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Targeting p53 in melanoma

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Melanoma is the sixth most common cancer in the United States with the numbers of annual cases increasing faster than any other tumor type. Approximately 50% of melanomas harbor oncogenic *BRAF* mutations, 90% of which are *BRAFV600E*. Recently, US Food and Drug Administration (FDA) approval of the selective *BRAFV600E* inhibitor, Zelboraf (vemurafenib), has inspired rapid development of new targeted therapeutic strategies for melanoma. Subsequently, the first FDA approved combination of molecularly targeted cancer therapies has emerged with GlaxoSmithKline's Tafenlar (dabrafenib) and Mekinist (trametinib) that inhibit *BRAFV600E* and the primary downstream target, MEK (mitogen-activated protein kinase kinase), respectively. As *BRAF* and MEK function within the same oncogenic RAS pathway, there is much anticipation about forthcoming clinical trials investigating molecularly targeted combination therapies that attack two or more different pathways.

Together with the extant inhibitor of mutationally activated KIT (Gleevec), *BRAF*-targeted therapy has allowed the development of a molecular classification of melanoma based on treatment strategy and companion diagnostic (Vidwans et al., 2011). This classification system promises tremendous advances in the management and treatment for metastatic melanoma. Vidwans et al. (2011) propose that the tumor suppressor *p53* may well-define new molecular tumor subtypes, where the presence of a *p53* mutation determines the therapeutic approach. Wild-type (WT) *p53* is a potent inducer of apoptosis, cell cycle arrest, and cellular senescence that is present at undetectable levels in normal tissues and is stabilized and activated in response to cellular stresses and stimuli such as ultraviolet light (UV) or oncogene activation. In most tumors, *p53* is mutated, which not only disables its tumor suppressor activity, but can confer an oncogenic potential. In melanoma, WT *p53* is found inactivated in some way in approximately 90% of cases with approximately 10% carrying disabling point mutations (Hocker and Tsao, 2007) although this number could be as high as 19% (Hodis et al., 2012). Based on the relatively low *p53* mutational rate and the success of *BRAFV600E* inhibitors, there has been great interest in re-examining the old paradigms of *p53* activation during conventional chemotherapy to determine whether the powerful antitumor functions of WT *p53* can be harnessed for clinical benefit. *p53* is a key target of many conventional chemotherapeutics which have proven generally ineffective as stand-alone agents. For example, dacarbazine, a powerful alkylating agent that induces *p53*-dependent death of tumor cells, has no ability to extend life in metastatic melanoma patients. Despite the poor clinical performance of dacarbazine as an antimelanoma agent, advances in our understanding of *p53* pathway regulation in cancer and the development of new

powerful preclinical reagents to study its regulation have prompted a re-examination of this area.

To gain a more complete view of the potential for WT p53 activation as a mode of therapy, the underlying biology of p53 regulation in melanocytes and melanoma cells must be understood. We and others have shown that these cells are relatively resistant to the proapoptotic effects of p53 once it is activated. Moreover, UV induces p53 expression in melanocytes much less efficiently than in adjacent keratinocytes in the basal layer (unpublished observation). Conventional wisdom suggests that resistance to p53 activation is an inherent property of melanocytes as they are programmed to survive for the entire life of the organism, even with p53 induction by highly mutagenic UV light, and by the oxidative stress of melanin production. In comparison, neighboring keratinocytes readily undergo apoptosis after UV exposure. Despite this hardwired resistance of melanocytes to p53 activation, virtually all melanomas likely need to further mute the activity of p53 to reach full growth and invasive potential. It may in fact be relatively easy to overcome residual p53 activity during progression and thus mutating *p53* may for the most part be unnecessary. At present, the major hypothesis explaining the low frequency of *p53* mutation in melanoma is that inactivation of the *CDKN2A* locus, encoding the dual tumor suppressors *p16INK4A* and *p14ARF*, makes mutation of *p53* unnecessary. In the presence of oncogenic activation (*BRAF* or *NRAS*), p14ARF acts to directly inhibit MDM2, the major ubiquitin ligase that degrades and inactivates p53. Therefore, in the presence of *p14ARF* deletions, p53 would remain maximally regulated by Mdm2 and would not play a role in counteracting tumor progression. While there is some evidence of increased mutation of *CDKN2A* in *p53* WT tumors (Hodis et al., 2012), this association does not persist when frequent *CDKN2A* deletions are considered. It is likely that other mechanisms determine which melanomas inactivate p53 through mutation and which ones functionally inactivate WT p53. Uncovering the mechanisms that determine the low *p53* mutation rate in melanoma and how WT p53 is functionally inactivated could lead to new approaches to reactivate p53 for melanoma therapy.

Recent studies performed by Lu et al. identified iASPP (inhibitor of apoptosis stimulating protein of p53), an important new player in p53 signaling during melanoma progression, as a potential new target in WT p53 reactivation (Lu et al., 2013). iASPP, encoded by the *PPP1R13L* locus, inhibits p53-dependent apoptosis preferentially through transcriptional regulation. Two different forms of iASPP were identified in melanoma cell lines: a cytoplasmic fast migrating (or lower molecular weight), and a nuclear slow migrating (high molecular weight) form. p53 selectively binds to the slower migrating iASPP that is enriched in metastatic melanoma and associated with reduced overall patient survival. Phosphorylation at serines 84 and 113 were the two main modifications responsible for high molecular weight iASPP and promoted its nuclear localization. Furthermore, cell cycle inhibitors such as nocodazole increased Cyclin B1 expression which in turn increased the formation and nuclear localization of slow-migrating iASPP through phosphorylation of S84 and S113. The importance of these two phosphorylation sites was confirmed with the creation of a phosphomimetic mutant iASPP (iASPP-S84D/S113D) that more efficiently bound to and inhibited p53 in cancer cell lines than WT iASPP. Specifically, inhibition of

p53 transactivation of pro-apoptotic promoters *PIG3*, *BAX* and *PUMA* was observed, but no inhibition of *CDKN1A* (*p21*) expression was seen. Studies in *Ppp1r13* l-null MEFs confirmed the preferential role of iASPP-S84D/S113D in inhibiting p53-dependent apoptosis. A screen for CDK1 inhibitors identified a drug, JNJ-7706621 (JNJ), which inhibits the nocodazole induced phosphorylation of iASPP and thus formation of slow-migrating and nuclear iASPP. In parallel, a panel of 18 melanoma cell lines was examined to quantify Cyclin B1, iASPP, p53, MDM2 and MDM4 levels. Approximately 70% of WT *p53* lines over-expressed both phosphorylated nuclear iASPP and MDM2, in agreement with frequent MDM2 over-expression found in metastatic melanoma. Further, the small molecule Nutlin-3a was designed to interfere with the MDM2-p53 interaction; however, treatment for WT *p53* melanoma cells with Nutlin-3a only resulted in modest reactivation of p53 consistent with cell cycle arrest rather than induction of apoptosis (Terzian et al., 2010). These lines were also used to test the therapeutic potential of a combination of JNJ and Nutlin-3a. Melanoma cells treated with Nutlin-3a or JNJ alone showed elevated p53 levels; however, only a small increase in apoptotic cells was observed. On the other hand, when Nutlin-3a and JNJ were used in combination, a dramatic increase in apoptotic cells was observed. To confirm in vivo, B16 mouse melanoma cells were injected into the flanks of syngeneic C57BL/6 mice, and the impact of different drug treatments on tumor formation measured. The JNJ and Nutlin-3a combination exhibited significantly greater effects on tumor engraftment and growth than was seen for either drug alone. This successful demonstration of preclinical efficacy was then followed with additional combination drug studies including vemurafenib and the MEK inhibitor UO126. In *BRAFV600E* mutant and WT *p53* cell lines, the greatest levels of apoptosis and growth suppression were observed with the triple vemurafenib/Nutlin-3a/JNJ combination. A similar effect was also observed for the triple UO126/Nutlin-3a/JNJ combination. Moreover, these data were validated with nude mouse xenografting studies where the vemurafenib/Nutlin-3a/JNJ cocktail resulted in the lowest tumor growth. The fact that the vemurafenib/Nutlin-3a/JNJ cocktail was superior to the vemurafenib/UO126 combination supports the idea that targeting two independent tumor pathways will be more effective clinically than targeting one pathway at different points (Figure 1). These findings greatly advance our understanding of the mechanisms by which melanoma cells maintain resistance to p53-dependent apoptosis, and promising preclinical findings suggest that this resistance may be overcome.

The observations of Lu et al. (2013) nicely complement functional murine studies focusing on the Mdm2 family member, Mdm4. Mdm4 binds directly to p53 to suppress its transactivation but in contrast to Mdm2, it lacks a ubiquitin ligase activity. The Tyr-HRAS transgenic mouse melanoma model exhibited a mild reduction in number of melanocytic nevi but a dramatic reduction in melanoma growth and no metastasis in the presence of *Mdm4* haploinsufficiency (thus higher p53 activity), suggesting that p53-dependent growth suppression may be one of the last barriers that a melanoma must overcome to reach its full proliferative potential (Terzian et al., 2010). In a subsequent study, 65% of melanomas are reported to over-express Mdm4, and treatment for melanoma cell lines with a stapled peptide inhibitor of Mdm4 increased apoptosis and impaired tumor formation in xenograft models (Gembarska et al., 2012). Melanoma cell lines with high Mdm4 protein levels were most responsive to Mdm4 inhibition; however, those with high Mdm2 may be more

susceptible to Nutlin-3a. Therefore, both Mdm2 and Mdm4 inhibition show promise for activating a p53-mediated clinical response in a subset of melanomas. Further studies are needed in this area to examine the efficacy of Mdm2 and Mdm4 inhibition in melanomas that are WT for *p53* and that have either low Mdm2, Mdm4 and p53, or those that have high Mdm2, Mdm4 and p53 protein levels.

While iASPP, Mdm2 and Mdm4 over-expression need to be investigated for clinical applications in melanoma therapy, they remain only a piece of the puzzle that is p53 regulation in melanocytes and melanoma. As both Mdm2 and Mdm4 are expressed at low levels in melanocytes, they may not explain the inherent resistance of melanocytes to stabilize and activate p53 after UV exposure or the inherent resistance of melanocytes to p53-dependent apoptosis. Similarly, sequence analysis of tumors with high Mdm2, Mdm4 and p53 did not identify any correlation with *p53* mutation, indicating that our understanding of the causes of this aspect of melanoma is limited indeed and that further work is needed. With these efforts, we anticipate that a targeted p53 therapy will take its place in the emerging cocktail of drugs that will ultimately be needed to treat metastatic melanoma successfully.

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Figure 1.

Targeting molecular pathways in melanoma. The oncogenic RAS pathway is inhibited by the clinically approved BRAF^{V600E} inhibitors zelboraf and tatinlar, while downstream MEK activity is inhibited by mekinist as a combination therapy with tatinlar. Experimental drugs that activate p53 in melanoma, including Nutlin-3a (Nut) that inhibits MDM2, the stapled peptide inhibitor of MDM4 (SAH-p53-8) and JNJ-7706621 (JNJ) that inhibits iASPP offer the possibility of developing combination therapies that target two separate pathways when used in combination with RAS pathway inhibitors [Adapted from Lu et al. (2013)].