was considerable with 47 patients (55 %) having grade 3 or 4 treatment-related adverse events.

3. *Carboplatin/taxol*: Very preliminary results of a randomized phase II trial comparing concurrent carboplatin plus paclitaxel and ipilimumab (four doses at 3 mg/kg) with sequential treatment of these agents were reported recently (Jamal et al. 2014). In 31 patients, response rates (RR) and disease control rates (DCR) for 14 evaluable patients at 24 weeks were 21.4 % and 42.9 % by mWHO, and 35.7 % and 64.3 % by irRC, respectively. Grades 3 to 4 AEs were observed in 63 % of patients.

17.2.3.2 Antiangiogenic Agents

Bevacizumab Four dosing cohorts of ipilimumab (3 or 10 mg/kg) with four doses at 3-week intervals and then every 12 weeks, and bevacizumab (7.5 or 15 mg/kg) every 3 weeks, were studied in 46 patients with metastatic melanoma (Hodi et al. 2014a). There were 8 PRs and 22 SDs, and a disease control rate of 67.4 %. Median survival was 25.1 months. Extensive CD8(+) and macrophage cell infiltration were observed in on-treatment tumor biopsies. From this initial experience, it appears that the combination of bevacizumab and ipilimumab can be safely administered. VEGF-A blockade influences inflammation, lymphocyte trafficking, and immune regulation that should be studied further.

17.2.3.3 Cytokines

1. *Interleukin-2 (IL-2):* The most mature data on the combination of IL-2 and ipilimumab regard 36 patients treated at the NCI Surgery Branch (Prieto et al. 2012). There were six complete responders (17 %), which was higher than the 6 % CR rate in 56 patients treated with ipilimumab alone and the 7 % CR rate among 85 patients who received ipilimumab with gp100 peptide vaccination. All CRs except one were ongoing at 54+ to 99+ months at the time of the report. The combination with IL-2 did not seem to increase toxicity. The combination with IL-2 should be explored further.
2. *Interferon-alpha (IFN):* The first phase II trial report on the combination of IFN was a study with the anti-CTLA4 drug tremelimumab (Tarhini et al. 2012). In this study, 37 stage IV melanoma patients received tremelimumab 15 mg/kg/course (three cycles [one cycle = 4 weeks]) intravenously every 12 weeks. High-dose interferon alfa-2b (HDI) was administered concurrently, at 20 MU/m^2/day i.v. for 5 days/week for 4 weeks followed by 10 MU/m^2/day s.c. three times a week for 8 weeks per course. In 35 evaluable patients, overall response rate was 24 % (four CRs and five PRs), 38 % SD, with a median progression-free survival of 6.4 months and a median overall survival of 21 months. These results seemed to indicate additive antitumor activity.

3. *Pegylated-IFN*: In 31 patients, ipilimumab (3 mg/kg for four doses) was administered in combination with peg-interferon alfa-2b at 3 mcg/kg weekly for up to 156 weeks (Kudchadkar et al. 2014). Among 26 evaluable patients, there were two CRs, nine PRs, three SDs, and twelve PDs. Peg-interferon alfa-2b added to ipilimumab resulted in a response rate of 42.3 % and was well tolerated except for a high grade 3 rash rate of 20 %. The combination warrants further exploration.
4. *GM-CSF:* In a randomized phase II trial, conducted by ECOG in 245 patients with unresectable stage III/IV melanoma, ipilimumab plus GM-CSF (sargramostim) treatment was compared with ipilimumab alone (Hodi et al. 2014b). Patients received ipilimumab at 10 mg/kg, intravenously on day 1 plus sargramostim, 250 μg subcutaneously, on days 1–14 of a 21-day cycle versus ipilimumab alone. Ipilimumab treatment included induction for four cycles followed by maintenance every fourth cycle. At a rather short median follow-up of 13.3 months, overall survival was superior for the combination treatment (17.5 months versus 12.7 months), the 1-year survival rates were 68.9 % versus 52.9 %. Surprisingly, no differences for PFS were observed (median PFS of 3.1 months for both treatment arms). The combination treatment was associated with less toxicity. Further studies are needed to elucidate these observations, which is true for all combinations with cytokines (Vacchelli et al. 2014b).

17.2.3.4 Vaccines

1. *gp100 vaccines*: Theoretically, a combination of a vaccine with anti-CTLA4 is very attractive. Yet the results from the RCT comparing ipilimumab versus ipilimumab plus gp100 vaccine versus gp100 vaccination alone did not show a benefit for the combination of ipilimumab plus the vaccine compared to ipilimumab alone (Hodi et al. 2010) and similar observations were made with the mature results of the NCI Surgery Branch experience (Prieto et al. 2012).
2. *Laherparepvec (T-VEC):* The first combination study of ipilimumab with the vaccine laherparepvec (T-VEC) was reported at the 2014 ASCO annual meeting (Puzanov et al. 2014a). In 17 patients, the response rate was 41 % (24 % CR, 18 % PR); and 35 % had SD. Median time to response was 2.9 months. No DLTs were reported. Grade 3/4 AEs occurred in 32 %, with only two patients having irAEs at grades 3/4. These very preliminary results seem promising, but more mature data are awaited.

17.2.4 BRAF and MEK Inhibitors

Combinations of BRAF inhibitors and MEK inhibitors with immune checkpoint inhibitors such as anti-CTLA are theoretically attractive, but have, in practice, proven to be not so simple to develop.

1. *Vemurafenib*: A phase I trial combining vemurafenib and ipilimumab was stopped early, after only 11 patients, because of several cases of grades 3–4 hepatitis (Ribas et al. 2013).

2. *Dabrafenib + Trametenib*: A phase I trial with dabrafenib + ipilimumab did not evoke a high rate of hepatitis, and an expansion cohort is ongoing (Puzanov et al. 2014b). However, the combination of dabrafenib + trametenib + ipilimumab phase I study was stopped because of life-threatening colitis in three of the first seven patients (Puzanov et al. 2014b).

17.2.5 Anti-PD1 and Anti-PDL1

PD1 protein is another immune checkpoint expressed in many tumor-infiltrating lymphocytes in response to inflammation. It has two ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). The engagement of PD1 on the lymphocyte surface by PD-L1 on melanoma cells delivers inhibitory signals down-regulating T-cell function (Topalian et al. 2012a). Remarkable results of phase I trials evaluating two anti-PD1 antibodies (nivolumab and pembrolizumab) reported response rates of 30 % (Topalian et al. 2012b; Robert et al. 2014). Anti-PD-L1 antibody also gave an encouraging long-term response rate of 17.3 % in melanoma patients in a phase I study (Brahmer et al. 2012). Importantly, the safety profile is very favorable compared to ipilimumab, with much lower rates of irAEs, in particular the troublesome colitis and hypophysitis. Both pembrolizumab and nivolumab have been reported to induce response rates around 30 % in advanced melanoma patients, even in patients that previously failed ipilimumab (Hamid et al. 2013; Topalian and Sznol 2014). Responses tend to be very durable, up to 2 years. Moreover, PDL-1 expression in the tumor is a good biomarker for response for monotherapy with either agent. Nivolumab proved to be vastly superior dacarbazine in first-line in a RCT in 418 patients with advanced non-BRAF-mutant melanoma (Robert et al. 2015a). Pembrolizumab proved to be superior to therapy of choice in ipilimumab failures (Ribas et al. 2015). Moreover, in a cohort of 655 patients treated with pemborlizumab it was demonstrated that response rates in BRAF wild-type patients and in BRAF-mutant patients are similar (45 % and 50 %, respectively) (Daud et al. 2015). Moreover, pembrolizumab has been shown to be superior to ipilimumab in a phase III trial (Robert et al. 2015b). Overall, it leads to the conclusion that anti-PD1 can be considered to be proposed to all patients with advanced melanoma in first-line, irrespective of mutational status, perhaps with the only exception of patients with bulky rapidly progressive BRAF-mutant melanoma. However, the incredible impact of anti-PD1 and anti-PDL1 monoclonal antibodies lies in its broad transversal impact in oncology, now with activity demonstrated against a wide panel of neoplasms other than melanoma, including lung cancer, renal cell cancer, bladder cancer, stomach cancer, head and neck cancer, ovarian cancer, and colorectal cancer with microsatellite instability and Hodgkin lymphoma (Lorenzo Galluzzi et al. 2014).

17.2.6 Anti-PD1 Plus Anti-CTLA4

Very impressive data have been reported on the efficacy of the combination of ipilimumab and nivolumab in the last 2 years (Wolchok et al. 2013; Sznol et al. 2014). The rationale to combine these two checkpoint inhibitors is that they have different

mechanisms of action, with anti-CTLA4 mainly acting at the central level in the lymph node compartment, perpetuating and/or restoring the induction and proliferation of activated T-cells, and with anti-PD1 mainly acting at the peripheral level at the tumor site, preventing the neutralization of cytotoxic T cells by PDL1 expressing tumor cells and PDL2 expressing plasmoid dendritic cells in the tumor infiltrate. Very deep and long-lasting responses are observed, and in the update on the current experience presented by Sznol et al. at the 2014 ASCO annual meeting, with impressive survival rates of >90 % at 1 year and >80 % at 2 years in advanced melanoma patients (Sznol et al. 2014). In 2015, the RCT comparing nivolumab + ipilimumab versus nivolumab versus ipilimumab in advanced melanoma patients was published and demonstrated that the combination is superior to either monotherapy and that nivolumab alone is superior to ipilimumab regarding PFS (Larkin et al. 2015). The trial is not mature regarding OS data. Importantly, patients with PDL1-positive tumors seemed to benefit equally from nivolimumab monotherapy compared with combination therapy. PDL-1-negative patients had the best results with the combination therapy. It will be very interesting to have the mature results of this trial in 1–1.5 years' time. Clearly, all these results are unprecedented in the melanoma world and demonstrate the power of the current concepts of breaking tolerance.

Immunotherapy combinations in general are expected to be perhaps the most dynamic drug development field for years to come. Once breaking tolerance is achieved, or even further improved with candidate molecules such as anti-LAG3 and others, the door is open to combine with agonists such as OX40, CD137, and others. Deepening breaking tolerance and combining with various agonistic approaches is a complex scenario to work out, but obviously, smart immune combos are the future (Eggermont and Robert 2014).

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A. Bosserhoff (ed.), *Melanoma Development*,
DOI 10.1007/978-3-319-41319-8

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