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REVIEW

Resistance to transforming growth factor β-mediated tumor suppression in melanoma: are multiple mechanisms in place?

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Resistance to transforming growth factor (TGF) β-mediated tumor suppression in melanoma appears to be a crucial step in tumor aggressiveness since it is usually coupled with the ability of TGFβ to drive the oncogenic process via autocrine and paracrine effects. In this review, we will focus mainly on the mechanisms of escape from TGFβ-induced cell cycle arrest because the mechanisms of resistance to TGFβ-mediated apoptosis are still essentially speculative. As expected, some of these mechanisms can directly affect the function of the main downstream effectors of TGFβ, Smad2 and Smad3, resulting in compromised Smad-mediated antiproliferative activity. Other mechanisms can counteract or overcome TGFβ-mediated cell cycle arrest independently of the Smads. In melanoma, some models of resistance to TGFβ have been suggested and will be described. In addition, we propose additional models of resistance taking into consideration the information available on the dysregulation of fundamental cellular effectors and signaling pathways in melanoma.

Role of transforming growth factor β in melanoma progression and metastasis

Transforming growth factor (TGF) β levels are elevated in the plasma of melanoma patients, especially those with metastatic lesions (1). In addition, TGFβ2 expression appears to be increased coincident with the development of invasive melanoma (2). Another study reported increased expression of TGFβ1, β2 and β3 proteins in invasive primary melanomas and in metastatic nodules as compared with normal skin melanocytes (3). Cultured melanoma cell lines derived from primary and metastatic tumors constitutively secrete all three TGFβ isoforms (4–7). Interestingly, treatment of a panel of human melanoma lines by exogenous TGFβ1 further increased the secretion of active TGFβs (TGFβ1 and β2), which was abrogated by the TGFβ type I receptor (TβR-I/ALK 5) inhibitor SB431542 (5). Thus, TGFβs induce their own expression, thereby setting up an autocrine loop.

TGFβ1, TGFβ2 and TGFβ3 inhibit normal melanocyte proliferation and DNA synthesis (6). TGFβ derived from the niche (cellular organization where somatic stem cells are present) is critical for induction of melanocyte stem cell quiescence and maintenance of melanocyte stem cell immaturity. However, TGFβ can also trigger melanocyte stem cell apoptosis in the context of Bcl2 deficiency (8). In contrast, melanoma cell lines are less responsive or completely resistant to the inhibitory effects of TGFβ (6,7,9,10). Therefore, the development and progression of malignant melanoma are characterized by resistance to TGFβ tumor-suppressive effects on the one hand and autocrine/paracrine activation of the TGFβ pathway on the other. High levels of TGFβ may provide a similar selective advantage to invasive and metastatic melanomas (11), as proposed for carcinomas (12). A number of studies have addressed the role of TGFβ in melanoma progression and metastasis. Melanoma cells can modulate their surrounding stroma through the paracrine activity of TGFβ1 (4). Interestingly, stable overexpression of the inhibitory Smad7 [involved in the negative regulatory feedback loop of the TGFβ signal transduction pathway; (13)] in 1205LU human melanoma cells inhibits their tumorigenicity *in vitro* and *in vivo* in nude mice (14). In a tail vein metastasis model system using the isogenic metastatic melanoma line 37–32, transgenic mice expressing a soluble TβRII receptor (trapping TGFβ) were protected against metastases at multiple organ sites (liver, lung, spleen and pancreas). This study reinforces the importance of TGFβ for melanoma metastasis (15). Moreover, the expression of interleukin 8, whose involvement in growth and metastasis of melanoma (as well as angiogenesis) has been well documented [for review, see ref. (16)], is induced by TGFβ in metastatic melanoma cells (17). Interestingly, microarray analysis of high- and low-pigment populations of melanoma cells revealed that TGFβ2 was upregulated in the poorly pigmented cells, characterized by a higher motility *in vivo*. In addition, TGFβ1 and TGFβ2 treatment of melanoma cells could reverse characteristics, such as pigment production and dendrite formation and increase cell motility (18).

TGFβ canonical signal transduction pathway and tumor-suppressive transcriptional responses to TGFβ

At the cell surface, TGF-β assembles a complex of transmembrane receptor serine/threonine kinases (types I and II) and induces transphosphorylation and activation of the type I receptor (TβR-I, ALK5) by the type II receptor kinase (TβR-II). The activated type I receptor phosphorylates the downstream effectors Smad2 and Smad3 at C-terminal serines (19–21). Smad2 and Smad3 then associate with a common Smad4, and these activated complexes translocate into the nucleus, where they regulate transcription of target genes (12,22). Smad2 and Smad3 activities mediate TGFβ growth inhibitory effects by (i) downregulation of c-myc, CDC25A and Id family members and (ii) upregulation of p15 and p21 cyclin-dependent kinase (CDK) inhibitors (Figure 1) (12,22). In addition to mediating the TGFβ growth inhibitory effects, Smads regulate the expression of several genes involved in the apoptotic machinery and thus mediate the proapoptotic effects of TGFβ. These genes include the TGFβ-inducible early response gene-1, the signaling factor growth arrest and DNA-damage-inducible 45 β, the proapoptotic factor Bim, the death-associated protein kinase and the death receptor Fas [ref. (13) for review]. Thus, there is also an apoptotic program controlled by the TGFβ/Smad signaling pathway. The TGFβ tumor-suppressive transcriptional programs have not been systematically investigated in melanocytic systems. However, one early study showed that in a melanoma cell line lacking p15INK4B, due to loss of chromosome 9 and rearrangement of the other chromosome, cooperation between $p21^{WAF1}$ and $p27^{KIP1}$ was necessary for these cells to undergo cell cycle arrest in the presence of TGFβ (23).

Resistance to TGFβ-mediated tumor suppression in melanoma

A small number of studies have addressed the possibility of a direct inactivation of TGFβ signaling intermediates (loss of expression or

Abbreviations: BRAF V600E, activated mutant form of BRAF with a substitution of valine by glutamate at codon 600; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; GSK, glycogen-synthase kinase; JNK, c-jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MIA, melanoma inhibitory activity; MITF, microphthalmia transcription factor; mTORC1, mammalian target of rapamycin complex 1; PAX, paired box; TGF, transforming growth factor.

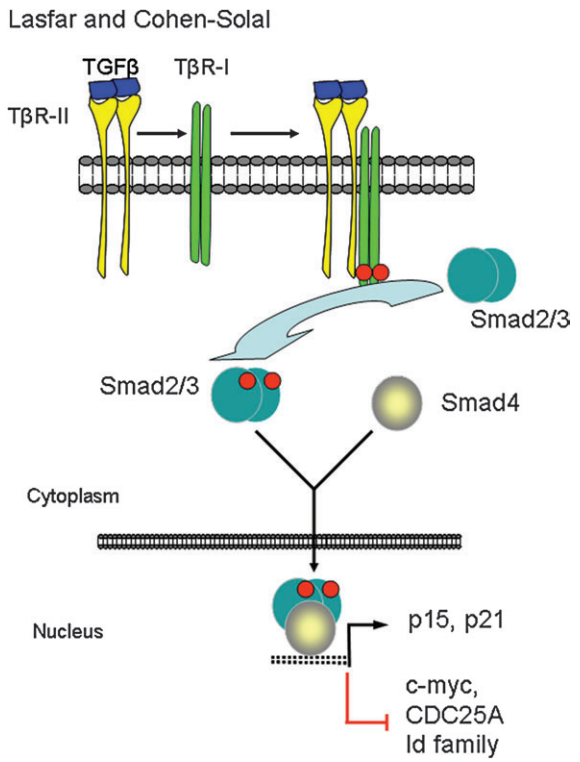

Fig. 1. TGFβ canonical signal transduction pathway and transcriptional responses mediating TGFβ growth inhibitory effects. At the cell surface, TGFβ assembles a complex of transmembrane receptor serine/threonine kinases (types I and II) and induces transphosphorylation and activation of the type I receptor (TβR-I, ALK5) by the type II receptor kinase (TβR-II). Activated TβR-I phosphorylates the main TGFβ downstream effectors, Smad2 and Smad3, at C-terminal serines. Activated Smad2 and Smad3 then associate with Smad4 and the complexes translocate into the nucleus and regulate transcription of target genes, involved in TGFβ-induced growth inhibition. Downregulation of c-myc, CDC25A and Id family members and upregulation of p15 and p21 CDK inhibitors are key events in this response.

mutation of TβR or Smads) to explain the resistance of melanoma cells to TGFβ inhibitory effects (10,24,25). From these studies, it appears that alterations in the components of the TGFβ signaling pathway do not occur in melanoma. The mechanisms of resistance of melanoma cells to TGFβ growth inhibition and apoptosis are probably unrelated to a global defect of the TGFβ signaling system because aggressive melanoma cells utilize TGFβ as a pro-oncogenic factor (11). Therefore, in melanoma, the strategies to escape from TGFβ-mediated tumor suppression have left intact the pathways necessary for TGFβ pro-oncogenic activities. In this review, we will specifically discuss different models of resistance to tumor suppression already suggested to operate in some melanoma cell systems as well as other potential mechanisms, based on knowledge of the multiple dysregulations of fundamental cellular effectors and signaling pathways found in melanoma.

Smads as mediators of resistance

Ski/SnoN. One indirect mechanism of resistance is the repressive effect of the oncoproteins Ski and SnoN on Smad2 and Smad3 activity (26,27). Ski protein levels were found to be elevated in 44 human melanoma tissues (28), as well as in melanoma cell lines (29,30). In addition, nuclear c-ski expression was associated with thicker and ulcerated tumors, whereas the percentage of SnoN positivity was found higher in ulcerated tumors and in tumors of patients with a positive sentinel node (31). Ski–Smad association in the cytoplasm was suggested to prevent Smad3 nuclear localization in response to TGFβ (28). Downregulation of Ski expression using antisense Ski vectors, restored TGFβ-mediated growth inhibition, apparently mediated by the upregulation of the CDK inhibitor p21 expression (28). In addition, knockdown of Ski by RNA interference in melanoma cells inhibited their growth in xenograft experiments (32). The proto-oncogene SnoN (a member of the Ski family) was expressed in nine melanoma lines with no expression of Ski (except for low levels in one line). SnoN expression was not found in melanocytes. Stable antisense SnoN-expressing cells were inhibited in their proliferation as compared with controls, though no mechanism was proposed for this finding (24). Although it seems established that Ski and SnoN are negative regulators of the TGFβ signaling pathway (26,27), some important questions, not specific to melanoma, remain unresolved: how can Ski/SnoN inhibit the tumor-suppressive arm of TGFβ without affecting its tumor-promoting effect if the function of these two oncogenes is to abrogate the function of activated Smads? How can high levels of Ski/SnoN be maintained if TGFβ is able to promote the degradation of Ski and SnoN (30,33)? How can we reconcile high levels of Ski expression with constitutively active TGFβ autocrine signaling in melanoma cells (5,10,34)? It is conceivable that within a melanoma lesion, concentrations of TGFβ vary from one site to the other and that the local variations of TGFβ lead to differential levels of Ski/SnoN within the tumor. That would explain why malignant melanoma lesions exhibit high levels of Ski/SnoN (28,31). It appears from these studies that the role of Ski/SnoN in melanoma, especially in relationship with the TGFβ signaling pathway and its pro-oncogenic activities, is still incompletely understood and requires further investigation (35).

Melanoma inhibitory activity (MIA), a secreted protein expressed in melanomas but not in normal melanocytes and involved in melanoma development and progression (36), has been studied as a clinical serum marker to monitor metastatic disease in melanoma patients (37). MIA has been proposed to positively regulate Ski and Sno expression and downregulate Smad2 and Smad3 expression in the metastatic melanoma-derived cell line, HMB2. MIA may, therefore, contribute to the resistance of the melanoma cells to TGFβ growth inhibition and/or apoptosis at multiple levels [(38) and references therein]. According to these results, a total disruption of TGFβ signaling would occur when MIA is overexpressed in melanoma cells. However, as previously mentioned, it seems unlikely that a total disruption of TGFβ signaling would be beneficial to melanoma cells at the invasive/metastatic stage.

Filamin. Another indirect mechanism of TGFβ regulation involves Filamin, a cytoskeletal actin-binding protein that appears to play a role in Smad-mediated signaling (39). TGFβ signaling was defective in filamin-deficient human M2 melanoma cells compared with a filamin-transfected subline, as determined by reporter gene activation (39). The defective TGFβ signaling in the M2 cells was associated with impaired TGFβ receptor I (ALK5)-mediated C-terminal phosphorylation of Smad2 and subsequent Smad2 nuclear translocation. The loss of expression of filamin could therefore represent a possible mechanism of TGFβ resistance in melanoma. In addition to Smad2, filamin appears to physically interact with Smad1, Smad4, Smad5 and Smad6. Surprisingly, the authors did not mention that filamin interacted with Smad3. Therefore, we don't know whether filamin did not physically interact with Smad3 or whether they did not test Smad3 as a possible filamin-interacting protein.

Smad2 and Smad3 linker phosphorylation. In epithelial systems, the linker region of Smad2 and Smad3, between the MH1 (N-terminal) and MH2 (C-terminal) domains, has been shown to be the target of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERKs), c-jun N-terminal kinases (JNKs) and p38, CDKs and glycogen-synthase kinase (GSK) 3β. Four sites within the linker region have been the main focus of intense study: threonine 220 and serines 245, 250 and 255 for Smad2 and threonine 179 and serines 204, 208 and 213 for Smad3 (40–50). Although it is now clear that modulation of Smad activity occurs through this linker region, the exact consequences of linker phosphorylation of Smad2 and Smad3 are still in debate. Some studies suggested that the MAPK- and CDK-mediated linker phosphorylation of Smad2 and Smad3 (i)

directly inhibits their activity on TGFβ-dependent promoters, such as p15 and p21 (activation), and c-myc (repression) resulting in escape from TGFβ-mediated tumor suppression (43,45–47,50–53) or (ii) interferes with the function of Smad2 and Smad3 as mediators of TGFβ-mediated tumor suppression in human cancer (49,51,54–56). Interestingly, several studies demonstrated that TGFβ itself was able to induce linker phosphorylation of Smad2 (41,51) and Smad3 (40,41,47–50), involving JNK (48,49), GSK3β (47,50) and CDK (40,41,50). These phosphorylation events negatively regulate Smad3 activity on endogenous p15 expression (50) and reduce growth inhibition by TGFβ (47,50).

In contrast to melanocytes of neonatal or adult origin, melanoma cell lines demonstrate constitutive linker phosphorylation of Smad2 and Smad3. The presence of Ski was required for a further increase of Smad3 linker phosphorylation by TGFβ (32). Therefore, TGFβ-induced linker phosphorylation observed in melanoma, in the context of autocrine TGFβ signaling, could play a role in inhibiting Smad3 antiproliferative activity in a sustained way. In addition, these results reinforce the idea that melanoma cells are still responsive to TGFβ with one consequence being the existence of a linker phosphorylated form of Smad3. The TGFβ-dependent increase in Smad3 linker phosphorylation was associated with TGFβ-mediated induction of the plasminogen activator inhibitor-1 in the melanoma cell lines (32). We also found constitutively high levels of linker phosphorylation of Smad2 and Smad3 in a panel of melanoma cell lines, in contrast to normal human melanocytes. In addition, we found that hyperactive MAPK and CDKs/GSK3 are involved in the constitutive linker phosphorylation of these two Smads. Our study further suggested that constitutive linker phosphorylation of Smad3 contributes to the resistance of melanoma cells to TGFβ-induced cell cycle arrest (Cohen-Solal,K.A., Merrigan,K.T., Dinh,K.G., Chan,J.L.-K., Goydos,J.S., Liu,F., Lasfar,A. and Reiss,M, submitted for publication). Interestingly, in non-melanocytic tumors, this linker phosphorylated form of Smad3 appears to be involved in both resistance to tumor suppression by TGFβ and promotion of TGFβ pro-oncogenic effects [(51) and references therein].

Smad-independent mechanisms involved in TGFβ resistance

c-myc expression in melanoma. Deregulation of c-myc expression by amplification at advanced melanoma stage (57–59), β-catenin-mediated transcriptional upregulation (60,61) and by the Raf-1/MEK/ERK pathway (62,63) appears to be an important oncogenic event in melanoma. C-myc overexpression is required for continuous suppression of oncogene-induced senescence in the context of activated $NRAS^{Q61R}$- or activated mutant form of BRAF with a substitution of valine by glutamate at codon 600 (BRAF V600E)-expressing melanoma cells (64). C-myc downregulation is an indispensable early step of the cytostatic transcriptional program to TGFβ, involving the upregulation of the CDK inhibitors of the INK4 or CIP/KIP families. The downregulation of myc is due to repression of the c-myc promoter, by a TGFβ-induced protein complex, containing Smad3 and Smad4 (22). The uncontrolled expression of c-myc in melanoma can represent an obstacle to the physiological response to TGFβ, leading to resistance to TGFβ-mediated cell cycle arrest in this disease.

The CDK inhibitor $p27^{KIP1}$. Two studies using the human melanoma line WM35, derived from a radial growth phase melanoma and sensitive to TGFβ-mediated growth inhibition, have suggested other possible mechanisms of resistance to TGFβ-mediated growth inhibition that could operate in melanoma (65,66). Using antisense p27 oligonucleotides to inhibit p27 expression, the first study showed that the loss of this CDK inhibitor conferred TGFβ resistance in the WM35 melanoma line, therefore pointing to p27 as an essential mediator of TGFβ-induced G_1 arrest in this line (65). This study raised the possibility that an abnormal p27 function during melanoma progression could contribute to the lack of response to TGFβ. In favor of this hypothesis, another study demonstrated that p27 phosphorylation by a downstream effector of mammalian target of rapamycin complex 1 (mTORC1), serum- and glucocorticoid-inducible kinase 1 (SGK1), resulted in p27 cytoplasmic mislocalization and TGFβ resistance (66). The PI3K/AKT signaling pathway (67) is frequently altered in melanoma, due to NRAS-activating mutation or phosphatase and tensin homologue deleted on chromosome 10-inactivating mutation or deletion (57). Therefore, it is conceivable that an activated PI3K/AKT pathway and consequently, activated mTORC1 (68) in melanoma leads to resistance to TGFβ-mediated growth inhibition through p27 phosphorylation and mislocalization. In addition to cytoplasmic mislocalization, melanoma cells may also have decreased levels of p27, with the same possible outcome being the resistance to TGFβ-mediated cell cycle arrest. Constitutively active ERK1/2 kinases were proposed to negatively regulate p27 in two cutaneous melanoma cell lines (69). Additionally, very low levels of p27, apparently associated with the activation of Raf-1 and the MEK/ERK effectors, were detected in proliferating human choroidal melanoma cells (62). In BRAF V600E-harboring melanoma cells, activated BRAF was sufficient to downregulate p27 levels (70). It was also shown that the melanocyte-specific transcription factor microphthalmia transcription factor (MITF) was required to suppress expression of p27 in melanoma cells, and that an inverse correlation between MITF and p27 existed *in vivo* in melanoma samples (71). In melanomas expressing high levels of MITF, by amplification (72) or other mechanisms, including β-catenin-mediated expression (73), it is therefore possible that low levels of p27 will be present. As previously mentioned, the absence or mislocalization of p27 could contribute to resistance to TGFβ-mediated cell cycle arrest.

The CDK inhibitor $p21^{WAF1}$. Immunostaining of melanoma lesions showed that all Radial Growth Phase (RGP) melanomas expressed p21 while most areas of advanced Vertical Growth Phase (VGP) melanomas lacked p21 expression (74). One possible mechanism to explain the absence of p21 expression involves Tbx2, a key developmental transcription factor, overexpressed in melanoma cell lines. Tbx2 has been demonstrated to repress the p21 promoter by a mechanism involving histone deacetylase 1 (75,76). Although it has not been definitively proven that the absence of p21 could result in resistance to TGFβ-mediated cell cycle arrest in melanoma, as shown for p27, we cannot exclude the possibility that the absence of induction of p21 by TGFβ in the course of the cytostatic program, could contribute to preventing inhibition of proliferation. In favor of this hypothesis, reducing the levels of the oncogenic Ski protein in melanoma cell lines, by antisense Ski vectors, restored TGFβ-mediated growth inhibition, associated with increased p21 levels (28).

The CDK inhibitor $p15^{INK4B}$. A recent study identified Id2, as a mediator of resistance to TGFβ-mediated cell cycle arrest. This study suggested that by preventing TGFβ-induced expression of p15, upregulation of Id2 was counteracting TGFβ-mediated growth inhibition in invasive melanoma cells (77).

By using High Density Single-Nucleotide Polymorphism Arrays, the high frequency of homozygous deletions of CDKN2A (encoding $p16^{INK4A}$; 43%, 33 of 76 cell lines) in melanoma was confirmed and extended to various neighboring genes on 9p22-p21, often including CDKN2B [encoding $p15^{INK4B}$; (78)]. Therefore, loss of p15 could constitute another way for melanoma cells to circumvent TGFβ-mediated inhibition of proliferation.

Cyclin D1 overriding the cytostatic effect of TGFβ. Cyclin D1 amplification in melanoma has been well documented (58,79–82). Sauter and coll. (81) also found that an additional 20 % of melanomas analyzed (137 invasive primary cutaneous melanomas) had cyclin D1 overexpression without amplification of the cyclin D1 locus. In melanoma cells harboring the activating BRAF V600E mutation, the high level of cyclin D1 was dependent on the presence of activated ERK in the nucleus, suggesting that the constitutively activated BRAF/MEK/ERK axis represents a possible inducer of cyclin D1 overexpression in melanoma (70). Cyclin D1 is a transcriptional target of c-Jun, whose transcription and activity are increased in melanoma with an activated

ERK pathway (83). Additionally, activated Wnt signaling alone with the resulting accumulation of β-catenin, would probably deregulate cyclin D1 expression in melanoma (60). From these data one can hypothesize that the increase in the stability and activity of the cyclin D-CDK complexes imposed by high cyclin D1 expression would lead to resistance to TGFβ-mediated cell cycle arrest, even in the presence of normal induction of the CDK inhibitors of the INK and CIP/KIP family by TGFβ. Such a scenario has been proposed for the A375 melanoma cell line in which cyclin D1 aberrant expression overrides the Tumor Necrosis Factor (TNF) alpha -induced increase in p21 levels, ultimately leading to resistance to TNF (84).

CDK4 activation in melanoma. In the vast majority of cutaneous melanomas, loss of the CDK inhibitor p16 function, resulting from homozygous deletion or methylation of the CDKN2A gene, or mutation of p16 [reviewed in ref. (57,85)] results in uncontrolled CDK4 activity. In addition, two types of CDK4 mutations affecting codon 24 have been found in the germline of melanoma patients. These two mutations abrogate the capacity of p16 to bind and inactivate CDK4, again leading to dysregulated CDK4 activity (86,87). More recently, human melanoma cell lines with both the BRAF V600E activating mutation and a CDK4 mutation in codon 24 have been identified (82). Finally, amplification of CDK4 has also been described in melanoma samples (79,88). In addition to contributing to Smad3 linker phosphorylation (see above) (45), aberrant CDK4 activity in melanoma could promote unregulated Rb hyperphosphorylation. In this context, the ability of TGFβ to impose cell cycle arrest would be compromised, in a way similar to cyclin D1 overexpression.

FoxO factors: where the cross talk between AKT and TGFβ signaling pathways could lead to resistance. The FoxO factors are important downstream targets of AKT and are currently being considered as new therapeutic targets in cancer therapy, including melanoma therapy (89). In epithelial cells, these transcriptional factors are directly involved in the expression of the two CDK inhibitors, p15 and p21 induced by TGFβ (22,90). In addition, TGFβ induces the expression of the proapototic factor Bim in a variety of cell types (91). The direct involvement of the FoxO factors in the regulation of the Bim promoter has been documented (92–94) although the exact role of FoxO in the TGFβ-induction of Bim expression is not completely understood. FoxO factors are impaired in their nuclear translocation upon phosphorylation by AKT on Thr 24, Ser256 and Ser319 (FoxO1) and Thr32, Ser253 and Ser315 (FoxO3), and therefore unable to act as transcriptional activators (95). It is therefore expected that in the context of activated AKT, the transcription of p15, p21, and possibly Bim would be prevented, counteracting the tumor-suppressive effects of TGFβ. In glioma, for example, a hyperactive PI3K/AKT pathway contributes to the prevention of p21 expression and cytostasis by the TGFβ/Smad-FoxO pathway (96).

The relevance of the link between AKT/FoxO and apoptosis in melanoma has been documented by two independent studies. Both showed that adenovirus-mediated transfer of constitutively active FoxO3 (triple mutant unable to be phosphorylated on the three AKT sites) induced apoptosis in the melanoma cell lines SK-MEL-2 and SK-MEL-28 (97) and A375, MeWo and WM9 (98), suggesting that AKT-mediated phosphorylation of FoxO3 in melanoma inhibits FoxO3's proapoptotic role. In addition, Bim was upregulated by the constitutively active FoxO3 mutant in A375 melanoma cells (98), suggesting that Bim expression resulting from AKT-insensitive FoxO activation could contribute to apoptosis in these cells. The missing pieces reside in the demonstration that in the context of an activated PI3K/AKT pathway, the exclusion of FoxO factors from the nucleus renders the melanoma cells incompetent for induction of p15, p21 or Bim in the presence of TGFβ, and therefore resistant to TGFβ-mediated tumor suppression.

The transcription factor PAX3. PAX3, a member of the paired box (PAX) family of transcription factors has been proposed as a survival factor in melanoma [(99) and references therein] and a possible mediator of resistance to TGFβ-mediated growth inhibition in melanoma. In human primary melanocytes, TGFβ represses the expression of PAX3. Ultraviolet irradiation represses expression of TGFβ in keratinocytes, and as a result, the repression of TGFβ leads to upregulated PAX3 expression in melanocytes. An ultraviolet-induced melanogenic response and consequent pigmentation are associated with the positive regulation of PAX3. The TGFβ-dependent negative regulation of PAX3 was not detected in two TGFβ resistant melanoma cell lines. In addition, when PAX3 was overexpressed in the TGFβ sensitive B16 melanoma cell line, these cells were less responsive to TGFβ-mediated growth inhibition (99).

MITF and resistance to TGFβ. Expression profiling of melanoma cell lines identified two transcription signatures associated with proliferative and invasive cellular phenotypes (9). An important feature of the cells harboring the proliferative signature was their increased

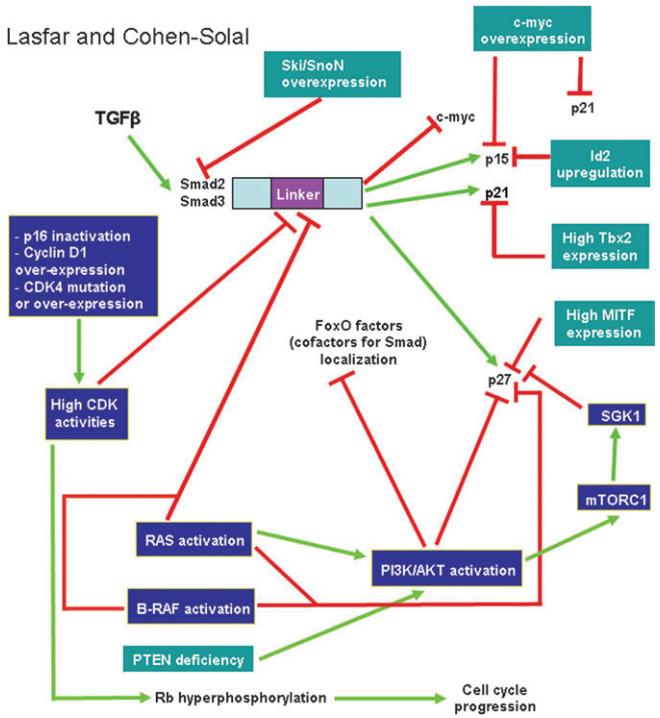

Fig. 2. Possible mechanisms of resistance to TGFβ-induced cell cycle arrest in melanoma. The most documented but still controversial model involves the oncogenes Ski/SnoN suppressing Smad-mediated transcription. In another model, a regulatory domain in Smad2 and Smad3, called the linker domain, could be the target of aberrant phosphorylations, via the hyperactive NRAS/MAPK signaling and high CDK activities. The linker phosphorylations would prevent Smad antiproliferative activity. NRAS activation and phosphatase and tensin homologue deleted on chromosome 10 deficiency result in PI3K/AKT activation. This activation is expected to result in the phosphorylation and cytoplasmic mislocalization of cofactors for Smad, called FoxO factors. FoxO factors are required for the transcription of p21, p15 crucial for the cytostatic effect of TGFβ. In the absence of nuclear FoxO factors, p15 and p21 would not be expressed, leading to resistance to TGFβ-induced cell cycle arrest. In addition, high c-myc expression level in melanoma could prevent p21 and p15 expression since it is a repressor for these two genes. High Id2, Tbx2 and MITF expression could, respectively, repress the expression of p15, p21 and p27, leading to resistance to TGFβ. Another mechanism involves the phosphorylation of p27 by AKT and by serum- and glucocorticoid-inducible kinase 1 (SGK1), a substrate of mTORC1, preventing p27 nuclear localization and triggering TGFβ resistance. In addition to phosphorylating the Smad linker region, uncontrolled CDK activity, resulting from p16 inactivation, cyclin D1 overexpression or CDK4 mutation or overexpression, would override TGFβ-mediated inhibition of proliferation since high CDK activity leads to sustained hyperphosphorylation of Rb and cell cycle progression.

susceptibility to TGFβ-mediated growth inhibition as compared with the cells harboring the invasive signature. In addition, the MITF, described as a lineage survival oncogene in melanoma (71,72,100), was shown to be a marker of the proliferative phenotype, with almost no expression in the invasive phenotype (71,101). The hypothesis that MITF expression could mediate the growth inhibitory effect of TGFβ in the proliferative signature melanoma cells, prompted Hoek *et al.* (101) to perform a knockdown of MITF expression in a melanoma line sensitive to TGFβ-mediated growth inhibition (proliferative signature). They observed that these cells became less susceptible to TGFβ-mediated growth inhibition upon knockdown of MITF expression. This study suggests a role for MITF in mediating the growth inhibitory response of TGFβ.

Simultaneous Smad-dependent and -independent mechanisms: the case for NRAS and MAPK signaling

The hyperactivity of MAPK signaling is observed in the vast majority of clinical melanoma specimens (102,103) and probably is responsible for multiple mechanisms of resistance to TGFβ-mediated tumor suppression that operate simultaneously. One of the mechanisms of MAPK hyperactivity resides in activating mutations of NRAS, most commonly the result of a substitution of leucine to glutamine at position 61 (103– NRAS-activating mutations have been reported in ~20% of sporadic cutaneous melanomas (57). Expression of activated NRAS in the WM35 melanoma line impaired TGFβ-mediated growth inhibition, by directly or indirectly preventing the accumulation of hypophosphorylated Rb (107), probably reflecting an impaired induction of the CDK inhibitors. However, no systematic examination of Smad activity was performed in the WM35 and WM35-NRAS melanoma cells to explain these observations. As previously mentioned, the linker region of Smad2 and Smad3, between the MH1 (N-terminal) and MH2 (C-terminal) domains, has been shown to be the target of MAPKs (ERK, JNK and p38) (42,43,46,48,49). One possible mechanism that we have already described above (Smad2 and Smad3 linker phosphorylation) is that the linker phosphorylated forms of Smad2 and Smad3 are less active or inactive on TGFβ-induced promoters involved in tumor suppression, such as p15, p21 and c-myc (43,46,47,50–53). As a result, escape from TGFβ-mediated cell cycle arrest would occur. An additional model suggested by studies in non-melanocytic cells proposes that the nuclear translocation of the linker phosphorylated Smad3 prevents Smad3 from being phosphorylated at the C-terminus by TβR-1 in the cytoplasm, a step required for p15 induction (48). Similarly, HRAS activation in normal gastric cells induced a high degree of JNK-dependent phosphorylation at the Smad3 linker region, indirectly suppressing Smad3 phosphorylation at the C-terminus of Smad3 (49). One could imagine that in the context of activated MAPK signaling in melanoma, linker phosphorylation of Smad3 could prevail and prevent an efficient C-terminal phosphorylation of Smad3, necessary for the expression of CDK inhibitors by TGFβ.

In addition to direct effects of activated MAPK signaling on Smads, a previously cited study on the TGFβ-sensitive melanoma cell line WM35 implies that activation of the PI3K/AKT pathway in melanoma cells by an activating NRAS mutation could lead to p27 phosphorylation and mislocalization and resistance to TGFβ-mediated growth inhibition (66). Moreover, in light of the above referenced studies, aberrant AKT phosphorylation of FoxO factors (cofactors for Smads) due to upstream NRAS activation could result in their exclusion from the nucleus suggesting an additional mechanism of resistance conferred by activated NRAS.

It is interesting to mention that normal human melanocytes exposed to the tumor-promoting phorbol ester, 12-*O*-tetradecanoylphorbol-13-acetate exhibited resistance to TGFβ-mediated inhibition of proliferation and DNA synthesis. The resistance was associated with downregulation of protein kinase C-alpha and suppression of Smad-mediated transcription (108). However, the activation of ERK and JNK activities by 12-*O*-tetradecanoylphorbol-13-acetate (109,110) were not explored in this study as a potential mechanism counteracting TGFβ-mediated cell cycle arrest in melanocytes.

Concluding remarks

In melanocytes and melanoma cells still sensitive to TGFβ, no systematic analysis of the mechanisms involved in TGFβ-mediated cell cycle arrest and apoptosis has been performed. To date, epithelial

Table I. Deregulations potentially playing a role in TGFβ resistance in melanoma

Deregulated proteins	Type of deregulation	Expected Effect	References
Transcription factors coactivators repressors			
Ski/SnoN	Upregulation	Impaired Smad3 nuclear localization	(28)
Smad2/Smad3	Linker phosphorylation	Impaired Smad transcriptional activity	(43,45–47,50–53)
FoxO factors	Phosphorylation on AKT sites	Impaired FoxO factors nuclear localization	(96)
c-myc	Upregulation	Derepression on p15 and p21 promoters prevented	(22,57–64)
MITF	Low expression level	Not precisely defined	(101)
PAX3	High expression level	Not precisely defined	(99)
Cell cycle G_1–S effectors			
Cyclin D1	Overexpression	High cyclin D1/CDK4/6 complex activity	(58,60,70,79–83)
CDK4	Activating mutation	High cyclin D/CDK4 complex activity	(82,86,87)
	Amplification		(79,88)
p16	Homozygous deletion	High cyclin D/CDK4/6 complex activity	(57,85)
	Methylation		
	Mutation		
p15	Loss of expression	High cyclin D/CDK4/6 complex activity	(77,78)
	Impaired TGFβ-induced p15 expression by Id2		
p21	Downregulation or loss of expression	High cyclin E/CDK2 activity	(74)
	Tbx2 repression of p21 promoter		(75,76)
p27	Downregulation	High cyclin E/CDK2 activity	(62,65,69,70)
	MITF repression of p27 expression		(71)
	Phosphorylation by AKT and serum- and glucocorticoid-inducible kinase 1 (SGK1)	p27 cytoplasmic mislocalization High cyclin E/CDK2 activity	(66)
Other types of proteins			
MIA	Upregulation	Upregulation of Ski and SnoN expression Downregulation of Smad2 and Smad3 expression	(38)
Filamin	Loss of expression	Impaired Smad2 C-terminal phosphorylation	(39)

systems have been the reference for the tumor-suppressive programs of TGFβ (13,22,111). Based on the knowledge gained from the studies on epithelial cells, some possible resistance mechanisms have been suggested that could occur in melanoma cells. It is expected that depending on the genetic and epigenetic background of each melanoma cell, and the identity of the deregulations of central cellular effectors (such as BRAF, NRAS, PI3K and phosphatase and tensin homologue deleted on chromosome 10) and key signaling pathways, the resistance mechanisms will differ between genetically/epigenetically different melanoma cells. In addition, we hypothesize that a combination of different mechanisms (Figure 2 and Table I) reinforces and strengthens this resistance in a particular melanoma cell in a unique microenvironment within a melanoma lesion. However, a central characteristic that seems to be shared by these mechanisms is that the escape from TGFβ-mediated growth inhibition is achieved though inactivation of the Rb pathway. This inactivation could involve direct deregulations of c-myc (increase), loss of expression (via Smad-dependent or independent mechanisms) or function (mislocalization of p27 for example) of CDK inhibitors or aberrant activation of CDK activity (via cyclin D1 overexpression or CDK4 mutation or overexpression).

As mentioned earlier in the section concerning Smad2 and Smad3 linker phosphorylation, we determined that hyperactive MAPK and CDK/GSK3 pathways are involved in the constitutive linker phosphorylation of these two Smads in melanoma, and our study further suggested that constitutive Smad3 linker phosphorylation contributes to the resistance of melanoma cells to TGFβ-mediated cell cycle arrest (submitted for publication). We could therefore envision that the precise identification of the kinases involved in Smad3 linker phosphorylation will be rewarding. Dephosphorylating the Smad3 linker region, using agents targeting these kinases, could potentially restore TGFβ-mediated growth inhibition, thereby interfering with melanoma development. Another therapeutic strategy would consist in restoring FoxO factors and p27 nuclear localization through PI3K/AKT/mTORC1 inhibition. However, we are fully aware that the dysregulations of fundamental cellular effectors and signaling pathways occurring in melanoma promote melanoma aggressiveness and dissemination through numerous mechanisms, in addition to resistance to TGFβ. Therefore, targeting these pathways (NRAS-MAPK, PI3K/AKT/mTORC1 and CDK) is expected to affect melanoma development beyond the restoration of the TGFβ-induced growth inhibition. As important as the mechanisms of escape from TGFβ-mediated cell cycle arrest are the molecular mechanisms of resistance of melanoma cells to TGFβ-induced apoptosis. Unraveling these mechanisms can be rewarding in that restoring the apoptotic response to TGFβ could block a critical step in melanoma progression.

Finally, it is essential to keep in mind that melanoma is also composed of the supporting stroma, which includes fibroblasts, endothelial cells, immune cells, soluble molecules (such as TGFβ) and the extracellular matrix (112). The cross talk between the tumor cells, the microenvironment and the immune system, is crucial for melanoma establishment and progression to aggressive phenotypes allowing tumor dissemination. Therefore, therapeutic strategies that combine the restoration of the TGFβ tumor-suppressive response in melanoma cells and the targeting of components of the melanoma tumor microenvironment (112), including immunosuppressive components, might be worth considering for this incurable disease.

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