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Simultaneous Knockdown of Mutant BRAF and Expression of INK4A in Melanoma Cells Leads to Potent Growth Inhibition and Apoptosis

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1. Introduction

Melanoma is the eighth most common cancer in the United States and incidence is increasing at a rate greater than all other cancers. Estimates indicate that in 2010 there were 68,130 new cases of melanoma in the United States and 8,700 deaths from the disease. Overall, the lifetime risk of developing melanoma is about 2% (1 in 50) for Caucasians, 0.5% (1 in 200) for Hispanics, and 0.1% (1 in 1,000) for African Americans (American Cancer Society). Malignant melanoma results from transformation of melanocytes with progression through histologically recognizable sequential steps including radial growth phase (RGP), vertical growth phase (VGP), and metastasis (Elder, 1999). In RGP, neoplastic cells are confined to the epidermis or with microinvasion into the dermis. In more advanced VGP, cancer cells expand in the dermis and generate tumor nodules and have a high potential for metastatic spread. In the metastatic phase, cancer cells disseminate to lymph nodes or distant organs (Clark & Tucker, 1998; Elder, 1999). Optimal treatment varies based on stage of disease. For the early diagnosed and localized melanomas, surgery is the treatment of choice and may be curative provided the lesion is excised completely. However, for invasive and metastatic melanomas, there is no effective treatment and many patients die within 6-8 months after diagnosis. The aggressive growth of melanoma cells and their intrinsic resistance to standard modalities of cancer treatment account for the dismal prognosis (Poulikakos & Rosen, 2011; Tawbi & Buch, 2010). Therefore, the ability to inhibit growth and overcome drug resistance is central to the effective treatment of melanomas.

Treatment options for advanced melanoma are currently limited to immunotherapy, single agent cytotoxic chemotherapies, and surgery. Recently, Yervoy (Ipilimumab), an inhibitor of cytotoxic T-lymphocyte antigen 4 (CTLA-4) gained Food and Drug Administration (FDA) approval to treat metastatic melanomas. It is the first drug shown to prolong the lives of patients with advanced melanoma (Weber, 2011). However, its effect is limited. Patients with metastatic melanoma treated with Yervoy lived a median of approximately 10 months compared with 6.4 months for patients in a control group. Treatment with interferon Alpha 2B has shown improvement in disease free survival although overall survival benefit results are conflicting (American Cancer Society, <http://www.cancer.org/>). Interleukin 2 has also shown activity against metastatic melanoma although patient populations are limited by severe toxicity. Dacarbazine is the most studied of single agent cytotoxic agents for

malignant melanoma and is considered gold standard for comparison of systemic chemotherapy. However, no survival benefit has been shown in randomized controlled trials. Complete response rates are 3-5%, and long term remission rates are less than 2% under systemic treatments for metastatic cutaneous melanoma. Combination chemotherapy involving dacarbazine increases toxicity without improvement in median survival compared to dacarbazine alone. Metastatic melanoma can sometimes be treated with surgery. Patients can achieve prolonged overall survival after complete resection of tumors. Unfortunately, beneficiary outcome of surgical resection is limited to single or solitary melanoma metastases compared with patients with disseminated disease (Sosman et al., 2011).

Results of the recent phase I clinical trial with PLX4032, a specific inhibitor of mutant BRAF, have generated great excitement because approximately 80% of BRAF mutant metastatic melanomas regressed in response to PLX4032 treatment. Though this trial was considered a true victory in the fight against melanomas, attention has been drawn to the fact that regressed tumors resurge more aggressively within 8 months after the start of therapy. Constitutive deregulation of BRAF-MEK-ERK and p16-CDK4-RB pathways occur at high frequencies in melanomas. We have shown that suppression of either BRAF-MEK or CDK4 activity inhibits cell growth, and that simultaneous inhibition of both BRAF-MEK and CDK4 activities compounds this effect and triggers significant apoptosis in melanoma cells. Our data suggest BRAF-MEK-ERK and p16-CDK4-RB pathways may act additively or synergistically in the malignant growth of melanoma cells, and could be jointly targeted for treatment of melanoma. We have recently reported that the expression of K type human endogenous retrovirus (HERV-K) correlates with ERK activation and p16 loss in melanoma cells and can be inhibited by MEK and CDK4 inhibitors, especially in combination. Given that HERV-K may drive malignant growth downstream of BRAF-MEK and CDK4, we hypothesize cells with activated HERV-K may escape the therapeutic effects of MEK and CDK4 blockers, and that triple inhibition of BRAF-MEK, CDK4, and HERV-K should be more effective than single or double inhibition.

2. Mutational activation of NRAS-BRAF-MEK-ERK pathway in melanoma cells

In a systematic genome-wide screen for gene mutations, Davies et al. (Davies et al., 2002) identified BRAF mutations in 70% of human malignant melanomas. A T1799A transversion in exon 15, resulting in a V600E substitution in the BRAF kinase domain, accounts for over 90% of BRAF mutations detected in melanoma samples. In addition to melanomas, BRAF mutations have been identified in several other tumor types including thyroid, ovarian, colorectal, and lung (Davies et al., 2002). BRAF, one of three members of the RAF family (ARAF, BRAF, CRAF), is a component of the RAS-RAF-mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) signaling pathway. RAF gene encodes cytoplasmic serine/threonine kinases that transduces regulatory signals from RAS to MEK-ERK (Mercer & Pritchard, 2003). RAF genes are differentially expressed with CRAF being ubiquitously expressed; ARAF predominantly found in urogenital tissue, and BRAF having highest expression in neural and testes (Mercer & Pritchard, 2003). Mutant BRAF has increased kinase activity causing intrinsic ERK activation in cultured NIH3T3, COS, and human melanocytes, and leads to elevated transforming activity of cultured NIH3T3 and human melanocytes (Davies et al., 2002; Dong et al., 2003; Satyamoorthy et al., 2003; Wellbrock et al., 2004). Suppression of mutant BRAF expression

has been reported to cause inhibition of melanoma cell proliferation and survival *in vitro* and *in vivo* (Hingorani et al., 2003; Karasarides et al., 2004; Rotolo et al., 2005; Sumimoto et al., 2004).

In addition to BRAF mutation, NRAS mutation occurs in about 10% of melanomas (Davies et al., 2002; Dong et al., 2003; Michaloglou et al., 2008) that can also lead to constitutive activation of the MEK-ERK pathway. Apart from NRAS and BRAF mutation, other factors have been identified leading to constitutive activation of the ERK signaling. For example, amplification and somatic mutations of KIT and constitutive expression of growth factors HGF and FGF (Fecher et al., 2008; Panka et al., 2006a). The RAF-MEK-ERK pathway conveys extra- and intracellular signals to transcription regulators that control gene expression in response to these signals. The pathway impinges on all the functional hallmarks of cancer cells including immortalization, growth factor independent proliferation, loss of responsiveness to cell cycle checkpoint signals, evasion of apoptosis; insensitivity to growth inhibitory signals, ability to invade and metastasize, and ability to attract blood vessels (Chang et al., 2003; Kolch, 2000; Pearson et al., 2001) and represents rich druggable targets for drug development (Fig. 1, <http://clinicaltrials.gov/>) (Fecher et al., 2008; Panka et al., 2006a).

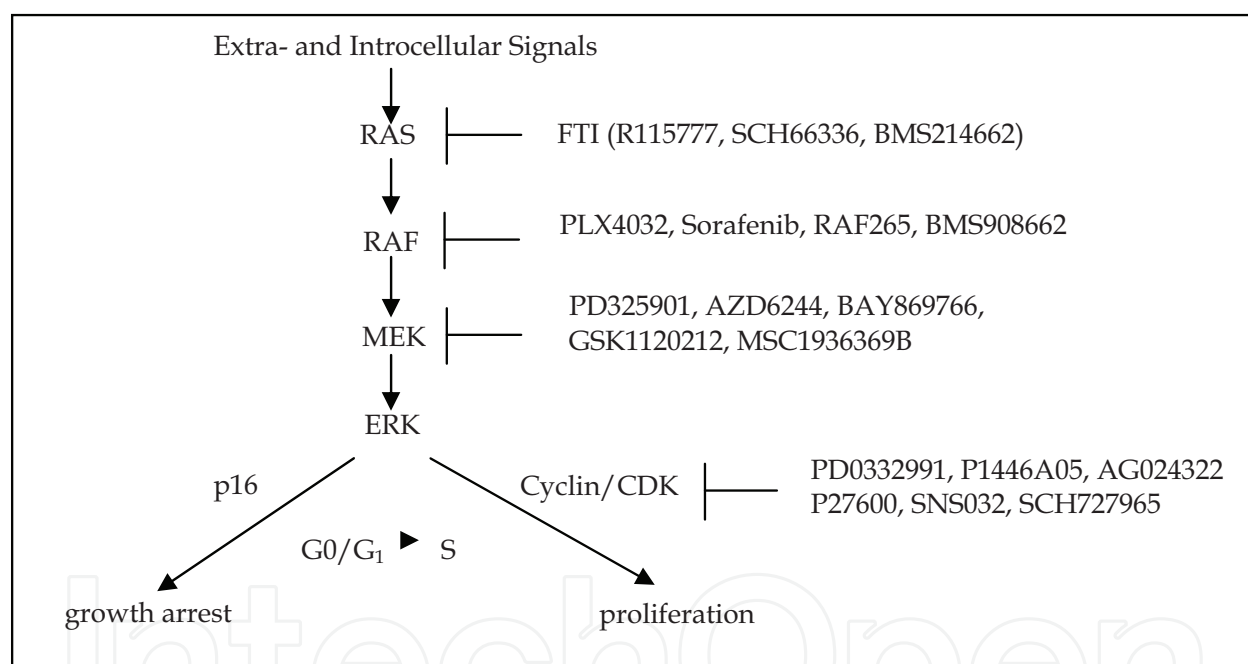


Fig. 1. RAS-RAF-MEK-ERK signaling pathway and specific inhibitors. RAF relays RAS signals through MEK to ERK. The activation of this pathway has multiple effects on cell proliferation, differentiation, and survival depending on the cellular contexts. Also shown are inhibitors to components of the pathway that are in active clinical trials (<http://clinicaltrials.gov/>). FTI, farnesyl transferase inhibitor.

3. Detection and specific inhibition of mutant BRAF

We performed research and validation studies to detect BRAF codon 600 mutations (Dong et al., 2003; Rotolo et al., 2005). As shown in Figure 2, a PCR and Sanger sequencing assay is used to detect BRAF codon 600 mutations. The common T1799A transversion changes wild-type GTG (valine) to GAG (glutamate) at codon 600 (V600E). In addition, a two-nucleotide

substitution, GTG to AAG (lysine), V600K, is occasionally identified in melanoma cells (Fig. 2A; Dong, 2003). BRAF proteins harboring V600E or V600K mutations, but not wild-type BRAF, are specifically inhibited by mutant specific BRAF inhibitor PLX4032 (Bollag et al., 2010; Flaherty et al., 2010; Rubinstein et al., 2010; Solit & Rosen, 2011). It is interesting to note that although most BRAF mutant alleles co-exist with wild-type copies, some melanoma cells show loss of the wild-type allele (Fig. 2A, A1799 LOH, AA1798-1799 LOH). Although rare, these cases suggest that loss of wild-type allele may offer growth advantage to cancer cells. We developed a PCR and restriction fragment length polymorphism (RFLP) assay (PCR-RFLP) to detect the common T1799A BRAF mutation (Fig. 2B). We designed a PCR forward primer (5'-GTA AAA ATA GGT GAT TTT GGT CTA GCT GAA G-3') to create, when the mutant A1799 follows in the DNA template, an MboII restriction site [GAAGA(N8)↓]. The reverse primer also has an MboII site for enzyme digestion and size control. PCR products are digested with MboII and restriction fragments separated by electrophoresis (Fig. 2B). The method is validated by direct sequencing of PCR products. Using this method, we identified melanoma cell lines that are wild-type (1363Mel), heterozygous (624Mel, WM35) or show LOH (A375) for the T1796A mutation (Rotolo et al., 2005).

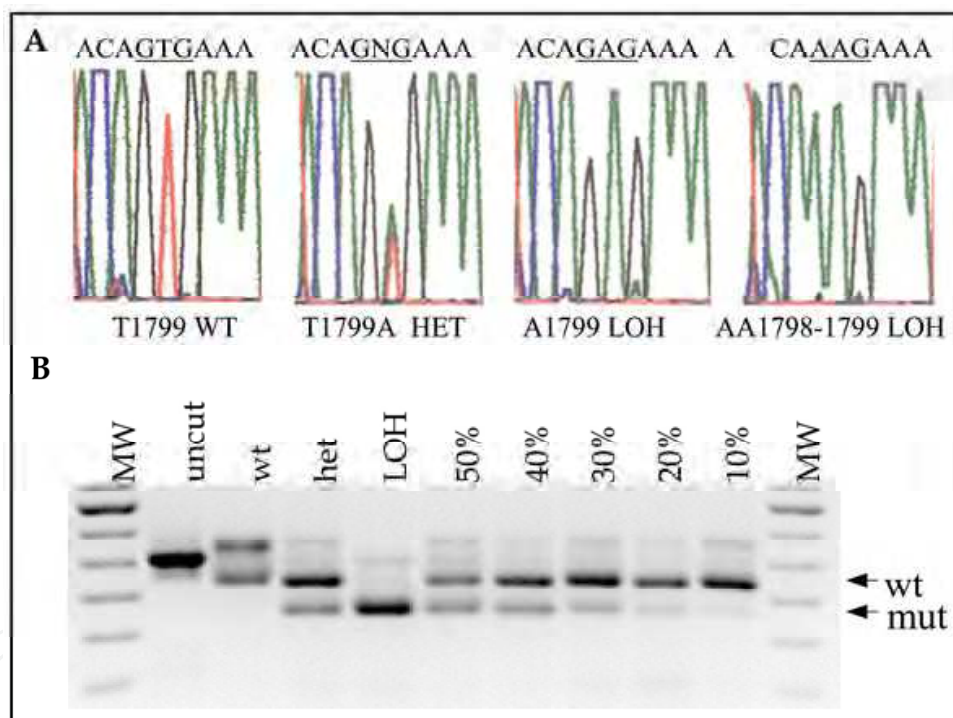


Fig. 2. BRAF codon 600 mutation analysis. (A) Wild-type (wt) and mutant (mut) alleles are detected by Sanger sequencing. The heterozygous (HET) sequence showed approximately 50:50 ratio of wt vs. mut alleles. Samples with no detectable wild-type allele are called loss of heterozygosity (LOH). A two-nucleotide substitution, GTG to AAG, is occasionally identified in melanoma specimens. (B) PCR-RFLP assay to increase sensitivity of detecting mutant allele (10% vs 30% by Sanger sequencing). Control and enzyme digested PCR amplicons are separated by electrophoresis on a 3% agarose or 10% polyacrylamide gel. The undigested PCR amplicon, digested wt and mut bands are 255, 218, and 178 bps, respectively. Serial dilutions containing 50%, 40%, 30%, 20%, and 10% of mutant alleles are used to determine assay sensitivity. Various heteroduplex bands are visible. MW, 50 bp molecular weight marker.

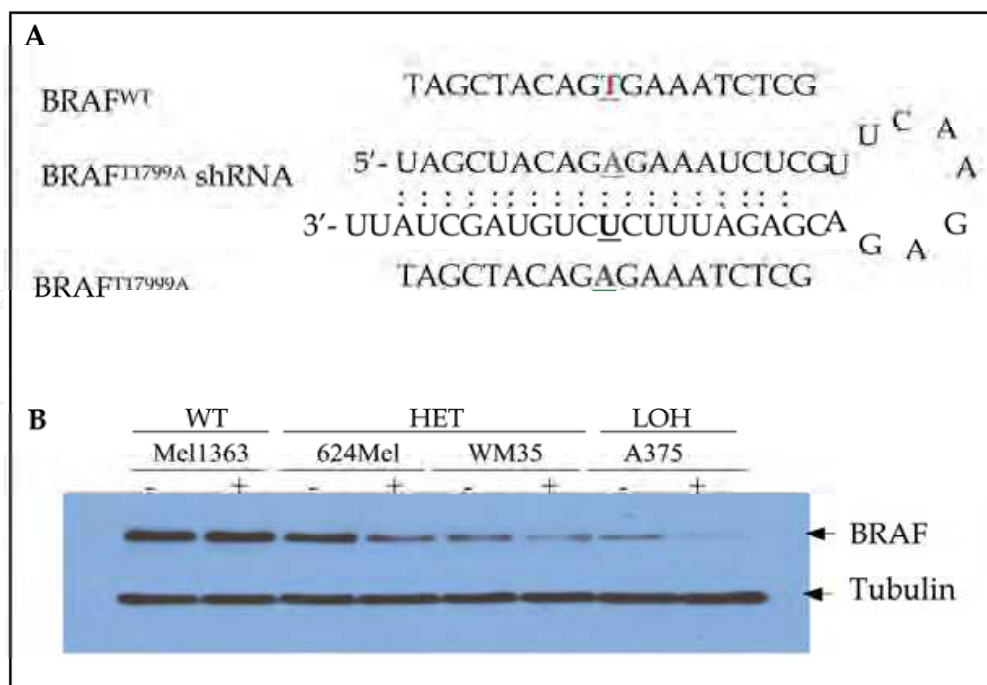


Fig. 3. T1799A mutant specific RNAi. Control and mut BRAF RNAi retroviruses are produced to infect melanoma cell lines. (A) The predicted BRAF^{T1796A} shRNA transcript encoded by pRS-BRAF^{T1799A}. Shown are sequences of the wt and T1799A mut alleles, and the predicted shRNA. (B) Western blot analysis of specific inhibition of mut BRAF by RNAi. Melanoma cells are stably infected with control (-) or pRS-BRAF^{T1799A}, a retrovirus expressing mut BRAF shRNA (+). Cell lysates are separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to membrane, and probed with BRAF and tubulin antibodies. BRAF is not significantly affected in Mel1363 cells that have wt BRAF, but inhibited in 624Mel and WM35 cells that are heterozygous for mut BRAF (HET). Mut BRAF RNAi almost completely inhibited BRAF expression in A375 cells that are LOH of mut BRAF (Dong et al., 2003; Rotolo et al., 2005).

Other methods including real-time PCR, pyrosequencing, and allele-specific hybridization can be used to detect BRAF codon 600 mutations (e.g., reagents from Qiagen, Valencia, CA, USA; EntroGen, Tarzana, CA, USA; and Trimgen Sparks, Maryland, USA). Using various mutation assays, we and others reported high frequencies (approximately 70%) of BRAF mutations in benign melanocytic nevi (Dong et al., 2003; Pollock et al., 2003). This information is important in understanding BRAF mutation in tumor biology. Since nevi are much more common in the general population compared with the rare occurrence of melanoma, BRAF mutations alone are insufficient to cause malignant transformation in nevus cells. It has been shown that oncogenic BRAF cause proliferative senescence instead of unlimited proliferation in nevus cells (Michaloglou et al., 2005).

We designed mutant specific BRAF RNAi (siRNA and shRNA) to specifically target the T1799A mutant allele (Rotolo et al., 2005). Shown in Fig. 3 is the predicted mutant specific shRNA transcript, pRS-BRAF^{T1799A}, expressed from a retroviral vector pRETRO-SUPER-puro (pRS) (Brummelkamp et al., 2002). When a pRS-BRAF^{T1799A} construct is stably expressed in cultured melanoma cells using empty vector and a 19 bp firefly luciferase (luc) sequence as controls, BRAF expression is reduced in T1799A BRAF-positive but not in BRAF wild-type melanoma cells (Fig. 3B). Further, inhibition of BRAF expression is greatest in

A375 melanoma cells that show LOH at the BRAF locus and thus express only the T1799A mutant BRAF (Rotolo et al., 2005).

4. Loss of wild-type p16 and exogenous expression of INK4A in melanomas

Genetic and epigenetic lesions in the tumor suppressor gene INK4A are commonly found in cancer cells (Hanahan & Weinberg, 2000; Lowe & Sherr, 2003). Most melanomas, but not nevi, have lost the expression of wild-type INK4A either through DNA deletion/mutation or promoter hypermethylation (Castellano et al., 1997; Funk et al., 1998; von et al., 1999; Welch et al., 2001; Zhang et al., 2002). INK4A shares coding sequence with ARF in the CDKN2A locus (Fig. 4), but the proteins are translated in different reading frames (Sherr, 2001). ARF up-regulates p53 by interfering with the p53 negative regulator MDM2, while p16 binds to and inhibits cyclin-dependent kinases 4 and 6 (CDK4/6) promoting cell-cycle arrest via the RB tumor suppressor pathway (Chang et al., 2003; Lowe & Sherr, 2003). Although deletions and mutations may affect both INK4A and ARF genes, several studies have identified mutations in melanoma specimens affecting only INK4A without ARF involvement (Chin et al., 2006; Fargnoli et al., 1998). Consistent with a tumor suppressor role of INK4A in melanomas, a germ line Arg24Cys (R24C) mutation in CDK4 has been identified in familial melanoma patients (Wolfel et al., 1995; Zuo et al., 1996). This mutation abolishes CDK4 inhibition by p16 and thus is believed to be functionally equivalent to p16 loss. Cyclin D1 and D3 are over-expressed in melanomas, which is required for growth and survival of melanoma cells *in vitro* and *in vivo* (Bartkova et al., 1996; Florenes et al., 1996; Polsky & Cordon-Cardo, 2003). Mutational inactivation of RB tumor suppressor is rare in melanomas (Bartkova et al., 1996; Maelandsmo et al., 1996).

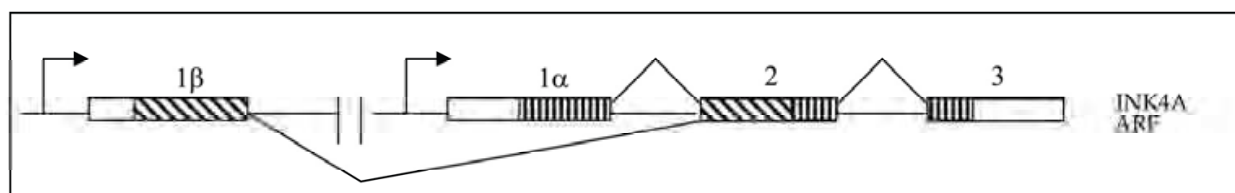


Fig. 4. Human CDKN2A locus. INK4A and ARF share sequences in the CDKN2A locus. The locus encodes two products, p16 and ARF (p14). Exons are shown as rectangles. Alternative first exons (1 α and 1 β) are transcribed from different promoters (arrows). The first exons are spliced to the same splicing acceptor site in exon 2 but are translated in alternative reading frames. INK4A coding sequences in exons 1 α , 2, and 3 and ARF coding sequences in exons 1 β and 2 are indicated by different shading patterns. Adopted from Sherr (Sherr, 2001).

We performed expression and mutation analyses of INK4A in melanoma cells. Immunohistochemical analysis shows that p16 is highly expressed in nevus cells (Fig. 5A), but expression decreases increasingly during tumor progression (Fig. 5B-D). Western blot analysis of cultured melanoma cells shows that p16 is barely detectable, if any, in melanoma cells (Fig. 5E). Mutation analysis of INK4A show that 624Mel cells have an 18 bp in-frame deletion of codons 32–37 (CTGGAGGCGGGGCGCTG) in exon 1 α . The deleted sequence is located in the first ankyrin repeat and encodes an evolutionarily conserved 6 amino acids (LEAGAL) (Greenblatt et al., 2003). Deletions and mutations affecting these amino acid have been reported in melanomas and other cancers and significantly affect the CDK- and cell cycle-inhibitory activities of p16 (Harland et al., 1997; Muzeau et al., 1997; Ruas & Peters,

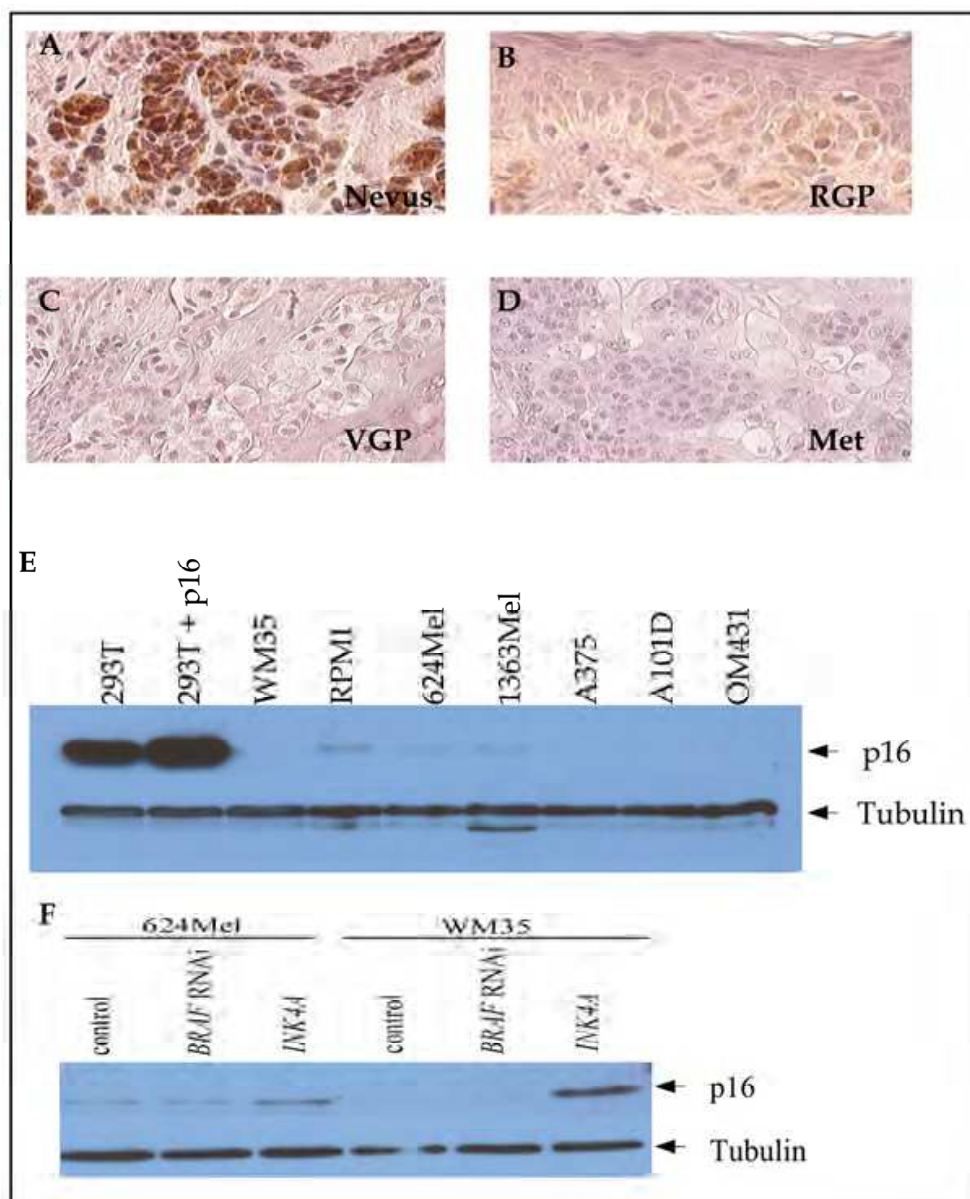


Fig. 5. Endogenous and exogenous expression of p16 in melanoma cells. (A-D) Immunohistochemical analysis of p16 expression in melanocytic lesions. Formalin-fixed and paraffin-embedded (FFPE) tissue sections of nevus (A), RGP (B), VGP (C), and metastatic (D) melanomas are stained with p16 antibody. All the samples are also positive for BRAF T1796A mutation (not shown). Note the strong expression of p16 in nevus but not in melanoma cells. Magnification $\times 100$. (E) Western blot analysis of p16 expression in melanoma cell lines. 293T control (293T), 293T transfected with INK4A cDNA (293T + p16) are used as controls. WM35, RPMI, 624Mel, 1363Mel, A375, A101D, and OM431 melanoma cell lines are cultured in regular media. Cell lysates are separated by SDS-PAGE. Western blot was probed with p16 and tubulin antibodies (arrows). WM35, RPMI, 624Mel, A375, A101D, and OM431 also have BRAF T1796A mutation. (F) Exogenous expression of wt INK4A in melanoma cells. Immunoblotting of 624Mel and WM35 controls (-), and cells stably infected with retroviruses expressing mutant BRAF RNAi (BRAFi RNAi) or INK4A cDNA (INK4A). Western blot is probed with p16 and tubulin antibodies (arrows). Note mutant BRAF inhibition has no detectable effect on endogenous (624Mel) or exogenous (WM35) p16 expression.

1998). The deletion shows LOH (data not shown), suggesting that either the wild-type copy of the gene is deleted or this is a homozygous deletion. Sequence analysis also shows that INK4A is wild-type in RPMI and 1363Mel and deleted in WM35 melanoma cells (data not shown). Mutant p16 is expressed at relatively lower levels in 624Mel cells compared to wild-type p16 in RPMI and 1363Mel cells (Fig. 5E). The expression of wild-type INK4A can be restored in melanoma cells by exogenous expression of INK4A cDNA (Rotolo et al., 2005; Zhao et al., 2008). As shown in Fig. 5F, 624Mel and WM35 melanoma cells are infected with either vector control or pBabe-neo-INK4A retroviruses. After G418 selection of mass culture, immunoblotting showed that p16 is expressed at approximately 3-5 fold more than endogenous p16 in 624Mel cells and at a higher level in WM35 cells (Fig. 5F).

5. Functional interaction of BRAF and INK4A lesions in melanoma cells

Activating BRAF mutations and loss of wild-type INK4A expression both occur at high frequencies in melanomas. However, BRAF and INK4A lesions can have overlapping roles in the regulation of the RB pathway (Fig. 6). As shown in Fig. 6, oncogenic BRAF can upregulate cyclin D through ERK pathway resulting in activation of CDK4/6. In contrast, p16 binds to and inactivates these CDKs. Therefore, CDK4/6 may be activated either by mutant BRAF through upregulation of cyclin D via ERK signaling or by loss of p16 activity. Activated CDKs phosphorylate and inactivate RB proteins resulting in the liberation of E2F transcription factors and cell cycle progression, which may contribute to the observed hyperphosphorylation of RB proteins and activation of E2F transcription in advanced melanoma cells (Halaban, 1999). There is also indirect evidence suggesting the cooperation between lesions of BRAF and INK4A in tumor development. In normal fibroblasts, oncogenic RAS and RAF have been shown to cause permanent growth arrest and/or senescence, rather than unrestricted proliferation, through a mechanism involving induction of INK4A expression (Lin et al., 1998; Zhu et al., 1998). Deficiencies in INK4A abrogate RAS-induced senescence, leading to transformation of human fibroblast cells (Brookes et al., 2002; Drayton & Peters, 2002; Drayton et al., 2003; Huot et al., 2002). In mice, neither oncogenic Ras nor Ink4a loss is sufficient to induce the development of melanomas. However, they generate spontaneous melanomas in combination (Chin et al., 1997).

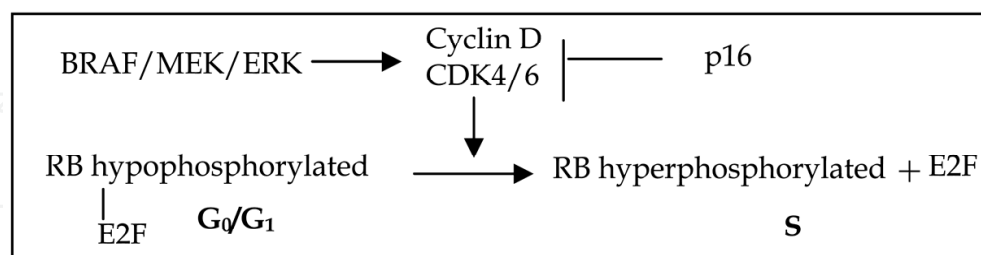


Fig. 6. The effects of BRAF and p16 can converge at the RB/E2F pathway through the opposite effects on cyclin-dependent kinases and cell cycle progression in the G₁/S phase.

6. Growth inhibition by either suppression of mutant BRAF or expression of wild-type INK4A

We performed experiments to examine the effects of inhibiting mutant BRAF or expressing wild-type INK4A in melanoma cells. We found that 624Mel, A101D and WM35 cells that harbor both T1796A and INK4A mutations have intrinsic MEK activation (data not shown) that is

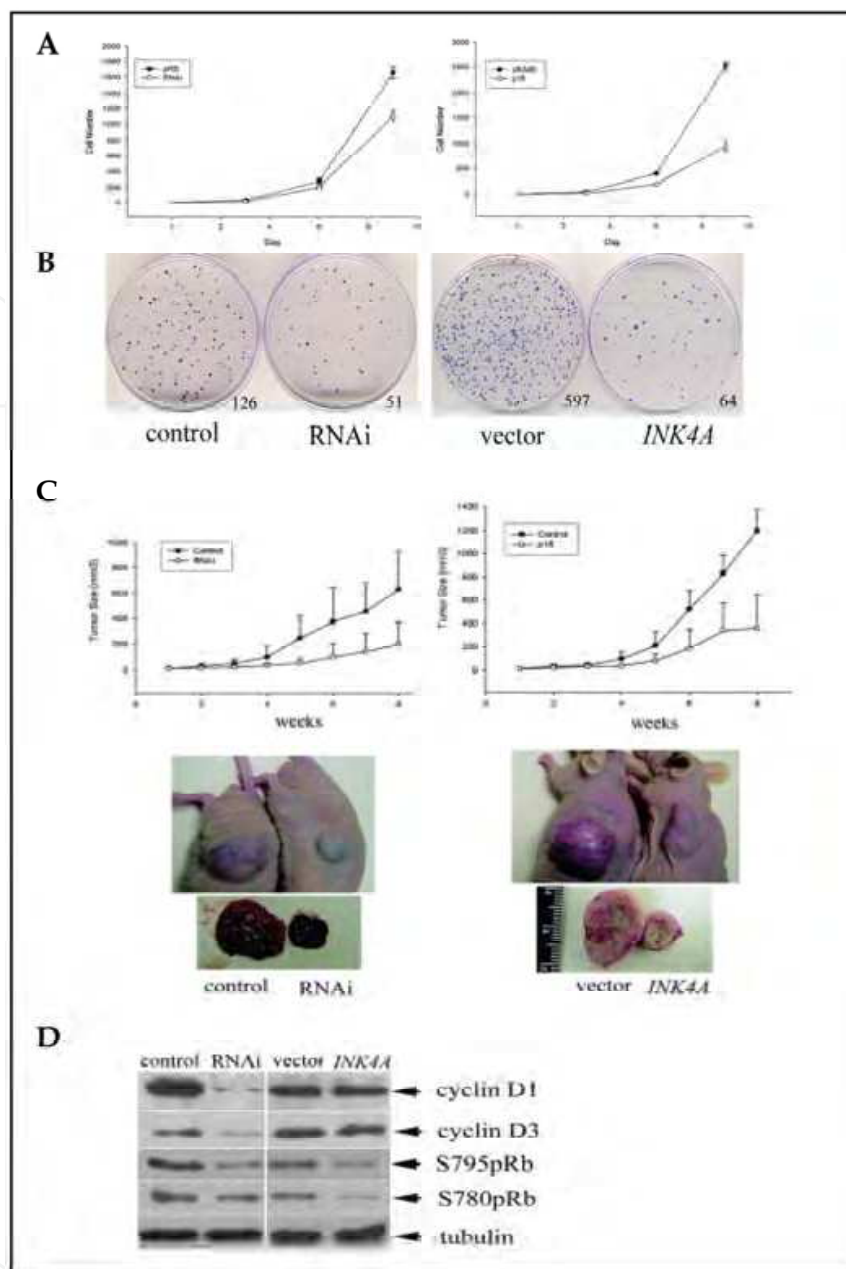


Fig. 7. Growth inhibition of melanoma cells by mutant BRAF RNAi or wild-type p16. Shown are *in vitro* and *in vivo* growth of 624Mel cells stably expressing either luc-RNAi (control) or mut BRAF RNAi (RNAi), vector control (vector) or wt INK4A (INK4A). (A) Cell counting. 5×10^4 624Mel cells are cultured and counted every 3 days using a hemocytometer ($P < 0.05$, Student *t* test of day 9 cells). (B) Colony formation assay. 1×10^3 624Mel cells are plated in triplicate in 35 mm diameter plates and grown for 2 weeks. Colonies are fixed and stained. The colony numbers shown are the average colony counts from three plates ($P < 0.001$, Two Poisson Parameters test). (C) Mouse xenograft assay. 5×10^5 624Mel cells are injected subcutaneously into flanks of nude mice ($n = 6$). Tumor growth is monitored. Pictures are taken 8 weeks after cell inoculation. The average tumor volumes are calculated and plotted ($p < 0.01$, *t* test). (D) Expression of cyclin D1/D3 and phospho-pRB. Western blotting is performed using cell lysates from 624Mel control cells and cells expressing mut BRAF RNAi or INK4A and probed with cyclin D1 and cyclin D3 or Ser795 and Ser780 phosphorylated pRB (Rotolo et al., 2005).

inhibited by mut BRAF RNAi (Rotolo et al., 2005), consistent with earlier reports (Hingorani et al., 2003; Karasarides et al., 2004; Sumimoto et al., 2004; Wellbrock et al., 2004). Both mutant BRAF RNAi and wild-type INK4A significantly inhibited the growth of 624Mel cells in tissue culture, as measured by cell count (Fig. 7A) and colony formation assay (Fig. 7B). Population doubling times of mut BRAF RNAi and wild-type INK4A expressing 624Mel cells are on average 36 hr and 50 hr, respectively, compared to controls of approximately 24 hr (Rotolo et al., 2005). The effect on tumorigenesis is examined in a nude mice xenograft assay. Tumor growth is significantly inhibited by both mut BRAF shRNA and wild-type INK4A in 624Mel (Fig. 7C) and A101D and WM35 cells (not shown). Consistent with the observed growth inhibitory effects and with an overlapping role of BRAF and INK4A lesions in RB protein and cell cycle regulation (Fig. 6), cyclin D1 and D3 are downregulated in cells expressing mut BRAF RNAi, and phosphorylation of S780 and S795 of pRB, known cyclin D1/CDK4 targets are suppressed by both BRAF RNAi and INK4A (Fig. 7D).

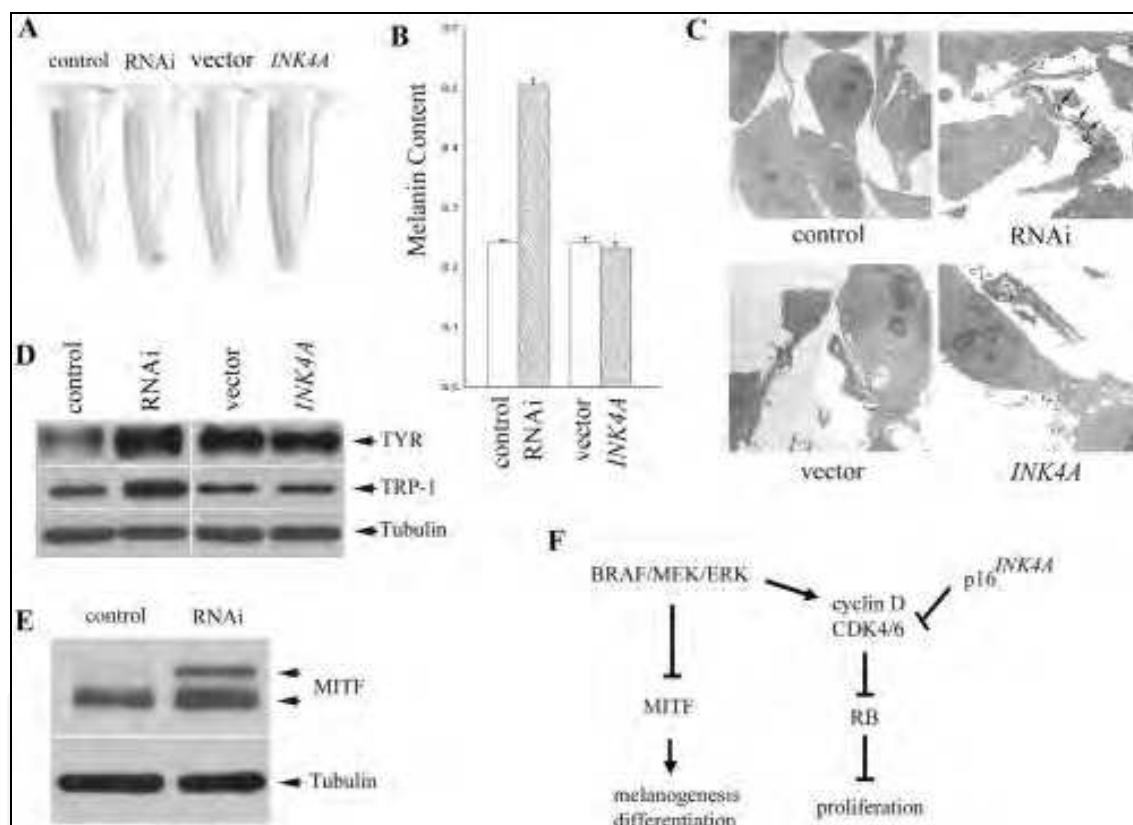


Fig. 8. Effect on melanogenesis. (A) Color of cell pellets. 5×10^6 624Mel controls and cells expressing mut BRAF RNAi or INK4A are pelleted. RNAi pellet shows a visible darker color. (B) Melanin contents; 5×10^6 cells are measured for melanin content. Data are means \pm SE from 3 experiments performed in triplicate ($p < 0.001$, t test). (C) Representative electron micrographs of cultured 624Mel controls and cells expressing mut BRAF RNAi or INK4A. Note the increased numbers of mature melanosomes in RNAi-expressing cells (arrows). Magnification $\times 2,000$. Similar changes are obtained using dissected xenograft tumors. (D) Western blot analysis of the expression of tyrosinase (TYR) and TRP-1 proteins. Western blot is probed with TYR, TRP-1 and α -tubulin antibodies. (E) Increased MITF in cells expressing T1796A BRAF RNAi, shown by Western blotting. (F) Model of separate regulation of proliferation and differentiation by mutant BRAF and p16 in melanoma cells.

Melanogenesis, a sign of melanocyte differentiation, is often changed in melanomas (Halaban, 2002). During normal development, differentiation stimuli trigger the activation of microphthalmia-associated transcription factor (MITF) to promote cell-cycle arrest and initiate the melanogenesis process (Fang & Setaluri, 1999; Setaluri, 2003; Widlund & Fisher, 2003). Melanogenesis is a multi-step biochemical process resulting in the formation of melanin in pigment cells. Melanogenic factors, tyrosinase (TYR), and tyrosinase-related protein-1 (TRP1) participate in the melanogenic pathway and are important melanocyte differentiation markers (Fang & Setaluri, 1999; Setaluri, 2003). Eberle et al (Eberle et al., 1995) compared the expression of TYR and TRP1 in cultured normal human melanocytes and melanoma cell lines by Northern blotting and reverse transcriptase-PCR (RT-PCR). They found that TYR and TRP1 genes are expressed in normal human melanocytes, but the expression is repressed in nearly all of the 14 melanoma cell lines examined and is completely absent in 4 of the 14 lines (Eberle et al., 1995). Hofbauer et al (Hofbauer et al., 1998) also found that tyrosinase expression level correlates inversely with clinical stage. These data suggest that the normal melanogenesis program is inhibited in melanoma cells. It is believed that differentiation and malignancy are inversely correlated and cancer is a disease of cell differentiation (Harris, 2004). Although unlimited proliferation and defects in cellular differentiation are characteristic of cancer growth, how the abnormal proliferation and pigmentation/differentiation are interconnected in the development of melanoma and other cancers is largely unknown (Coffman & Studzinski, 1999; Halaban, 2000).

Suppression of mutant BRAF inhibited ERK signaling, which may be attributed to the observed melanogenic effects. However, both activation and inhibition of ERK signaling can cause increased pigmentation. For example, although melanogenesis in melanocytes is suppressed both *in vitro* and *in vivo* by exogenous expression of Ras oncogene (Dotto et al., 1989; Tsukamoto et al., 1992; Wilson et al., 1989), transgenic mice expressing Ras under a mouse tyrosinase promoter demonstrated hyperpigmentation and melanocytic hyperplasia (Powell et al., 1995). Activation of ERK signaling by the c-Kit receptor plays a crucial role in the differentiation of pigment cells during development (Lassam & Bickford, 1992), whereas constitutive ERK signaling in melanoma cells is inhibitory to melanogenesis and inhibition of intrinsic ERK signaling in melanoma cells causes induction of melanogenesis and cellular differentiation (Englaro et al., 1998; Kim et al., 2004). Although our results demonstrate a melanogenic effect by inhibiting mutant BRAF, it is worth noting that activating BRAF mutations have been found in 70–80% of benign nevi that are mostly dark in color, and some malignant melanomas that are highly pigmented may have BRAF mutation. Thus, although BRAF mutation is important in blocking the melanogenic process in melanoma cells, it is not sufficient by itself to cause depigmentation. It is clear that the roles of BRAF mutation and ERK signaling in melanogenesis and melanocytic differentiation depend on the cellular context. Precisely what role is played by BRAF mutation and how it interacts with other signaling pathways in the determination of pigmentation phenotype needs to be further studied.

Since restoring p16 expression does not increase pigmentation, though cell proliferation is also suppressed, the differentiation effect of BRAF inhibition is not merely the result of general growth suppression. Rather, mutant BRAF actively participates in the differentiation program while simultaneously inducing proliferation. It is well known that malignant cells, including those of melanomas, maintain their differentiation program and sensitivity to differentiation modulators (Leszczyniecka et al., 2001). Therefore, understanding mutant BRAF in the regulation of the differentiation process may provide strategies by targeting

mutant BRAF to reverse melanoma cells to a more mature and less malignant state. Melanogenesis is induced by inhibition of mutant BRAF but not by expression of wild-type p16, suggesting the existence of different mechanisms and nonoverlapping roles of the two most common genetic lesions in the malignant transformation of melanoma.

7. Growth inhibition and apoptosis by simultaneous inhibition of mutant BRAF and expression of INK4A in melanoma cells

Aberrant BRAF and INK4A often co-exist in melanoma and the regulatory relationship between BRAF and INK4A is unclear. It is possible that the co-occurrence is a result of genetic changes necessary for melanoma development without functional significance in melanoma cells. For example, in benign nevi, mutant BRAF turns on the expression of p16 resulting in proliferative senescence to counteract BRAF's proliferative drive (Bennett, 2008). Thus, loss of wild-type p16 is permissive to BRAF initiated melanoma development (Bennett, 2008). If mutant BRAF is still capable of turning on INK4A in melanoma (as in benign nevi), suppression of mutant BRAF, which has been actively explored to treat melanoma, may actually reduce the expression of any remaining functional p16 in melanoma cells and counteract the inhibitory effect of the treatment. On the other hand, the non-overlapping roles in melanogenesis suggest non-epistatic and additive/synergistic activities by the co-existing lesions. We hypothesize that simultaneous inhibition of BRAF-MEK and INK4A-CDK4 pathways is more effective than either alone in melanoma treatment. To test this hypothesis we performed experiments to co-express mut BRAF RNAi and INK4A in melanoma cells. Our initial studies revealed a sequence dependent difference when mut BRAF shRNA and wt INK4A are stably expressed in melanoma cells; the orders of introducing mut BRAF shRNA and wt INK4A generate different outcomes (Table 1, Table 2). 624Mel and WM35 cells stably expressing mut BRAF RNAi followed by wt INK4A expression (Set 1 in Table 1) are lethal although control 1363Mel cells are viable (Set 1 in Table 2). Whereas 624Mel and WM35 viable lines are eventually generated after weeks of tissue culture in experiments performed in the reverse order (Set 2 in Table 1 and Table 2). These results indicate that suppression of mut BRAF followed by restoration of wt INK4A generates more inhibitory effect than experiment performed in the reverse order in cells that harbor BRAF and INK4A lesions. We propose that inhibition of mut BRAF induces melanoma cells to a more differentiated state more susceptible to suppression by INK4A, whereas prior expression of INK4A interferes with differentiation program by mut BRAF inhibition. Therefore, the order of interventions could be important when targeting both BRAF and INK4A lesions in melanoma treatment.

Set 1*	Established line	pRS-puro	pRS-puro BRAF shRNA
	2 nd control	pBabe-neo	pBabe-neo
	2 nd Infection	pBabe-neo-INK4A	pBabe-neo-INK4A
Set 2*	Established line	pBabe-neo	pBabe-neo-INK4A
	2 nd control	pRS-puro	pRS-puro
	2 nd Infection	pRS-puro BRAF shRNA	pRS-puro BRAF shRNA

Table 1. Generation of melanoma cell lines expressing both mutant BRAF RNAi and INK4A

Cell line*	Set 1*		Set 2*	
624Mel	control	viable	control	viable
	BRAF shRNA + INK4A	lethal	INK4A + BRAF shRNA	viable
WM35	control	viable	control	viable
	BRAF shRNA + INK4A	lethal	INK4A + BRAF shRNA	viable
1363Mel	control	viable	control	viable
	BRAF shRNA + INK4A	viable	INK4A + BRAF shRNA	viable

*Set 1 and Set 2 experiments are performed in the reverse order. (Set 1) Cells infected and selected to stably expressing mut BRAF shRNA (pRS-puro BRAF RNAi) are subsequently infected with retrovirus pBabe-neo-INK4A. Cells are then under puro- and G418 double selection. (Set 2) Cells infected and selected to stably expressing pBabe-neo-INK4A are subsequently infected with retrovirus pRS-puro BRAF shRNA, and then double selected with puro- and G418. 624Mel and WM35 cells are positive for both BRAF and INK4A mutations, whereas 1363Mel is wt for both BRAF and INK4A.

Table 2. The sequence of expression of mut BRAF RNAi and wt INK4A affects cell survival

Melanoma cells under lengthy puro and G418 double selection seem to undergo various changes (e.g., change in ploidy as measured by flow cytometry, data not shown). We therefore choose to examine the more direct effect using transient transfection experiment. As described in Fig. 2 and Fig. 5, human melanoma cell line 624Mel is heterozygous for BRAF T1799A mutation (the ratio of T1799:A1799 alleles is about 1:1) and the cells have a six amino acid deletion in the exon 2 of INK4A although the smaller mutant protein is still detectable by Western blotting. Expression of BRAF RNAi and p16 cDNA each individually caused comparable levels of growth inhibition in melanoma cells through down-regulation of RB phosphorylation at the CDK4/6 sites (Fig. 7). 624Mel cells are transiently transfected with scrambled siRNA (5'-AAG UCC AUG GUG ACA GGA GAC-3') and pBabe vector (control), BRAF siRNA (5'-AAG UGG CAU GGU GAU GUG GCA-3') and pBabe vector (siRNA), scrambled siRNA and INK4A cDNA (INK4A), and BRAF siRNA and INK4A cDNA (siRNA-INK4A). The transfection efficiencies in 624Mel cells are approximately 30-50%, and the effects of transfection are assessed on unselected mass cultures. The RAS-RAF-MEK-ERK signaling pathway is viewed as upstream of the cyclin D-CDK4/6-p16-RB cascade; consistently, only melanomas with wild-type NRAS/BRAF have amplified CDK4 and cyclin D1 genes (Curtin et al., 2005). Therefore, expression of p16 may be epistatic to down-regulation of BRAF. If so, we would expect similar effects in cells expressing BRAF siRNA, INK4A cDNA, or BRAF siRNA plus INK4A cDNA. However, we found that simultaneous expression of BRAF siRNA and wild-type INK4A (siRNA-INK4A cells) inhibit cell growth more than either alone (siRNA or INK4A cells) as measured by cell counting (Fig. 9A, $p < 0.0001$, ANOVA; Tukey's Studentized range (HSD) test at 0.05 significance level) (Zhao et al., 2008) and colony formation (Fig. 9B).

Expression of BRAF siRNA and INK4A cDNA caused corresponding changes on levels of BRAF and p16 as detected by immunoblotting (Fig. 9D). BRAF levels are reduced in cells expressing BRAF siRNA (siRNA) and BRAF siRNA plus INK4A cDNA (siRNA-INK4A) but not in cells expressing INK4A cDNA (INK4A). The expression of p16 is increased in INK4A-transfected cells, and not in cells transfected with BRAF siRNA alone (Fig. 9D). Expression of BRAF siRNA caused reduction of MAPK signaling as indicated by ERK phosphorylation relative to ERK abundance (Fig. 9D), suggesting that the effects of BRAF inhibition are mediated by the BRAF-MEK-ERK signaling cascade. Since CDK4/6 kinases can be activated not only by RAF-MEK-ERK signaling, but also by other signaling pathways such

as PI3K-AKT (Meier et al., 2007), exogenous p16 may generate broader inhibition of CDK4/6 activity not limited to that regulated by RAF-MEK-ERK. BRAF siRNA, on the other hand, may aid in p16 growth inhibition through reduced baseline activity of cyclin D-CDK4/6. Additionally, we found that levels of p27KIP1 are increased in siRNA, INK4A, and siRNA-INK4A cells (Fig. 9D). The changes of p27KIP1 are in line with the observed growth inhibition and with the well-established role of p27KIP1 in cell cycle regulation. p27KIP1, a negative regulator of cyclin E-CDK2, together with the reduced CDK4/6 activity as a result of BRAF siRNA and p16 expression, may block RB phosphorylation at both the CDK4/6 and CDK2 sites (note CDK2 level is not significantly altered, Fig. 9D), allowing RB to form complex with E2F and block cell cycle progression. p27KIP1 is regulated through different mechanisms (Bhatt et al., 2005), and further studies are required to understand the molecular mechanisms of the growth inhibition by BRAF siRNA and INK4A.

Induction of apoptosis by BRAF siRNA has been observed in some melanoma cells (Hingorani et al., 2003; Sumimoto et al., 2004), whereas p16 may act to block apoptosis (Maddika et al., 2007). To test whether expression of INK4A cDNA interferes with apoptosis induction by BRAF siRNA, we performed a TUNEL assay 72 h after transfection of 624Mel cells with BRAF siRNA and/or INK4A cDNA. Neither inhibition of BRAF siRNA nor expression of INK4A cDNA lead to significantly higher apoptosis than control (Fig. 10C, Tukey's Studentized range (HSD) test at 0.05 significance level does not detect statistically significant difference of apoptosis between siRNA, INK4A and control). However, coexpression of BRAF siRNA and INK4A cDNA triggered statistically significant difference of apoptosis (Fig. 9C, siRNA-INK4A in 624Mel cells generated significantly higher apoptosis than control or siRNA or INK4A, ANOVA $p = 0.0003$ and $p < 0.0001$, respectively; Tukey's Studentized range (HSD) test at 0.05 significance level). Note that there is no selection for transfected cells, so the maximum expected apoptosis is 50% (equivalent to the maximum transfection efficiency). The apoptotic effect, together with the enhanced growth inhibition (Fig. 9A and 9B), are consistent with the difficulty in generating stable cell lines expressing both BRAF short hairpin RNA and INK4A cDNA (Table 1 and Table 2). An anti-apoptotic effect of p16 has been previously reported that may be functionally equivalent to activation of RB since functional RB is known to suppress apoptosis (Harbour & Dean, 2000; Pucci et al., 2000). However, RB phosphorylation should be further inhibited in siRNA-INK4A expressing cells. The observed apoptosis may be mediated by other molecule(s) that over-ride or modify the anti-apoptotic effect of RB. Proteins in the BCL2 family are known to be critical regulators of apoptosis (Adams & Cory, 2007) which is in line with our observation that apoptosis is associated with the pro-apoptotic protein BIM (BCL2 interacting mediator of cell death (Collins et al., 2005), and BCL2, a critical prosurvival factor (Fig. 9D). The pro- and antiapoptotic counterparts of the BCL2 protein family can form heterodimers and neutralize each other's functions, suggesting that their relative concentrations play a pivotal role in the execution of programmed cell death. In BRAF siRNA cells, only an increase in BIM is detected, whereas in siRNA-INK4A cells, increased BIM occurs together with a decreased level of BCL2 (Fig. 9D). This could further offset the balance toward activation of apoptosis. The decrease in the levels of BCL2 protein is observed only in siRNA-INK4A cells but not in siRNA or INK4A cells suggesting a functional interaction between BRAF and p16 in the regulation of this pro-survival protein. Given the critical role of p53 in apoptosis, we performed sequencing analysis of p53 cDNA and found that 624Mel cells harbor a T1076G (Cys275Trp) mutation in the DNA binding domain that is likely to compromise the transcription and apoptosis function of p53 (Petitjean et al., 2007), suggesting that the observed apoptosis may not necessarily involve p53.

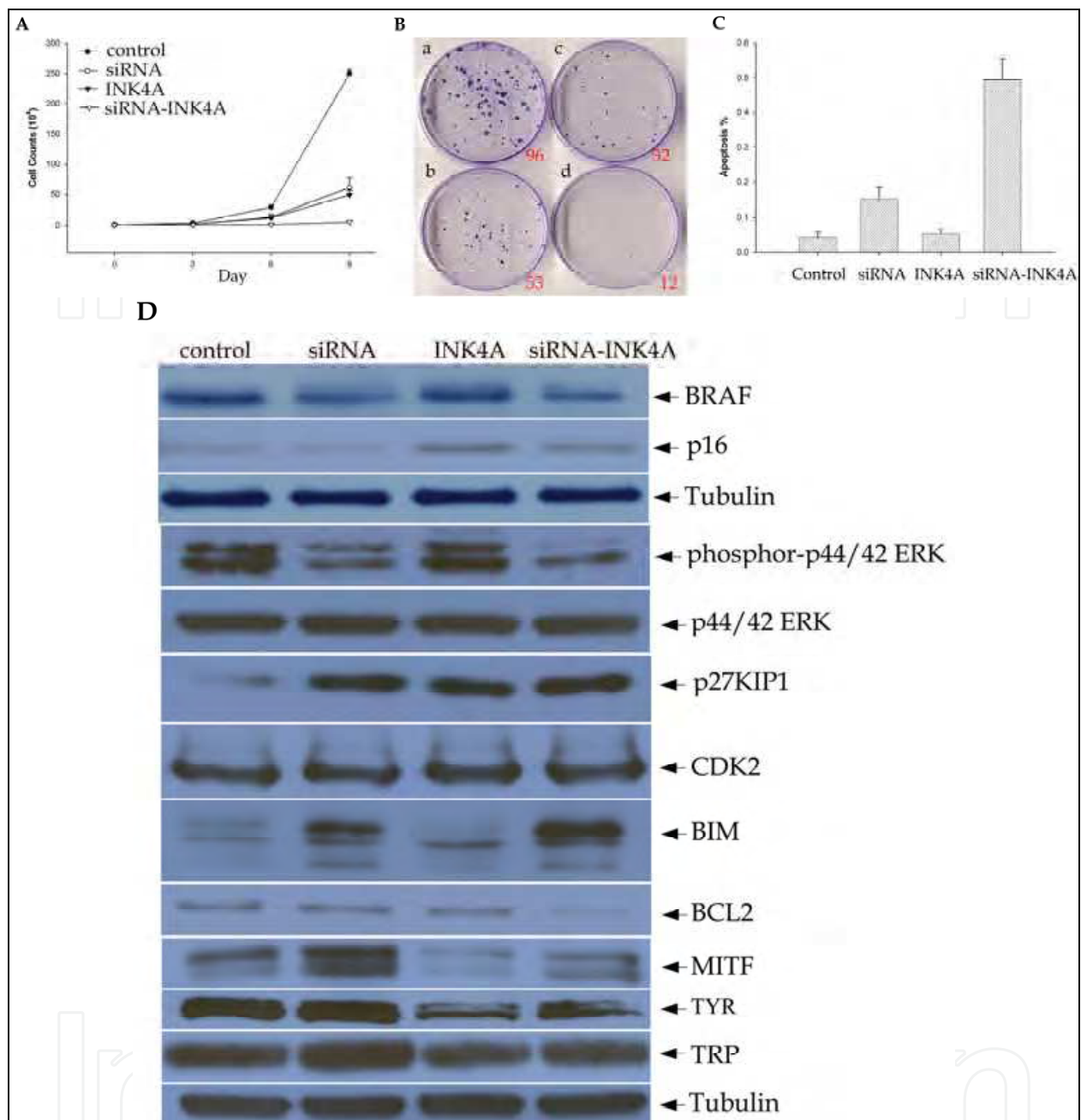


Fig. 9. Effects of simultaneous inhibition of BRAF by siRNA and expression of INK4A cDNA in 624Mel melanoma cells. 624Mel melanoma cells are transiently transfected with scrambled siRNA and pBabe vector (control), BRAF siRNA and pBabe vector (siRNA), scrambled siRNA and INK4A cDNA (INK4A), and BRAF siRNA and INK4A cDNA (siRNA-INK4A). (A) Growth inhibition measured by cell counting at day 3, 6, and 9 after transfection. (B) Growth inhibition measured by colony formation 2 weeks after transfection. (C) Apoptosis detected by TUNEL assay 72 h after transfection. (D) The expression of BRAF, p16 and several other proteins that are key regulators of proliferation (phosphor-p44/42 ERK, p27KIP1, CDK2), apoptosis (BIM, BCL2), and melanocytic differentiation (MITF, TYR, TRP1) are examined by Western blotting 48 hr after transfection. Tubulin is used as loading control (Zhao et al., 2008).

The mechanisms involved in the observed combinatory inhibition of proliferation and survival needs to be further explored. As the induced apoptosis is concomitant with growth arrest, it is possible that they are related. Alternatively, different cell populations, such as cells in different phases of the cell cycle, may respond differentially to BRAF siRNA and p16 under the same experimental condition. The observed growth inhibition and apoptosis may not simply be a quantitative/additive effect on CDK-RB since BRAF-MEK-ERK signaling has multiple targets that are not limited to CDK-RB regulation. Additionally, BRAF inhibition and p16 expression have different effects on melanogenesis (Fig. 8), suggesting that p16 may have qualitatively different activities not necessarily overlapping with BRAF, which may be the basis for the observed combinatory effects. In summary, our results show that simultaneous inhibition of mutant BRAF and expression of wild-type p16 cooperates in the inhibition of proliferation and enhances apoptosis, suggesting that BRAF and INK4A lesions - two of the most common genetic abnormalities in melanomas, interact functionally in melanoma cells and that strategies designed to correct both lesions could be effective for melanoma treatment.

8. Inhibition of MEK and CDK4 in melanoma cells

It has been shown that BRAF and INK4A may have activities independent of the corresponding canonical ERK and RB pathways, and the two pathways also mediate cellular signals independent of aberrant BRAF and INK4A. For example, RAF can act through apoptosis signal-regulating kinase-1 (ASK1)/c-Jun-NH2-kinase or mammalian sterile 20-like-kinase 2 (MST2) pathways (O'Neill et al., 2005); cyclin D:CDK4/6 can be activated by enhanced phosphatidylinositol 3-kinase (PI3K) and WNT signaling pathways in melanomas (Delmas et al., 2007; Schmitt et al., 2002). To examine whether the combinatorial effects of BRAF RNAi and INK4A cDNA in melanoma cells is specific to BRAF and INK4A or can be generalized to other components of the ERK and RB pathways, we tested PD98059 and 219476, commercially available inhibitors of MEK and CDK4, respectively, in human melanoma cells. Of note, deregulation of the RAS-RAF-MEK-ERK (ERK) and p16-cyclin D:CDK4/6-RB (RB) pathways are common in human malignancies and appears to be important for melanoma development. As shown in Fig. 1, chemotherapeutic agents targeting components of both pathways have been developed but clinical studies with monotherapy have been disappointing.

Human melanoma cell lines 624Mel, A101D, and A375 harbor heterozygous BRAF T1799A mutation and loss of wild-type p16 (Rotolo et al., 2005). The cells are treated, alone or in combination, with MEK inhibitor PD98059 (Waters et al., 1995) and CDK4 inhibitor 219476 (Zhu et al., 2003). As anticipated, ERK phosphorylation is reduced in cells treated with PD98059 and PD98059 plus 219476 (Fig. 10A). Levels of p27KIP1, a negative regulator of cyclin E:CDK2, are increased in cells treated with either PD98059 or 219476, and further increased in cells with combinatorial treatment (Fig. 10B). Phosphorylation of S780, S795, and S807/811 of RB, known cyclin D:CDK4 and cyclin E:CDK2 targets (Halaban, 2005), is decreased in cells treated with either PD98059 or 219476 (except S780 and S807/811 in OM431 cells), and further reduced in cells with combinatorial treatment (Fig. 10C). Of note, total RB is decreased under combinatorial treatment with PD98059 and 219476 in all three melanoma cells (Fig. 10C).

PD98059 and 219476 inhibit tumor cell growth in a dose dependent manner (Krasilnikov et al., 2003; Zhu et al., 2003). In order to make it possible to monitor the additional therapeutic

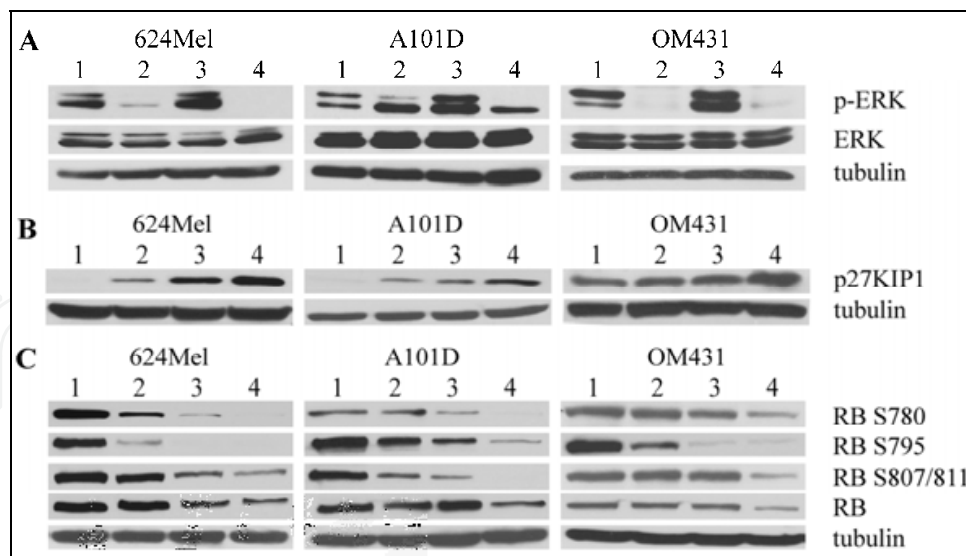


Fig. 10. Regulation of ERK phosphorylation, p27KIP1 expression, and RB phosphorylation by PD98059 and 219476, alone and in combination. Human melanoma cell lines 624Mel, A101D, and OM431 are treated with either vehicle solvent (1), PD98059 (2), 219476 (3), or PD98059 plus 219476 (4) (Li et al., 2010a). Western blot is performed using 20 μ g total cell lysates. Tubulin is used as the loading control.

effects of the combinatorial treatment, both chemicals are used at dosages lower than that which would lead to maximal effect by either agent. The cytotoxicity of PD98059 and 219476 is measured using the MTS assay that measures the dehydrogenase enzyme activity found in metabolically active cells. In all the three cell lines, a significant difference in MTS counts exists for the control, PD98059, 219476, and PD98059 plus 219476 groups ($p < 0.0001$, R-Square 0.981444, 0.956956, and 0.991102 in 624Mel, A101D, and OM431, respectively, ANOVA). Further analysis shows that simultaneous treatment with PD98059 and 219476 results in significantly reduced numbers of cell survival than control or mono-treatment as measured by MTS in all the three cell lines (Fig. 11A, Tukey's Studentized Range (HSD) Test at 0.05 significance level). TUNEL DNA fragmentation assay is used to identify loss of viability due to programmed cell death. As shown in Fig. 11B, at the drug concentrations used, significantly different levels of apoptosis exist among control for PD98059, 219476, and combinatorial treatment groups ($p < 0.0001$, R-Square 0.973862, 0.990697, and 0.987900 in 624Mel, A101D, and OM431, respectively, ANOVA). Treatment with PD98059 alone results in no difference in apoptosis over controls in all three cell lines; 219476 enhances apoptosis in OM431 but not in the other two cell lines. However, combined treatment dramatically increases apoptosis over that seen for the control and mono-treatments (Fig. 11B, Tukey's Studentized Range (HSD) Test at 0.05 significance level).

We examined the expression of several pro- and anti-apoptotic proteins. Mono-treatment with PD98059 or 219476 causes a decreased or no change in the expression of anti-apoptotic proteins BCL2, BCL2L1, and BIRC5. While there are variations in the patterns of expression of BCL2, BCL2L1, and BIRC5 among the different cell lines, combinatorial treatment causes a comprehensive down-regulation of the proteins in all three cell lines (Fig. 12). In addition apoptosis facilitator BIM-EL is increased following treatment with PD98059 and PD98059 plus 219476 in all three cell lines. It is also increased in OM431 cells following treatment with 219476 (Fig. 12). Consistent with increased apoptosis, caspase 3 is activated by simultaneous

treatment with PD98059 plus 219476 in all three cell lines, as shown by decreased pro-caspase 3, increased levels of the active form of caspase 3 (cleaved caspase 3), and degradation of PARP, a direct substrate of active caspase 3 (Fig. 12).

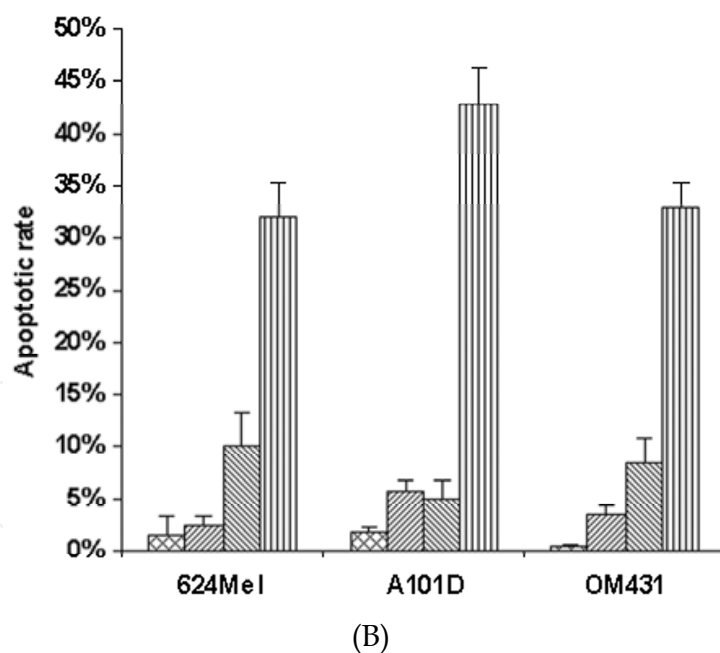
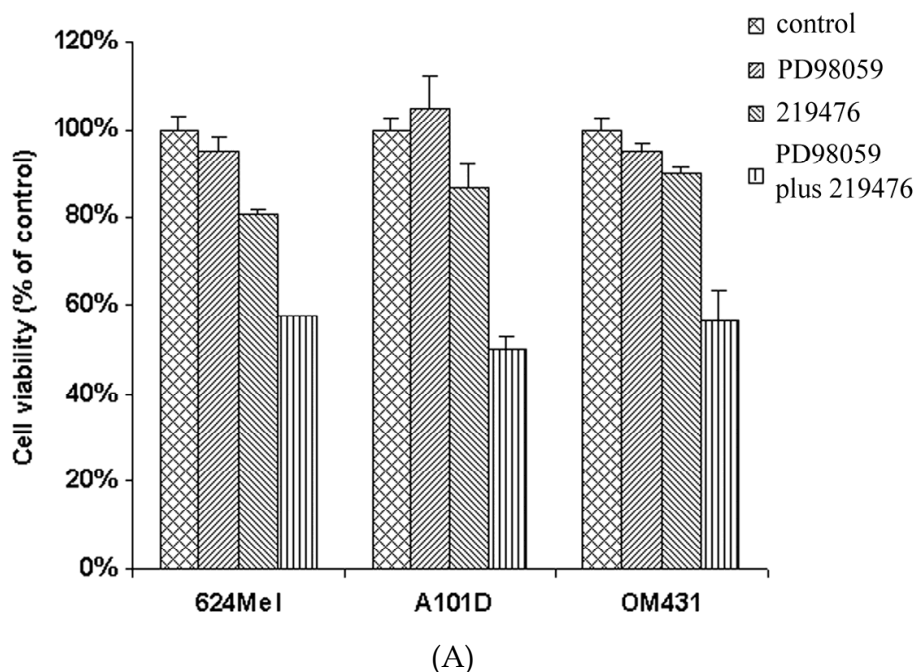


Fig. 11. Cytotoxicity by MEK inhibitor PD98059, CDK4 inhibitor 219476, and combinatorial treatment. (A) MTS cytotoxicity assay is performed in 624Mel, A101D and OM431 cells after 48h treatment in medium supplemented with 0.5% FBS. The results are given as means \pm SD from three independent tests. (B) MEK and CDK4 inhibitors induce apoptosis of melanoma cells. TUNEL assay is performed in 624Mel, A101D and OM431 cells after 48h treatment with vehicle solvent, PD98059, 219476, or PD98059 plus 219476 in medium with 0.5% FBS. The results are given as means \pm SD from three independent assays.

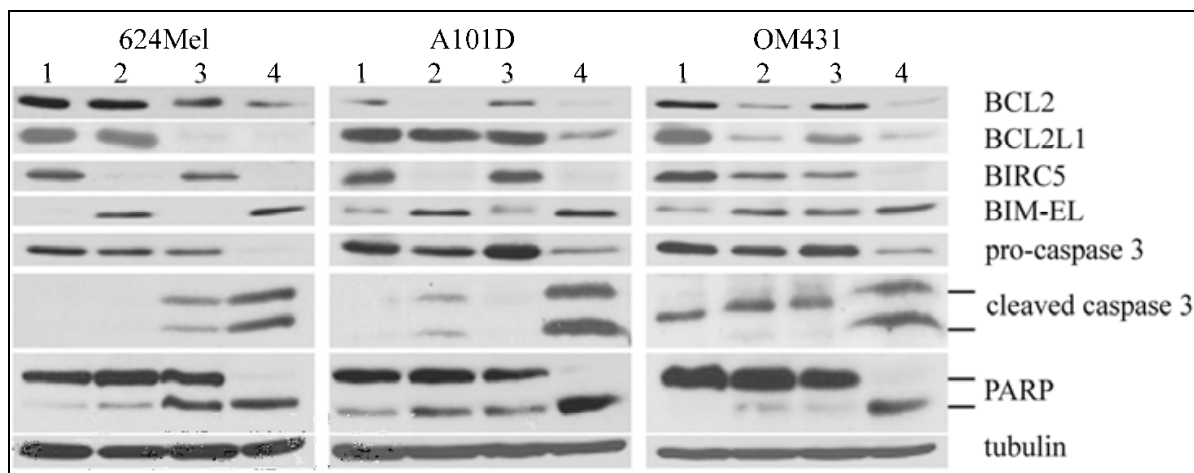


Fig. 12. Western blot analysis of changes in the expression of pro-survival and pro-apoptotic proteins. Cells are treated with solvent vehicle control (1), PD98059 (2), 219476 (3), and PD98059 plus 219476 (4) for 48 h in medium containing 5% FBS. 20 μ g total cell extracts from 624Mel, A101D and OM431 cells are separated by SDS-PAGE and blotted using BCL2, BCL2L1, BIRC5, BIM, caspase-3, and PARP antibodies. Tubulin is used as loading control.

It has been well-established that constitutive activation of the ERK signaling induces the expression of cyclin D (Michaloglou et al., 2008; Rotolo et al., 2005), which binds to and activates CDK4 leading to the phosphorylation of RB protein facilitating cell cycle entry (Halaban, 2005). Consistent with an epistatic regulation between ERK pathway and cyclin D:CDK4, amplification of cyclin D1 and CDK4 genes have been identified mainly in melanomas that harbor wild-type NRAS and BRAF (Curtin et al., 2005; Smalley et al., 2008). Additionally, cyclin D:CDK4 can mediate resistance to inhibitors of the ERK signaling pathway (Smalley et al., 2008). Therefore, the enhanced apoptosis and decreased proliferation by simultaneously inhibiting ERK and RB pathways could result from the double hitting of ERK-cyclin D:CDK4-RB that regulate cell cycle progression and cell survival. Alternatively, in support of our previous results that BRAF and INK4A have a non-linear functional interaction (Rotolo et al., 2005; Zhao et al., 2008), additional cellular processes could be affected when cells are exposed to both PD98059 and 219476. ERK pathway has pleiotropic activities that regulate cell proliferation, survival, and differentiation through both cyclin D:CDK4 dependent and independent routes (Fecher et al., 2008; Panka et al., 2006a; Sekulic et al., 2008). Likewise, cyclin D:CDK4 can be regulated and converges multiple cellular signals. For example, while PI3K signaling can activate CDK4 through down-regulation of INK4A and up-regulation of cyclin D (Schmidt et al., 2002), WNT signaling can turn on CDK4 through suppression of INK4A transcription (Delmas et al., 2007). It is conceivable that inhibition of MEK and CDK4 not only affects ERK and RB pathways, but also PI3K, WNT, and other ERK signaling activities not mediated through the RB pathway. Therefore, simultaneous targeting of both ERK and RB pathways can generate enhanced effects by targeting both linear and non-overlapping activities.

Our results show that simultaneous inhibition of MEK and CDK4 using pharmacological inhibitors PD98059 and 219476 leads to significantly increased apoptosis compared to control and single agent treatment (Figs. 11, 12). This effect is consistent with results observed after simultaneous knockdown of mutant BRAF using siRNA and expression of INK4A cDNA in melanoma cells (Fig. 9). The apoptotic effect is associated with changes of

apoptosis related proteins (Figs. 9 and 12). BCL2L1 and BIRC5 are highly expressed in melanoma cells, and increased expression correlates with tumor progression (Piras et al., 2007; Zhuang et al., 2007). A straightforward explanation for the observed apoptosis is that changes in the pro- and anti-apoptotic factors offset the balance and lead to apoptosis (Adams & Cory, 2007). Sequencing analysis of TP53 cDNA (Zhao et al., 2008) showed that 624Mel and OM431 cells harbor a T1076G (Cys275Trp) and a G1048A (Gly266Glu) mutations, respectively, in the DNA binding domain that is likely to compromise the transcriptional and apoptotic function of p53 (Petitjean et al., 2007). No TP53 mutation has been detected in A101D cells. Although apoptosis is enhanced in all three cell lines, it is somewhat more pronounced in A101D than 624Mel and OM431 cells treated with PD98059 and 219476 (Fig. 11), suggesting that TP53 status may influence the magnitude of apoptosis. Combinatorial treated cells have further inhibited phosphorylation of ERK and RB, reduced total RB, and increased expression of p27KIP1 (Figs. 9 and 12). Yu et al. demonstrated that loss of Rb causes apoptosis without effecting cellular proliferation (Yu et al., 2003) and Wang et al. found that over-expression of p27KIP1 leads to apoptosis in melanoma cells (Wang et al., 1997). The mechanisms of these changes in relationship to each other and to the observed cooperative effects need to be further investigated.

Apoptosis resistance is a critical factor for therapy failure in melanoma patients. Encouragingly, our results demonstrate that an increase in apoptosis can be achieved through combinatorial targeting of BRAF-MEK-ERK and p16-CDK4-RB pathways. Deregulation of the RAS-RAF-MEK-ERK and p16-cyclylin D:CDK4/6-RB pathways are common in human malignancies and appears to be important for melanoma development. There has been significant effort to target components of these pathways in cancer treatment. Pharmacologic agents targeting components of the ERK and RB pathways have been developed. However, clinical studies using monotherapy show that the clinical responses have failed expectations and maximum tolerated doses are often reached before reaching clinical efficacy (Burdette-Radoux et al., 2004; Fecher et al., 2008; Panka et al., 2006b; Sekulic et al., 2008). Our study suggests that combination targeting of ERK and RB pathways is a promising strategy for melanoma treatment and should encourage further in-depth investigations.

9. HERV-K expression correlates with MEK and CDK4 activation

The K type human endogenous retroviral sequence (HERV-K) is expressed in melanoma cells but not in melanocytes (Muster et al., 2003). Through millions of years of evolution and natural selection, HERVs have become indispensable parts of the human genome. For example, syncytin-1 and syncytin-2, encoded by the envelope (ENV) genes of HERV-W and HERV-FRD, respectively, mediate intercellular fusion of trophoblast cells to form syncytiotrophoblast as well as prevent maternal immune attack against the developing embryo, thereby facilitating implantation of the embryo (Krone & Grange, 2010; Rote et al., 2004). It is estimated that 8% of the human genome consists of retroviral elements (Singh et al., 2009). A complete HERV sequence is composed of GAG, POL, and ENV genes flanked by two long terminal repeats (LTRs), similar to exogenous retroviruses such as human immunodeficiency virus (HIV) (Ahn & Kim, 2009). HERVs can be classified into over 20 families based on tRNA specificity of the primer binding site used to initiate reverse transcription; thus, HERV-K would use lysine and HERV-W tryptophan if they were replicating viruses. Increased HERV expression has been found under pathological conditions, particularly in cancer and inflammatory disease (Voisset et al., 2008). Unlike

most HERVs that harbor defective mutations, the HML-2 group of HERV-Ks has open reading frames that code for functional viral proteins, which may form noninfectious particles (Beimforde et al., 2008; Turner et al., 2001). We have demonstrated recently that expression of HERV-K correlates with ERK activation and p16 loss in human melanoma specimens, and that inhibition of MEK and CDK4, especially in combination, suppresses HERV-K expression (Li et al., 2010b), which suggests that HERV-K may mediate BRAF-MEK-ERK and p16-CDK4-RB signaling pathways in melanoma cells.

It is interesting to note that the growth characteristics of melanoma cells that can be modified by HERV-K activation (e.g., changes in cell shape, loss of melanin, anchorage-independent growth) (Serafino et al., 2009) overlap with those that can be blocked by suppression of the BRAF-MEK-ERK signaling pathway, especially with simultaneous restoration of p16 or inhibition of CDK4 (Li et al., 2010a; Rotolo et al., 2005; Zhao et al., 2008). This observation, together with the knowledge that aberrations in BRAF-MEK-ERK and p16-CDK4 pathways are early events and often co-exist during melanomagenesis, and the evidence that the RAF-MEK-ERK signaling pathway is required for the completion of HIV-1 reverse transcription (Mettling et al., 2008), prompted us to hypothesize that HERV-K is regulated by BRAF-MEK-ERK and p16-CDK4 pathways. We examined the expression of HERV-K GAG and ENV proteins, the active form of ERK (phospho-ERK, p-ERK), and p16 in a panel of human melanocytic specimens including 38 benign nevi and 34 melanomas. As summarized in Table 3, both HERV-K GAG and ENV proteins are largely cytoplasmic with occasional nuclear staining observed, whereas p-ERK is typically co-expressed in the nucleus and cytoplasm. Wild-type INK4A is expressed in both nucleus and cytoplasm, whereas mutant p16, if expressed, is either nuclear or cytoplasmic (Ghiorzo et al., 2004). HERV-K GAG cytoplasmic staining is over 10-fold more frequent in melanoma than in nevi (38% of melanomas vs. 3% of nevi, $p < 0.001$) (Table 3). Similarly, HERV-K ENV immunoreactivity is detected in the cytoplasm in 44% of melanomas and 11% of neval specimens (a 4-fold difference, $p = 0.003$). The nuclear staining of GAG and ENV are infrequently detected in both nevi and melanomas, and the differences do not reach statistical significance (Table 3). p-ERK staining is 5-fold more often positive in melanomas than in nevi (68% and 13%, respectively, $p < 0.001$). p16 staining, both in the cytoplasm and

Antigen	Positivity (%)		p value
	Nevi [n=38]	Melanoma [n=34]	
HERV-K GAG, cytoplasmic	3	38	$p < 0.001^{**}$
HERV-K GAG, nuclear	0	6	$p = 0.493$
HERV-K ENV, cytoplasmic	11	44	$p = 0.003^{**}$
HERV-K ENV, nuclear	8	12	$p = 0.7$
p-ERK, cytoplasmic and nuclear	13	68	$p < 0.001^{**}$
p16, cytoplasmic	79	50	$p = 0.014^*$
p16, nuclear	79	15	$p < 0.001^{**}$

Protein immunoreactivity, cytoplasmic or nuclear, is dichotomized as negative/decreased (<30% of cells staining positively) or positive ($\geq 30\%$ of cells staining positively).

** Difference is significant at the ≤ 0.01 level (2-tailed).

* Difference is significant at the ≤ 0.05 level (2-tailed).

Table 3. Expression of HERV-K GAG and ENV, p-ERK and p16 in neval and melanoma specimens

nucleus, is more frequently observed in nevi than in melanomas (cytoplasmic, 79% vs. 50%, $p = 0.014$; nuclear, 79% vs. 15%, $p < 0.001$) (Table 3). Figure 13 demonstrates representative staining patterns of HERV-K GAG, HERV-K ENV, p-ERK, and p16 in melanoma and neval specimens. Further analysis shows that the expression of HERV-K GAG in the cytoplasm of melanoma is positively correlated with p-ERK ($p = 0.005$) and negatively correlated with p16 cytoplasmic expression ($p = 0.012$) (Table 4). The expression of HERV-K ENV in the cytoplasm of melanomas is positively correlated with p-ERK ($p < 0.001$) and negatively correlated with p16 nuclear expression ($p = 0.046$) (Table 4).

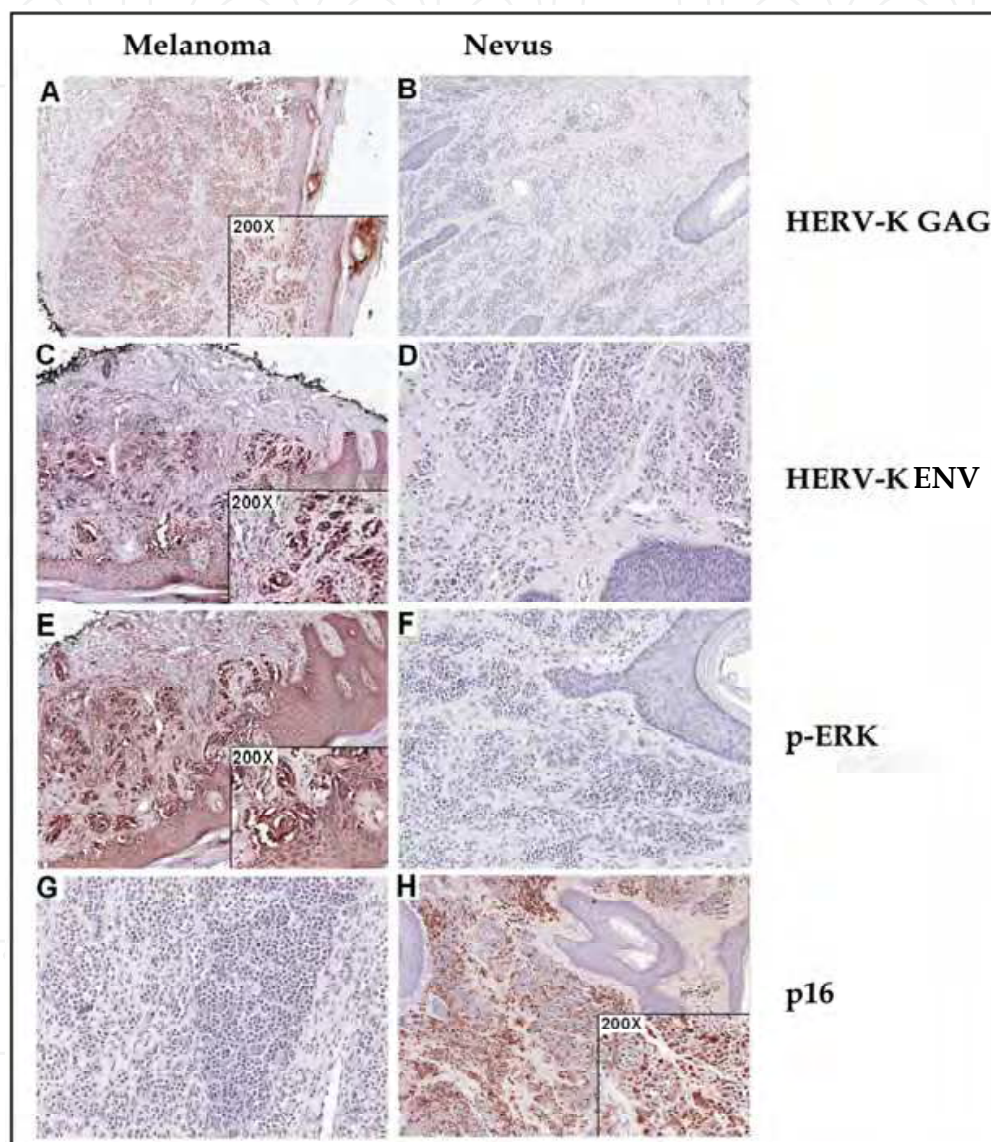


Fig. 13. Expression of HERV-K GAG and ENV, p-ERK and p16 in neval and melanoma specimens. Formalin-fixed and paraffin-embedded microscopic sections of melanoma (A, C, E, G) and nevus (B, D, F, H) are analyzed by immunohistochemical staining using HERV-K GAG (A, B), ENV (C, D), p-ERK (E, F), and p16 (G, H) specific antibodies. Shown is a representative staining pattern. HERV-K GAG and ENV are mostly detected in melanoma cells (A and C), but rarely expressed in neval cells (B and D). p-ERK is mainly detected in melanoma cells (E) but rarely found in neval cells (F). p16 is not as prominently expressed in melanoma (G) as in neval cells (H). Magnification: $\times 100$ and $\times 200$ (insets).

	HERV-K GAG cytoplasmic	HERV-K GAG nuclear	HERV-K ENV cytoplasmic	HERV-K ENV nuclear
p-ERK, cytoplasmic and nuclear	$p = 0.005^{**}$	$p = 0.093$	$p < 0.001^{**}$	$p = 0.2$
p16, cytoplasmic	$p = 0.012^*$	$p = 0.058$	$p = 0.114$	$p = 0.608$
p16, nuclear	$p = 0.123$	$p = 0.378$	$p = 0.046^*$	$p = 0.251$

Note: ** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Table 4. Associations of the expression of p-ERK, p16, and HERV-K GAG and HERV-K ENV

The expression of HERV-K has been reported in several melanoma cell lines (Buscher et al., 2006; Buscher et al., 2005; Muster et al., 2003; Serafino et al., 2009). We examined HERV-K in four melanoma cell lines (624Mel, A375, A101D, and OM431) that have constitutive activation of p-ERK and loss of wild-type p16 with corresponding hyper-phosphorylation of RB protein (Li et al., 2010a; Rotolo et al., 2005; Zhao et al., 2008). HERV-K expression is detected using a specific HERV-K ENV antibody, as described (Muster et al., 2003; Serafino et al., 2009), that recognizes a 37 Kd spliced transmembrane domain of ENV protein (Buscher et al., 2006; Buscher et al., 2005; Muster et al., 2003). HERV-K ENV protein is prominently expressed in 624Mel and A375 cells, weakly positive in A101D cells, but barely detectable in OM431 cells (Fig. 14A). We extracted total cellular RNA from all the four cell lines and performed conventional RT-PCR using specific HERV-K POL and ENV primers as described (Serafino et al., 2009). Gel electrophoresis of RT-PCR end-products shows similar levels of POL and ENV RNAs in the four cell lines, in contrast to the dramatic differences in the case of ENV protein (data not shown). Direct sequencing of RT-PCR amplicons and NCBI BLAST analysis show that expressed sequences share 96%-98% overall homology with Group N HERV-K (Romano et al., 2006) (data not shown), as reported in other melanoma cells (Hirschl et al., 2007).

Multiple endogenous and exogenous factors have been linked to the activation of HERVs including hormones, cytokines, and cytotoxic chemicals (Taruscio & Mantovani, 2004). To our knowledge, a direct association between ERK and p16-CDK4 pathways and HERV expression has not been reported previously. It has been shown that HERV-K sequences, as other host genes, are regulated by DNA methylation in the promoter/enhancer sequences located in the 5'-LTR regions (Lavie et al., 2005). Since RB protein, a downstream mediator of BRAF-MEK-ERK and p16-CDK4 signaling pathways, is a key regulator of DNA methylation (Montoya-Durango et al., 2009), it is conceivable that the observed association between HERV-K, p-ERK, and p16-CDK4 may act through RB, a notion that will surely prompt further investigation. It is worth noting that the four melanoma cell lines examined, 624Mel, A375, A101D, and OM431, all have BRAF T1799A mutation, constitutive activation of ERK, loss of wild-type p16, over-expression of phospho-RB protein (Rotolo et al., 2005), and have comparable levels of HERV-K RNA transcripts (not shown). However, only 624Mel and A375 cells express high levels of HERV-K ENV protein (Fig. 14A), suggesting that HERV-K ENV protein is regulated by mechanisms in addition to ERK and p16-CDK4 pathways. Alternatively, A101D and OM431 cells may harbor mutant HERV-Ks affecting EVE expression, a notion that needs to be further investigated.

We examined whether HERV-K ENV expression is suppressed by MEK and CDK4 inhibitors using established experimental conditions (including time course and dose-

response curve) for PD98059 and 219476, specific inhibitors of MEK and CDK4, respectively (Li et al., 2010a). As expected, treatment with PD98059 inhibited ERK phosphorylation in both 624Mel and A375 cells (Fig. 14B, lane 2). Phosphorylation of serine 780, a CDK4 target in the RB protein, is reduced by PD98059 and 219476, especially in combination, in both cell lines (Fig. 14B). Similarly, HERV-K ENV expression is inhibited by either PD98059 or 219476, especially when used in combination (Fig. 14B). The results are consistent with findings of the association between HERV-K expression and p-ERK and p16 in melanocytic specimens (Table 4).

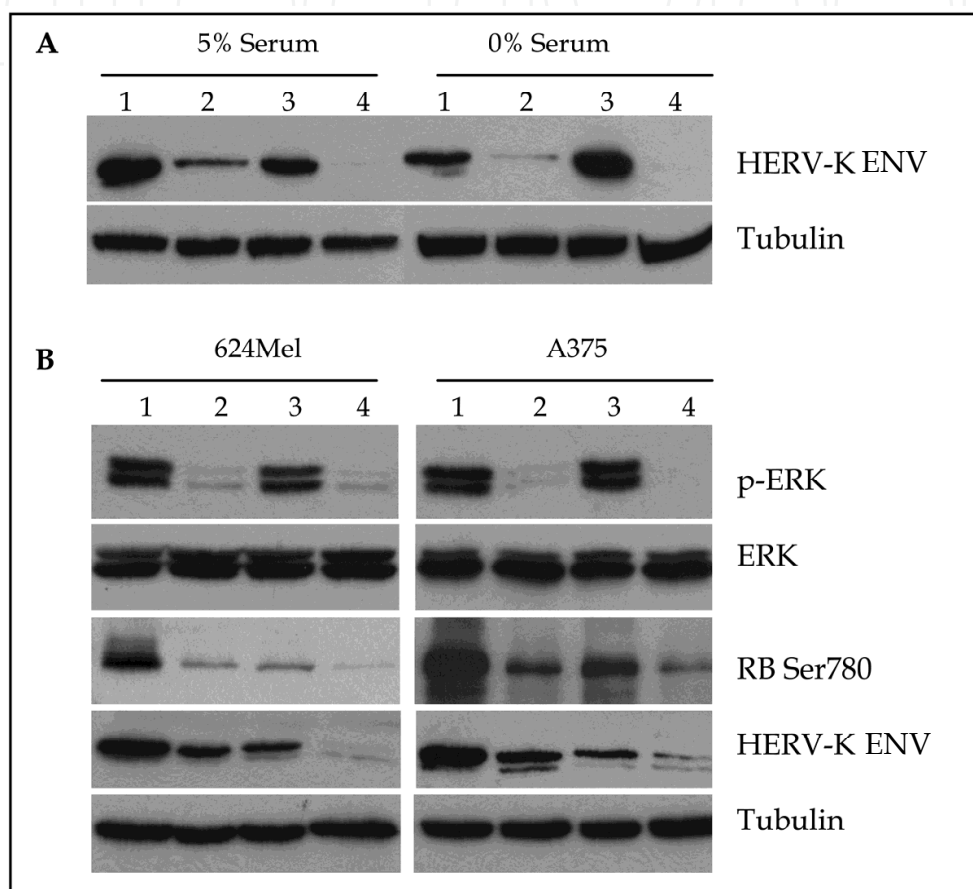


Fig. 14. MEK and CDK4 inhibitors suppress HERV-K ENV protein expression. (A) 624Mel (1), A101D (2), A375 (3), and OM431 (4) melanoma cells are cultured in DMEM with 5% serum or serum starved overnight, and cell lysates collected for Western blotting. (B) 624Mel and A375 melanoma cells are treated with solvent vehicle control (1), 25 μg MEK inhibitor PD98059 (2), 1 μg CDK4 inhibitor 219476 (3), and 25 μg PD98059 plus 1 μg 219476 (4) for 48 h under serum starvation, and cell lysates collected for Western blotting. Western blotting is performed using 50 μg total cell extracts and commercially available HERV-K ENV antibody that recognizes a 37 Kd spliced transmembrane domain of ENV protein. Tubulin is used as loading control.

HERVs have been implicated in the etiology of various diseases including cancer and chronic inflammation (Voisset et al., 2008), and emerging data support a role of HERV-K in melanomagenesis. For example, HERV-K is expressed in melanomas but not in normal melanocytes (Buscher et al., 2005; Muster et al., 2003), and specific inhibition of HERV-K by

RNAi suppresses melanoma cells *in vitro* and *in vivo* (Mangeny et al., 2005; Oricchio et al., 2007; Pothlichet et al., 2006; Serafino et al., 2009). There are several potential mechanisms to explain the role of HERV-Ks in melanomagenesis. First, HERV-K sequences may jump around by retro-transposition leading to mutagenesis and chromosomal abnormalities (Pothlichet et al., 2006; Tchenio & Heidmann, 1991) which may drive the evolution of aggressive clones. Second, HERV-K ENV, which is homologous to syncytin, may have fusogenic activity to mediate melanoma-melanoma and melanoma-target cell intercellular fusions and, therefore, can be the molecular link in the melanoma-normal cell fusion theory of metastasis (Carter, 2008; Pawelek & Chakraborty, 2008). Third, HERV-K proteins can be immunosuppressive (Mangeny et al., 2005) and may facilitate tumor progression by providing a critical survival/escape mechanism for tumor invasion and metastasis (Singh et al., 2009; Voisset et al., 2008), especially in the blood/lymph stream where circulating tumor cells are under attack by the host immune system.

HERV-K may prove to be a key mediator of BRAF-MEK-ERK and p16-CDK4-RB pathways during melanoma pathogenesis. Activation of BRAF/ERK is recently shown to drive chromosome abnormality and aneuploidy in melanocytes (Cui et al., 2010). It is conceivable that the effect may be mediated, at least partly, through HERV-K sequences that are capable of jumping around by retro-transposition leading to mutagenesis and chromosomal abnormalities (Pothlichet et al., 2006; Tchenio & Heidmann, 1991). It is possible that the observed growth promotion and anti-apoptotic effects of activated ERK and CDK4 (Li et al., 2010a; Zhao et al., 2008) can be mediated, at least in part, by HERV-K since HERV-K has been shown to directly affect melanoma cell proliferation, differentiation, and anchorage related survival (Oricchio et al., 2007; Serafino et al., 2009). Importantly, if HERV-K drives melanomagenesis downstream of the BRAF-MEK-ERK and p16/CDK4 pathways, when HERV-K is already turned on, cells may escape the inhibitory effects of therapies targeting BRAF-MEK-ERK and p16-CDK4. Triple therapies, such as simultaneous targeting of HERV-K, BRAF/MEK, and CDK4, may be necessary to produce more effective and long-lasting therapeutic effects than single or, as we have proposed previously, double inhibition of MEK-ERK and CDK4 (Li et al., 2010a; Zhao et al., 2008). This strategy is analogous to HIV “cocktail” therapy that disrupts HIV at different steps of replication and brought many AIDS patients from death to fairly normal and productive lives (Henkel, 1999).

10. Conclusion

Melanoma is the most lethal skin malignancy notorious for aggressive growth and therapeutic resistance. Multiple genetic and environmental factors have been linked to the development of melanoma. Approximately 60% of melanomas harbor mutations in v-raf murine sarcoma viral oncogene homolog B1 (BRAF) that lead to constitutive activation of the mitogen activated protein kinase/ERK kinase (MEK)-extracellular signal regulated kinase (ERK) signaling pathway. Results of the recent clinical trials with PLX4032, a specific inhibitor of mutant BRAF, have generated great excitement because approximately 80% of BRAF mutant metastatic melanomas regressed in response to PLX4032 treatment. Though this trial is considered a victory in the fight against melanomas, attention has been drawn to the fact that regressed tumors may resurge more aggressively within 8 months after the start of therapy indicating more research is needed to conquer melanoma. Most melanoma cells, but not nevi, have lost the expression of p16 inhibitor of CDK4 (INK4A), either through DNA mutation or promoter hypermethylation, resulting in a deregulated cyclin dependent

kinase 4 (CDK4)-retinoblastoma (RB) pathway. We found that simultaneous inhibition of mutant BRAF and expression of wild-type INK4A or combinatorial application of MEK and CDK4 inhibitors in melanoma cells significantly inhibits growth and enhances apoptosis. Our results support the hypothesis that simultaneous targeting of BRAF-MEK-ERK and p16-CDK4 pathways may be a promising strategy for melanoma treatment. Additionally, we found that HERV-K activation in melanoma cells is associated with BRAF-MEK-ERK and p16INK4A-CDK4 pathways. Our results should help the better understanding of BRAF-MEK-ERK and p16INK4A-CDK4 pathways, as well as HERV-K involvement in melanomagenesis and facilitate the design of novel management strategies. Further studies are warranted to examine the molecular mechanisms and biological consequences of these associations.

11. References

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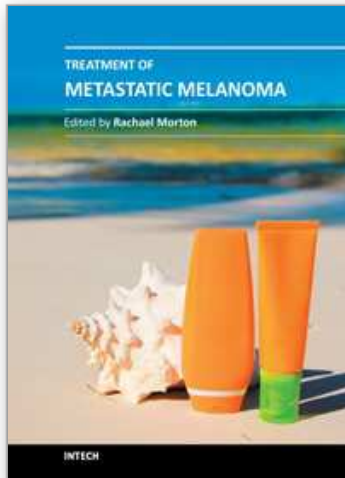
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Surgery continues to be the mainstay treatment for melanoma localized to the primary tumor and/or lymph nodes. Results from randomized controlled trials indicate that sentinel node biopsy for the treatment of cutaneous melanoma of intermediate thickness has a beneficial effect on recurrence rates, and adjuvant radiotherapy to regional lymph node fields following surgical resection reduces loco-regional recurrence in patients at high risk of relapse. Isolated limb perfusion, electrochemotherapy, and photodynamic therapy continue to be evaluated for treatment of stage IV disease. However, the greatest excitement in new treatment has been with targeted therapies for genetic mutations. In particular, the promising results of partial and complete tumor response in stage IV disease from early phase trials of the B-RAF kinase inhibitors. This book provides a contemporary insight into the therapeutic treatment options for patients with metastatic melanoma and is relevant to clinicians and researchers worldwide. In addition, an update on current clinical trials for melanoma treatment has been included, and two chapters have been reserved to discuss the treatment of oral and uveal melanoma.

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