Chapter 10
The Role of TGF-β in Cutaneous Melanoma Biology

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Abstract Ample evidence indicates that transforming growth factor-β (TGF-β) signaling plays a major role at various stages of carcinogenesis. While it may represent a tumor suppressor pathway at early stages of cancer progression, essentially due to its cytostatic activity, it has also become clear that TGF-β may act as a potent tumor promoter via both autocrine and paracrine mechanisms: TGF-β enhances tumor cell migration and homing to various metastatic sites, allows tumor escape from the immune system and promotes peri-tumoral vasculogenesis. This chapter reviews the current literature on the implication of TGF-β signaling in melanoma.

Keywords Invasion • Melanoma • Metastasis • TGF-β

10.1 Introduction

Melanomas arise from the transformation of melanocytes, cells that derive from melanoblasts in the neural crest that migrate and differentiate before localizing essentially in the hair follicles and in the epidermis. They produce melanin and are responsible for the pigmentation of hair and skin. Premalignant melanocytic lesions include clinicopathologic entities such as lentigo, nevi, or dysplastic nevi (Chin et al. 2006) Melanoma is the most lethal skin cancer and its incidence increases faster than any other malignancy. Although early stage melanoma patients can be treated successfully by surgical resection of the primary tumor, the prognosis for metastatic disease is dismal with an overall 5-year mortality rate of 90 %: melanomas are highly aggressive tumors that exhibit strong metastatic potential together

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with high resistance to common anticancer treatments, including chemotherapy, interleukin-2-based immunotherapy, and a combination of chemotherapy and immunotherapy.

The key malignant transition is thought to occur from radial growth phase (RGP) to vertical growth phase (VGP) of primary melanoma lesions, which results in expansive infiltration of the dermis and its constituent structures, such as blood and lymphatic vessels, presaging distant metastasis.

Genetic alterations occur early during melanoma development and contribute to melanoma development. They include inactivation of the *INK4a/ARF* melanoma susceptibility locus, activating mutation of *NRAS*, *BRAF*, and *cKIT* oncogenes, alteration/loss of *PTEN*, *p53* mutations and *CDK4* mutations [see Table 10.1 and (Dutton-Regester and Hayward 2012; Flaherty et al. 2012; Tsao et al. 2012)].

Targeted therapeutic strategies have emerged over the recent years, accompanied with new hopes to cure patients with advanced melanoma. For example, Vemurafenib (Plexxikon/Roche) or GSK2118436 (GlaxoSmithKline) are highly selective and very potent mutated BRAF inhibitors. BRAF is the most commonly activated oncogene in melanoma, with approximately 50 % of advanced melanomas harboring such mutations. About 80–90 % of the BRAF mutations found in melanoma consist of an activating substitution of glutamate for valine at position 600 of the kinase domain within the BRAF amino-acid sequence ($BRAF^{V600E}$). In a randomized phase III trial comparing Vemurafenib to the commonly used chemotherapeutic drug, Dacarbazin, Vemurafenib treatment led to a decrease of 63 % for relative risk of death and to a significant increase in overall survival (+6 months). However, some patients presented resistance to this BRAF inhibitor and new devastating progression of the disease. Identification of drug resistance mechanisms developed by tumors and rapid preclinical and clinical testing of strategies to overcome such resistance are under investigation. Ongoing trials are combining BRAF and MEK inhibitors with the goal of delaying or overcoming resistance as a result of reactivation of the MAPK pathway [reviewed in (Flaherty et al. 2012), ASCO 2012, http://chicago2012.asco.org/Abstracts.aspx]. Another drug, Ipilimumab, a blocking antibody targeting CTLA4 (Cytotoxic T lymphocyte-associated antigen4), a protein involved in the differentiation of immunosuppressive regulatory T cells, has been tested in stage IV melanoma patients. Initial results indicate a clear increase in overall survival (ASCO 2012, http://chicago2012.asco.org/Abstracts.aspx).

Given the heterogeneity of genetic alterations found in melanoma patients and the weak success of actual therapeutic treatments coupled with exacerbated resistance mechanisms, further efforts have to be realized in developing targeted therapies. In this context, numerous preclinical studies have provided convincing evidence that TGF-β and its cognate signaling cascade play a major role in the progression of malignant melanoma to metastasis and could therefore represent a relevant target for preventing melanoma development. This chapter summarizes the literature on the pleiotropic activities of TGF-β in cutaneous melanoma biology (Fig. 10.1).

Table 10.1 Selected genetic alterations in malignant melanoma thought to be involved in melanomagenesis

Gene	Functions/pathways affected	Alteration in melanoma (frequency)
Signaling factors		
BRAF	Oncogene/MAPK signaling	Point mutation (50 %)
NRAS	Oncogene/MAPK signaling	Point mutation (20 %)
MEK	Oncogene/MAPK signaling	Point mutation (1–2 %)
KIT	Oncogene/MAPK and PI3K signaling	Point mutation (<1 %)
ERBB4	Oncogene/PI3K signaling	Point mutation (15–20 %)
PTK2B	Oncogene/MAPK signaling	Point mutation (2.5 %)
NEDD9	Melanoma metastasis gene	Amplification (55–60 %)
AKT1, AKT2, AKT3	Oncogene/PI3K signaling	Point mutation (<1 %) or amplification (25 % for AKT3)
PTEN	Tumor supressor/PI3K signaling repression	Point mutation (50–60 %)/ hemizygous deletion (50–60 %) homozygous deletion (10 %)
CTNNB1	Cell adhesion/transcriptional co-activator	Point mutation (30 %)
EGFR	Oncogene/β-catenin and MAPK signaling	Amplification associated with polysomy 7 (80 %)
MET	Oncogene/promoter of cellular invasion	Amplification (47 %)
Cell cycle and apoptosis regulators		
CDK4	Promoter of cell proliferation	Point mutation or amplification (5 %)
CDKN2A/INK4A	Tumor supressor-cell cycle inhibitor	Point mutation or deletion (30 %)
CCND1	Promoter of cell proliferation	Amplification (10 %)
TP53	Tumor supressor- cyle inhibitor/ apoptosis inducer	Point mutation (5 %)
APAF1	Apoptosis inducer	Silenced/promoter inactivation (40 %)
Transcription factors		
MITF	Melanocyte-specific lineage transcription factor	Amplification (20 %)
ETV1	Oncogene/MAPK signaling and MITF induction	Amplification (10–20 %)
MYC	Oncogene/Promoter of cell proliferation	Amplification (20 %)

From Flaherty et al. (2012), Meyle and Guldberg (2009), Miller and Mihm (2006)

10.2 TGF-β Signaling Molecules in Melanoma

Increased expression and secretion of the different TGF-β isoforms in melanoma cell lines when compared with normal melanocytes has been reported by several studies (Albino et al. 1991; Krasagakis et al. 1994; Rodeck et al. 1991; 1994).

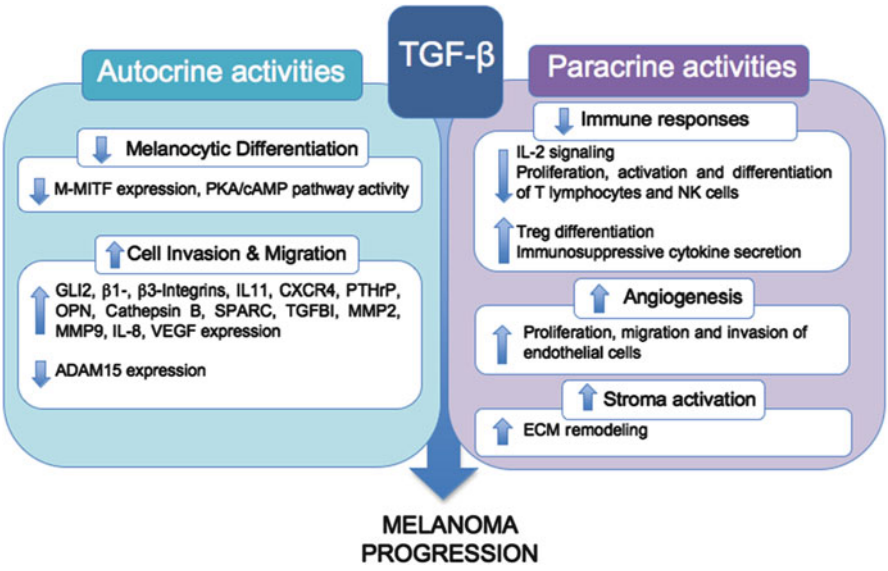

Fig. 10.1 Multiple roles of TGF-β in promoting melanoma development

In situ, TGF-β1 is secreted by normal melanocytes and melanomas at various stages, while TGF-β2 and TGF-β3 are not expressed in normal melanocytes but only heterogeneously in nevi and melanoma. Expression of TGF-β2 and TGF-β3 seems to appear early in melanoma progression and to increase with tumor progression (Van Belle et al. 1996). A correlation between TGF-β2 expression levels and tumor thickness has also been reported (Reed et al. 1994). Similarly, increased TGF-β1 and TGF-β2 plasma levels are observed at later stages of tumor development, while no significant differences have been reported between those of healthy patients and those from patients with primary or locally invasive melanoma (Krasagakis et al. 1998). Thus despite some discrepancies, all these studies point toward an increase in TGF-β expression levels that correlates with tumor progression.

Interestingly, TGF-β treatment of melanoma cells induces expression of TGF-β1 and TGF-β2 in a positive amplification loop. Moreover, patients with MMP-1 positive metastases had significantly shorter disease-free survival compared to patients with MMP-1 negative metastases (median 11.2 vs. 17.0 months, $p = 0.0383$) (Nikkola et al. 2002) and MMP-1 secreted by melanoma cell was shown to be involved in the activation of TGF-β from its latent form (Iida and McCarthy 2007).

Expression of TGF-βII receptor (TβRII) mRNA is much more heterogeneously distributed in primary melanomas compared with benign melanocytic nevi. Melanoma progression appeared not to be associated with a complete loss of *TβRII* gene expression, since all skin metastases revealed clearly detectable although heterogeneous levels of TβRII mRNA expression (Schmid et al. 1995). Melastatin (TRPM1), a member of the transient receptor potential (TRPM) cation channel

family, is robustly expressed in benign and dysplastic nevi and in melanomas in situ, but it is only variably expressed in invasive melanomas. Melastatin expression levels show widespread downregulation in melanoma metastases and are inversely correlated with metastatic potential and good prognosis in melanoma (Duncan et al. 1998; 2001). Intron 6 of the *TRPM1* gene hosts the gene for miR-211, a microRNA whose expression is restricted to the melanocyte lineage (Gaur et al. 2007). miR-211 and melastatin share the same promoter and are expressed coordinately in melanocytes and melanomas. It has been described that TβRII is one of the direct targets of miR-211 (Levy et al. 2010), suggesting that epigenetic events may control the expression and activity of the TGF-β in melanoma cells during disease progression.

CD105/endoglin, a co-receptor associated with TβRII that modulates the activity of the latter, is heterogeneously expressed in small clusters of tumor cells within primary melanomas and in intradermal nevi and metastatic melanoma lesions (Altomonte et al. 1996; Pardali et al. 2011).

Several studies have described a functional Smad signaling in melanoma. Basal phosphorylation of Smad3 is detectable in vitro by western blot analysis of whole melanoma cell extracts, demonstrating a constitutive activation of the TGF-β receptors. Furthermore, a pan-TGF-β antibody was shown to inhibit basal Smad3/Smad4-dependent transcriptional activity in melanoma cells (Javelaud et al. 2007; Rodeck et al. 1999). Also, exogenous TGF-β was shown to induce further a Smad3/Smad4 transcriptional response, demonstrating the functionality of the entire signaling cascade (Javelaud et al. 2007; 2008; Rodeck et al. 1999). Frequent nuclear phosphorylation of Smad2 has been detected in clinical specimens of benign and malignant cutaneous melanocytic neoplasms (Lo and Witte 2008), suggesting that the TGF-β pathway is constitutively activated in melanomas. Yet, another study showed that Smad2 phosphorylation correlated with low tumor thickness but not with overall survival and development of metastases suggesting that it would not be a useful prognostic marker (Mnich et al. 2007). This indicates the need for further studies to characterize both the phosphorylation and cellular localization of Smad2, Smad3, and Smad4 in clinical samples to determine their exact activation state throughout the various stages of melanoma progression. Interestingly, it has also been shown that phosphorylation of Smad2 in melanoma cells is associated with Smad2 phosphorylation in neighboring keratinocytes, indicating that melanoma-derived TGF-β affects the microenvironment and adjacent cells in a paracrine fashion (Mnich et al. 2007). Genome-wide expression analysis of nearly a hundred human melanoma cell lines has identified two groups with very distinct gene expression profiles: the first one is characterized by high expression of neural crest and melanocytic differentiation markers, the other one is characterized by the expression of a number of genes associated with a more aggressive phenotype, whose concomitant expression is reminiscent of a TGF-β signature (Hoek et al. 2006). These two groups present very distinct behaviors: the melanocytic differentiation signature is associated with strong proliferation but weak invasive capacity, while the TGF-β signature characterizes highly invasive melanoma cell lines with low proliferation rate (Hoek et al. 2006).

10.3 Resistance to TGF-β Induced Cell Cycle Arrest in Melanoma Cells: A Complex, Somewhat Unresolved Issue

TGF-β exerts potent anti-proliferative activity on normal melanocytes but has weak or no effect on melanoma cells. During tumor progression, melanoma cells acquire a growing resistance to TGF-β-dependent growth inhibition (Krasagakis et al. 1999). The latter may be explained by attenuation or inhibition of the TGF-β pathway in these cancer cells. For example, CD105 expression is correlated with cell sensitivity to TGF-β treatment, suggesting that expression of this co-receptor may modulate the cellular response to TGF-β (Altomonte et al. 1996). Recently, Yilmaz et al. described that TGF-β induction of DLX-2 (Distal-less homeobox) attenuates growth suppressive TGF-β signaling in a negative feedback loop (Yilmaz et al. 2011). Expression of filamin, a cytoskeletal actin–binding protein, has also been suggested to repress TGF-β signaling by direct interaction with Smad2 and Smad4 (Sasaki et al. 2001). SKI and SNON oncoproteins are direct repressors of Smad2/3 transcriptional activity and are highly expressed in melanoma cells lines as well in melanoma samples (Javelaud et al. 2011; Reed et al. 2001). In some melanoma cell lines, downregulation of SKI expression using antisense SKI vectors or shRNA restored TGF-β-mediated growth inhibition through $p21^{CIP1}$ induction and inhibited subcutaneous tumor growth in xenograft experiments (Chen et al. 2009; Reed et al. 2001). MIA (melanoma inhibitory activity) is a secreted protein expressed in melanomas but not in melanocytes and is associated with tumor progression in vivo (Bosserhoff et al. 1997). Rothhammer et al. have shown that MIA induces SKI and SNO expression, leading to reduced Smad2/3 expression in melanoma cells (Rothhammer and Bosserhoff 2006).

Despite overexpression of different proteins known to repress TGF-β signaling, mechanisms of resistance to TGF-β-induced cell growth arrest are likely more complicated than due to a single inhibitory protein. Autocrine activation of the pathway in melanoma cell has been documented in a variety of settings (Lo and Witte 2008; Rodeck et al. 1999). High phosphorylation levels of Smad3 and Smad2 linker domain phosphorylation have been described in both melanoma cell lines and clinical melanoma specimens (Cohen-Solal et al. 2011). The latter phosphorylation event specifically inhibits the expression of the cell cycle inhibitors $p21^{CIP1}$ and p15, and resulting cell growth arrest by TGF-β, but does not alter the induction of other TGF-β target genes such as PAI-1 (Cohen-Solal et al. 2011). At this stage, the mechanisms responsible for Smad2 linker domain phosphorylation are not fully understood and several studies have implicated high levels of SKI, GSK3, CDK, and MAPK (p38, JNK and ERK) in the phosphorylation of Smad2/3 linker domains (Cohen-Solal et al. 2011; Lin et al. 2010). In particular, melanomas are characterized by hyperactivity of the MEK/ERK cascade, often induced by activating mutations of the BRAF and NRAS oncogenes (Chin et al. 2006; Flaherty et al. 2012; Tsao et al. 2012). ERK activation may contribute to the phosphorylation of Smad2/3 linker domains and elicit TGF-β-induced growth arrest resistance.

Shellman et al. have shown that forced overexpression of NRAS in primary melanoma cell lines counteracts TGF-β-induced growth inhibition by preventing the accumulation of hypophosphorylated Rb protein (Shellman et al. 2000).

Another important protein that may be implicated in the loss of growth inhibition by TGF-β is Id2, a positive regulator of cell growth and plays a critical role prompting G1/S cell cycle progression. Id2 inhibits CDK inhibitors expression by interfering with bHLH, ETS and PAX transcription factor activity and growth suppressing activity of pRb by direct interaction. Id2 is negatively regulated by TGF-β, yet Schlegel et al. have shown a differential inhibition of Id2 by TGF-β in melanoma cells, depending on whether they are of the proliferative or invasive type (Schlegel et al. 2009).

During melanoma initiation and progression, a number of genetic and epigenetic alterations occur, including amplification of c-MYC or cyclin D1 overexpression, hyperactivation of CDK4 due to loss of the CDK inhibitor p16 function or activating mutations in the CDK4 gene, alterations in the activity of CDK inhibitors such as $p21^{CIP1}$, p27, or p15. All of them may participate in a cellular activation state whose endpoint is an attenuation of the TGF-β anti-proliferative effects.

PAX3, a paired-box transcription factor involved in melanocyte differentiation and survival, could also participate in resistance to TGF-β-induced cell growth arrest. Its forced overexpression in melanoma cells attenuates TGF-β anti-proliferative activity and TGF-β represses PAX3 expression (Yang et al. 2008).

10.4 Promotion of Melanoma Aggressiveness by Autocrine TGF-β Signaling

Melanoma cells secrete high levels of TGF-β ligands and most of them are largely insensitive to their anti-proliferative activity. Autocrine TGF-β is also involved in a number of additional cellular processes, as it can act on tumor cells and their microenvironment by autocrine and paracrine mechanisms that contribute to tumor development and aggressiveness.

Cell autonomous effects of TGF-β in epithelial tumor cells have been long identified. In particular, this pathway is involved in the promotion of epithelial to mesenchymal transition (EMT), remodeling of extracellular matrix, cell migration, and cell survival (Tian et al. 2011). EMT takes place during essential processes of embryogenesis such as gastrulation. Coordinated modifications in gene expression promote cell migration through changes in the expression of cell–cell adhesion molecules of the cadherin family. Consequently, epithelial cells acquire a fibroblast-like phenotype (Davies et al. 2002). This phenomenon is also observed during the metastatic development of epithelial tumors. Melanoma derives from the neuroectoderm, yet melanoma cells undergo gene expression changes and acquire a fibroblast-like phenotype similar to what is observed in epithelial tumor. This melanoma-specific phenomenon may be qualified a pseudo-EMT.

In melanoma, TGF-β has been shown to promote tumor invasion via induction of β1- and β3-integrins and inhibition of *CDH1*, promoting pseudo-EMT (Janji et al. 1999). There is also strong evidence for a direct role of autocrine TGF-β signaling in melanoma development and progression. For example, overexpression of the inhibitory Smad7 in melanoma cells reduces their capacity to form colonies in an anchorage-independent manner, dramatically inhibits the secretion of matrix metalloproteinases MMP-2 and MMP-9 and their capacity to invade Matrigel, and delays tumor growth in a subcutaneous injection model in *nude* mice (Javelaud et al. 2005). Furthermore, inhibition of constitutive TGF-β activity by Smad7 overexpression dramatically reduced experimental bone metastasis development in mice, by preventing TGF-β induction of genes known to mediate osteolytic breast cancer bone metastases, such as IL-11, PTHrP, CXCR4, and OPN (Javelaud et al. 2007). In a model of in vivo human skin grafting onto mice, it has been observed that overexpression of Smad7 in melanoma cells blocks invasion with melanoma cells being kept in close proximity to the dermal-epidermal junction (DiVito et al. 2010). Smad7 was found to increase N-cadherin expression in melanoma cells, thus promoting homotypic interactions between melanoma cells and dermal fibroblasts. Smad7 overexpression also inhibited β-catenin T41/S45 phosphorylation, resulting in the stabilization of β-catenin (DiVito et al. 2010). Of note, increased cytoplasmic/nuclear β-catenin expression has been linked with good prognosis and increased survival of melanoma patients (Chien et al. 2009).

Treatment of melanoma cell lines with SD-208, a highly specific small molecule inhibitor of TβRI, blocks in vitro TGF-β induction of Smad3 phosphorylation, TGF-β induced Smad3/Smad4–driven transcription, as well as invasion through Matrigel. SD-208 was also shown to reduce the development of experimental osteolytic bone metastases following intracardiac inoculation of tumor cells in *nude* mice (Mohammad et al. 2011). SD208 was also found to reduce the growth of established bone metastases in the same model. Together with the data obtained with Smad7 overexpression, these experiments demonstrate that inhibition of constitutive activation of TGF-β signaling may represent a valuable therapeutic option to prevent melanoma progression to metastases and inhibit the growth and dissemination of existing metastases.

We have identified the transcription factor GLI2 as a direct transcriptional target of TGF-β signaling in a variety of normal and transformed cell lines, including melanoma (Dennler et al. 2007; 2009). GLI2 is known to be a mediator of the Sonic Hedgehog pathway and numerous studies have established its role in cancer development (Javelaud et al. 2012). In melanoma cells, we found that GLI2 regulates the invasive capacity via downregulation of E-cadherin, a protein that plays a critical role in maintaining melanocyte interactions with epidermal keratinocytes, and upregulation of metalloproteinases, MMP2 and MMP9, that contribute to basement membrane invasion by tumor cells (Alexaki et al. 2010). GLI2 has also been shown to induce the expression of proteins known to participate in the metastatic process, such as WNT5a (Dissanayake et al. 2008; Reddy et al. 2001) and PTHrP (Sterling et al. 2006).

Numerous studies described modulation of gene expression by the TGF-β pathway, participating in melanoma development. For example, TGF-β induces expression of integrins, proteases such as Cathepsin B, and that of matrix remodeling proteins such as SPARC involved in the promotion of cellular invasion and migration, and represses the expression of ADAM15 (A Disintegrin And Metalloproteinase) in cooperation with IFN-γ (Ungerer et al. 2010). ADAM15 is expressed in melanocytes and endothelial cells of benign nevi and melanoma tissues and is downregulated in melanoma metastases compared to primary melanoma lesions (Ungerer et al. 2010). It seems to have tumor suppressor function in melanoma, as tail vein injection of recombinant disintegrin domain of ADAM15 together with mouse melanoma cells reduces the formation of lung metastases in mice (Trochon-Joseph et al. 2004). Furthermore, overexpression of ADAM15 has been shown to reduce migration and invasive capacity of melanoma cells in vitro, probably driven by induction of α5β1 integrin expression and increased adhesion to fibronectin (Chen et al. 2008; Ungerer et al. 2010).

TGFBI (transforming growth factor-β induced) is a TGF-β1-inducible ECM protein that plays a role in the invasive growth of melanoma cells (Nummela et al. 2012). It is highly expressed during melanoma VGP and in metastatic cell lines, has anti-adhesive properties and promotes the growth of subcutaneous tumor xenografts in mice. TGFBI localizes at tumor edges, together with fibrillar fibronectin/tenascin-C/periostin structures that characteristically surround melanoma cells at the invasive front. Both in tumors generated in nude mice and in human melanoma metastases.

Genome wide expression analysis of a large series of human melanoma cell lines revealed distinct groups characterized by specific invasive and proliferative behaviors. On the one end, highly proliferative cell lines that express neural crest and melanocytic differentiation markers, such as MITF, TYR, and MLANA, were found, while another group was composed of cell lines that grow slower than the former, are more invasive, and display high expression of a set of genes controlled by the TGF-β pathway, including TGFBI, SERPINE1, and CTGF, to cite a few (Hoek et al. 2006). These extreme phenotypes were indistinguishable by their BRAF or NRAS mutation status. It thus appeared from this broad study that cell autonomous TGF-β activation is associated with a characteristic TGF-β gene signature together with an invasive cell behavior. The genetic context may still contribute to TGF-β-induced invasive behavior of melanoma cells, as it has been shown that forced TGF-β pathway activation in immortalized melanocytes via overexpression of a constitutively activated TβRI only promotes invasion into the dermis of organotypic human skin cultures in the presence of mutated $BRAF^{V600E}$ and PTEN deficiency (Lo and Witte 2008). This was also associated with increased levels and activities of MMP-2 and MMP-9.

One important point to keep in mind is that human melanoma tumors are, in essence, highly heterogeneous and present both invasive and proliferative regions simultaneously. These behaviors are described as reversible and may be under the control of signals originating in the tumor microenvironment (Hoek et al. 2008).

Both primary and metastatic melanomas contain a heterogeneous mix of more or less differentiated cells. Strikingly actively disseminating cells are more uniform with low levels of pigment and high levels of TGF-β2 expression (Pinner et al. 2009). Several lines of evidences have suggested that TGF-β ligands negatively control melanocyte differentiation. Melanocytes in the epidermis rarely proliferate and produce melanin pigments that protect the skin from deleterious ultraviolet light (UV), while melanocytes in the hair follicles repeatedly proliferate and differentiate for hair pigmentation in every hair cycle. The hair follicle repeats its cyclic regeneration and regression with alternating phases, anagen (growing phase), catagen (regressing phase), and telogen (resting phase) to regrow new hair (Fuchs 2007). The fates of follicular melanocytes, including their proliferation, differentiation, and death all happen in synchronization with hair cycle progression (Foitzik et al. 2000; Nishimura et al. 2002). The catagen phase is an apoptosis-driven process accompanied by terminal differentiation, proteolysis, and matrix remodeling. TGF-β plays an important role in catagen regulation as an inhibitor of keratinocyte proliferation and as an inducer of apoptosis (Foitzik et al. 2000; Soma et al. 2003; Tumbar et al. 2004). Melanocyte stem cells reside with keratinocyte stem cells in the hair follicle bulge enriched in active TGF-β. Melanocyte stem cell quiescence is preceded by activation of TGF-β signaling, loss of Ki67 expression, downregulation of melanogenic gene expression, and dramatic morphologic changes from dendritic shape into a slender, oval shape with shrinkage resulting from an increased nuclear/cytoplasmic ratio (Nishimura et al. 2010). Upregulation of TGF-β1/2 expression in the niche area and phospho-Smad2 expression were detected prior to the morphologic changes, downregulation of melanogenic genes, and loss of Ki67 expression by melanocyte stem cells. Activation of TGF-β signaling plays dual roles in melanocyte stem cell maintenance: (a), through inhibition of stem cell differentiation in the stem cell niche and (b), via induction of stem cell quiescence that requires BCL2 expression for cell survival (Nishimura et al. 2010). TGF-β may also prevent melanocyte maturation through a direct repression of M-MITF expression (Kim et al. 2004; Pierrat et al. 2012). M-MITF is a master transcription factor of the melanocytic lineage that controls cell survival, migration, and differentiation. This factor directly activates expression of melanogenic genes such as those encoding tyrosinase, TRP1, and TRP2. TGF-β inhibits M-MITF promoter activity by two different mechanisms: repression of CREB activity through an inhibition of cAMP/PKA signaling and induction of GLI2, the latter directly binding to the MITF promoter to repress transcription by mechanisms that remain to be identified (Pierrat et al. 2012). Proteolysis of tyrosinase has also been involved in melanogenesis inhibition by TGF-β (Martinez-Esparza et al. 1997).

TGF-β also induces dedifferentiation of melanoma cells and promotes cell migration and invasion, thereby promoting metastasis. This dedifferentiation is reversible, transient, and dynamic and probably dependent upon hypoxia and the tumor microenvironment (Hoek et al. 2008; Pinner et al. 2009).

10.5 The Role of Tumor-Derived TGF-β on the Microenvironment

Paracrine effects of TGF-β on surrounding cells in the tumor microenvironment may be advantageous for melanoma cells. Two main roles are to be pointed out. Suppressive effects of TGF-β on the immune system may allow tumor cells to escape from immune surveillance, and pro-angiogenic properties of TGF-β could support nutrition of the tumor and facilitate metastasis. In addition, stimulation of stromal cells by TGF-β leads to increased production of reciprocally paracrine-acting growth factors. Also, melanoma cells can modulate their surrounding stroma for their own benefits through the paracrine activity of TGF-β. Stimulation of production of ECM proteins such as collagen, fibronectin, tenascin, and α2-integrin, by stromal fibroblasts provides a scaffold for melanoma cells to adhere and migrate, leading to increased survival and metastasis formation (Berking et al. 2001). Finally, modulation of the expression of proteases and their inhibitors by TGF-β likely facilitates invasion by means of stromal remodeling.

Aberrant TGF-β signaling in mice results in large vascular and endothelial defects, demonstrating the central role of TGF-β in embryonic vascular morphogenesis and in the establishment and maintenance of vessel wall integrity (Goumans et al. 2003). TGF-β can regulate the initiation and development of new blood vessels as well as the maturation of newly formed vessels. These processes involve not only stimulation of proliferation, migration, and invasion of endothelial cells during initiation of angiogenesis but also increase in endothelial cell adhesion, basement membrane deposition and recruitment of pericytes, and vascular smooth muscle cells during vessel maturation (Tian et al. 2011). In melanoma cells, TGF-β enhances angiogenesis by activating the expression of proangiogenic factors such as Interleukin (IL)-8 and VEGF (Liu et al. 2005).

TGF-β exerts immunoregulatory functions by inhibiting the proliferation, activation, and differentiation of lymphocytes, as well as the natural killer and dendritic cell functions, all these events leading to the suppression of the antitumor immune response (Arteaga et al. 1993; Letterio and Roberts 1998; Wrzesinski et al. 2007). Noteworthy, transplantation of murine bone marrow expressing a dominant-negative TβRII in a syngeneic mouse model of melanoma leads to the generation of mature leukocytes capable of a potent antitumor response (Shah et al. 2002).

TGF-β also exerts repressory effects on IL-2 activity, the main activator of immune response: TGF-β inhibits the phosphorylation and activation of components of the JAK/STAT cascade downstream of the IL-2 receptor and expression of IL-2 targets genes such as c-MYC and Cyclin D2 (Bright et al. 1997; Nelson et al. 2003). TGF-β is also an important differentiation factor for regulatory T cells (Foxp3+ Treg), a subset of strongly immunosuppressive T cells often found in tumors, including melanoma.

MCP-1 (monocyte chemoattractant protein-1), a potent chemokine that recruits macrophages, is expressed by malignant but not normal melanocytes (Nesbit et al. 2001). Monocytes and macrophages infiltrate melanomas and produce factors

promoting tumor development and invasion, including immunosuppressive cytokines and angiogenic factors (Dirkx et al. 2006). IL-10 is also expressed in primary and metastatic melanoma lesions (Dummer et al. 1996) and elevated levels of IL-10 have been measured in sera of patients with advanced melanoma that correlate with tumor progression (Moretti et al. 2001; Nemunaitis et al. 2001). TGF-β increases both MCP-1 and IL-10 expression in melanoma cells, by means of a crosstalk between the Smad, AKT, and MAPK/ERK pathways. Thus, TGF-β enhances MCP-1 mediated monocyte/macrophage migration and IL-10 immunosuppressive functions (Diaz-Valdes et al. 2011).

10.6 Other TGF-β Family Members in Melanoma

While the above paragraphs have focused on TGF-β *stricto sensu*, other members of the TGF-β family of growth factors are also expressed and/or contribute to melanoma development. A brief overview of the cognate literature is provided below.

It has been shown that Activin inhibits the proliferation and induces apoptosis of primary melanocytes. Expression of Activin and its receptors has been detected in melanoma cell lines that show a response to exogenous Activin. Yet those cell lines were found to be resistant to Activin's anti-proliferative activity (Stove et al. 2004).

Melanoma cells, unlike normal melanocytes, express and secrete high level of Follistatin. Follistatin is a secreted protein that binds to activin and blocks activin-induced signaling (Stove et al. 2004). It was suggested that early during melanoma progression, secretion of Follistatin may protect against the cytostatic function of activin while, on the other hand, melanoma cells at a later stage may use activin, either autocrine or paracrine, to maintain a state of dedifferentiation and create a microenvironment supportive of melanoma growth.

Expression of multiple BMPs (bone morphogenetic proteins) including BMP-2, -4, -7, is upregulated in melanomas compared to nevi (Rothhammer et al. 2005), and expression of BMP-4 and BMP-7 was found to increase during disease progression. BMP-4 was found to be implicated in the control of the migratory and invasive properties of melanoma cells, as inhibition of BMP signaling by means of overexpression of the BMP inhibitor chordin, or by specific downregulation of BMP4 expression, resulted in a strong reduction of both invasive and migratory capacities of melanoma cells (Rothhammer et al. 2005), possibly via reduced MMP-1, 2, 3, and 9 expression (Rothhammer et al. 2008). Dido1 (Death inducer-obliterator 1) was recently identified as a direct and specific BMP target gene. This protein was first described as an early apoptosis regulator protein and as a tumor suppressor gene in hematological myeloid neoplasm (Jilaveanu et al. 2009). Upon apoptosis induction, Dido-1 is translocated into the nucleus and activates pro-caspase expression (Garcia-Domingo et al. 2003). It is highly expressed in the nucleus of melanoma cell lines derived from metastases (Braig and Bosserhoff 2013). Dido1 expression induced by BMP-4 signaling regulates anchorage independent growth, apoptosis resistance as well as the migratory and invasive capacities of melanoma

cells (Braig and Bosserhoff 2013). In melanocytes, BMP-4 inhibits melanogenesis by blocking Tyrosinase expression (Yaar et al. 2006). Accordingly, overexpression of Noggin, a BMP antagonist, in the epithelium of hair follicles in mice, induces a darker coat color compared to wild-type mice (Sharov et al. 2005). Inversely, BMP-2 promotes melanogenesis by inducing the expression of Tyrosinase (Bilodeau et al. 2001). In astrocytoma cells, BMP-2 also inhibits the expression of HGF (hepatocyte growth factor) (Chattopadhyay et al. 2004), a potent mitogenic, motogenic, and morphogenic cytokine for melanoma cells (Noonan et al. 2003).

While BMP7 is overexpressed in melanoma tissues, it may exert antitumor activities. For instance, it has been described that BMP7 inhibits the proliferation of both normal and malignant melanocytes, an effect that is alleviated by concomitant overexpression of Noggin (Hsu et al. 2008). Noggin expression is also correlated with an induction of Nodal and VEGF expression in a subset of but not all melanoma cells lines. Induction of these growth-promoting factors may participate in the restoration of proliferation (Hsu et al. 2008). Another study only found a minor effect of BMP7 on melanoma proliferation while potently inhibiting of both the migratory and invasive capacities of melanoma cells (Na et al. 2009).

Nodal expression is restricted to embryonic tissues, epithelial stem cells, and cancer cells. Nodal is absent in normal skin and only rarely detected in poorly invasive radial growth phase melanomas. Yet, it is observed in up to 60 % of cases of vertical growth phase primary melanoma lesions and melanoma metastases. Its expression correlates with melanoma progression and experimentally, its depletion in melanoma cells decreases anchorage-independent growth and plasticity, concomitant with a marked abrogation of tumorigenicity (Topczewska et al. 2006; Yu et al. 2010).

Cripto-1, a Nodal co-receptor, is also expressed in melanomas and contributes to invasion and proliferation of melanoma cells (De Luca et al. 2011). In addition to mediating Nodal signaling, Cripto-1 modulates signaling of other TGF-β family ligands, including GDF-1, GDF-3, Activin, and TGF-β.

MIC-1 (macrophage inhibitory cytokine-1)/GDF-15 (growth differentiation factor-15) is expressed in a high proportion of melanoma cell lines compared to normal melanocytes and was detected in all examined metastatic melanoma biopsies (Boyle et al. 2009). GDF-15 levels are also increased five- to sixfold in sera from patients with an advanced melanoma compared to healthy donors (Huh et al. 2010). Affymetrix analysis of microdissected fresh frozen melanocytic nevi and melanoma samples showed upregulated expression of GDF-15 in primary and metastatic melanomas compared to melanocytic nevi (Mauerer et al. 2011). Interestingly, progression-free survival in melanoma patients with low GDF-15 staining was significantly higher compared to patients with high GDF-15 expression (Mauerer et al. 2011). Inhibition of GDF-15 expression with a specific shRNA strongly reduced tumor growth in an in vivo mouse xenograft model (Boyle et al. 2009; Huh et al. 2010). Inhibition of GDF-15 was found to delay melanoma tumor vascular development, subsequently affecting tumor cell proliferation and apoptosis. Melanoma cells secrete GDF-15 that promotes directional blood vessel development, in combination with VEGF (Huh et al. 2010). Expression of GDF-15 in melanoma cells is directly controlled by the transcription factor M-MITF (Boyle et al. 2009).

GDF-3/ Vgr-2 expression is also correlated with the metastatic capacity of melanoma cell lines and its overexpression promotes the growth of implanted melanoma tumors in a syngeneic mouse model. Moreover, GDF-3 expression is accompanied by an increased expression of CD24/CD44, markers of melanoma stem cell-like cells/melanoma-initiating cells (Ehira et al. 2010).

Together, these studies demonstrate the complexity of the TGF-β ligand/signaling network in melanoma and warrants further investigations.

10.7 Therapeutic Targeting of TGF-β to Fight Melanoma

There is broad experimental evidence for the potential benefit of targeting TGF-β signaling for cancer treatment. Different strategies have been developed to inhibit the TGF-β pathway: inhibition of TGF-β ligands expression with antisense molecules, blocking of ligands with ligand traps, and interference with receptor signaling by means of small molecules. These have been extensively reviewed over the last few years (Connolly et al. 2012; Hawinkels and Ten Dijke 2011; Wrzesinski et al. 2007).

For melanoma treatment, several anti-TGF-β therapies have been studied and they clearly present promising results. Antisense molecule targeting mRNA encoding the TGF-β2, called Trabedersen or AP12009, has been tested in clinical phase I/II study in patients with advanced pancreatic, malignant melanoma, or colorectal cancer. Traberdersen is safe and well tolerated and has shown promising efficacy. For instance, enrolled melanoma patients ($n = 14$) had reached a median overall survival of 9.3 months at the time of publication (Oettle et al. 2012).

Because TGF-β is abundantly secreted by tumor cells or by their microenvironment during disease progression, monoclonal TGF-β neutralizing antibodies and soluble TβRII or TβRIII have been elaborated to trap TGF-β ligands. In preclinical studies, this strategy has shown encouraging results in particular for breast cancer treatment (Connolly et al. 2012). An anti-TGF-β monoclonal neutralizing antibody, GC1008 (Fresolimumab) is under evaluation in a clinical phase I/II study in patients with advanced melanoma. In 2008, 5/22 patients with advanced melanoma achieved stable disease or partial responses. The most frequently reported drug-related side effects were skin rash/lesions including eruptive non-malignant keratoacanthomas and squamous cell carcinomas (Morris et al. 2008).

A number of small molecule inhibitors have been developed that target TGF-β receptor kinases. The majority of TβRI/ALK5 inhibitors also block the related activin and Nodal receptors ACVR1B/ALK4 and ACVR1C/ALK7, but with reduced affinity (Fu et al. 2008; Inman et al. 2002; Tojo et al. 2005). Several studies have illustrated the power of these inhibitors in the cancer treatment. As an example, in a mouse model of melanoma derived-bone metastasis, we have demonstrated that treatment with SD-208, a TβRI inhibitor, significantly reduces the incidence and size of osteolytic lesions in mice with established bone metastases compared to vehicle-treated mice (Mohammad et al. 2011). Unfortunately, a major drawback of these pharmacologic compounds is their high cardiotoxicity in humans, which has taken most of them out of clinical trials (Orphanos et al. 2009).

As discussed above, TGF-β plays a central role in allowing tumor immune escape. Some new antitumor strategies therefore consist in a combination of anti-TGF-β treatment and a vaccine, as blockade of TGF-β activity markedly enhances immunotherapy in preclinical models (Penafuerte and Galipeau 2008). It has been shown that TGF-β inhibits GM-CSF-induced maturation of bone marrow-derived dendritic cells, as well as expression of MHC class II and co-stimulatory molecules. Also, GM-CSF-secreting autologous immune vaccines have shown very good response in terms of immune stimulation and survival duration. The efficacy of these vaccines has been improved with a combination of TGF-β inhibition by means of TGF-β2 anti-sense oligonucleotides or specific shRNA targeting the proprotein convertase furin to prevent the conversion of latent TGF-β into an active growth factor (Olivares et al. 2011; Senzer et al. 2012).

High doses of IL-2 are a somewhat conventional treatment for metastatic melanoma in the USA despite limited response. Interestingly, nanoscale liposomal polymeric gel (nanolipogels) delivery of a TGF-β inhibitor together with IL-2 in a sustained fashion to the tumor microenvironment was found to significantly delay tumor growth and increase survival in tumor-bearing mice, accompanied with an increase in NK and TIL (tumor infiltrating lymphocytes) activity (Park et al. 2012).

10.8 Concluding Remarks

There is broad evidence underlying the involvement of TGF-β signaling in melanoma progression and metastasis development. TGF-β acts not only on the tumor cells but also on their microenvironment including stromal fibroblasts, endothelial and immune cells. Preclinical studies have reported very potent effects of anti-TGF-β approaches for cancer therapy. However, the effects of anti-TGF-β drugs have been less robust than hoped for in the clinical setting, one reason being their strong cardiotoxic activity. As numerous studies have exemplified the crosstalks between MEK/ERK and TGF-β pathways in epithelial cancers, it will be interesting to study the effect of TGF-β inhibitors in combination with the new melanoma drugs specifically targeting mutant BRAF or MEK. One may hope that combinatorial therapies will allow lower dosage to be used to circumvent drug toxicity and increase the clinical efficacy of TGF-β inhibitors.

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