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Cooperative interactions of PTEN deficiency and RAS activation in melanoma metastasis

Cristina Nogueira[†,1,2,7], **Kwan-Hyun Kim**[†,3], **Hyeran Sung**[3], **Kim Paraiso**[3], **Jan-Hermen Dannenberg**[1,7], **Marcus Bosenberg**[4,7], **Lynda Chin**[1,5,6,*], and **Minjung Kim**[1,3,7,*]

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, 02115

[2]Institute of Molecular Pathology and Immunology of the University of Porto, (IPATIMUP)/Medical Faculty, University of Porto, Porto, Portugal

[3]Molecular Oncology Department, Comprehensive Melanoma Research Center, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL, 33612

[4]Department of Pathology, University of Vermont, Burlington, Vermont

[5]Belfer Institute for Applied Cancer Science, Dana-Farber Cancer Institute, Boston, Massachusetts, 02115

[6]Department of Dermatology, Harvard Medical School, Boston, Massachusetts, 02115

Summary

MAPK and AKT pathways are frequently co-activated in melanoma through overexpression of receptor tyrosine kinases, mutations in their signaling surrogates, such as RAS and BRAF, or loss of negative regulators such as PTEN. Since RAS can be a positive upstream regulator of PI3-K, it has been proposed that the loss of PTEN and the activation of RAS are redundant events in melanoma pathogenesis (Tsao *et al.*, 2000). Here, in genetically engineered mouse models of cutaneous melanomas, we sought to better understand the genetic interactions between HRAS activation and PTEN inactivation in melanoma genesis and progression *in vivo*. We showed that HRAS activation cooperates with *Pten+/-* and *Ink4a/Arf-/-* to increase melanoma penetrance and promote metastasis. Correspondingly, gain- and loss-of-function studies established that Pten loss increases invasion and migration of melanoma cells and non-transformed melanocytes, and that such biological activity correlates with a shift to phosphorylation of AKT2 isoform and E-cadherin down-regulation. Thus, Pten inactivation can drive the genesis and promote the metastatic progression of RAS activated Ink4a/Arf deficient melanomas.

[*]Address correspondence to Lynda Chin and Minjung Kim, Lynda_Chin@dfci.harvard.edu; Minjung.Kim@moffitt.org; Mailing address: 44 Binney Street, Dana-Farber Cancer Institute, Boston, Massachusetts, 02115, Phone: 617-632-6091, Fax: (617) 582-8169.

[7]Present addresses: Whitehead Institute for Biomedical Research, Cambridge, MA. (C.N.); Department of Dermatology, Yale University School of Medicine, New Haven, CT (M.B.); Division of Molecular Genetics, Netherlands Cancer Institute, Amsterdam, The Netherlands (J-H.D.); Molecular Oncology Department, H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL. (M.K)

[†]These authors contributed equally.

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Keywords

melanoma; PTEN; RAS; E-cadherin; AKT2; mouse model

Introduction

RAS activation is a common and potent oncogenic event in human solid tumors, including melanoma. The relevance of RAS-RAF-MAPK pathway in melanoma pathogenesis is evidenced by high frequency BRAF mutations (Davies *et al.*, 2002) and less common yet reciprocal NRAS mutations (Tsao *et al.*, 2004). Activating mutations of NRAS are found in as many as 56% of congenital nevi, 33% of primary and 26% of metastatic melanoma samples (Chin *et al.*, 2006). Although, NRAS is the most commonly mutated RAS family member, mutations on KRAS (2%) and HRAS (1%) are also observed in human melanomas (Forbes *et al.*, 2008). In particular, HRAS mutation was reported to be found in 7.7% of the nodular melanoma subtype (Jafari *et al.*, 1995).

Genetic evidence of a pathogenetic role for the RAS-RAF-MAPK pathway in melanoma derives from a melanoma-prone condition in mice transgenic for melanocyte-directed $HRAS^{V12G}$ or $NRAS^{Q61K}$ alleles and null for $p16^{INK4a}$ and/or $p19^{ARF}$ tumor suppressors (Ackermann *et al.*, 2005; Chin *et al.*, 1997; Chin *et al.*, 1999; Sharpless *et al.*, 2003). Additionally, BRAF, a key effector of RAS signaling, is the most frequently mutated gene in human melanocytic neoplasms with frequencies of 82% of benign nevi and 66% in melanomas (Davies *et al.*, 2002; Pollock *et al.*, 2003). Melanocyte-directed expression of $BRAF^{V600E}$ can induce nevoid hyperpigmentation phenotype in mice (Dankort *et al.*, 2009; Dhomen *et al.*, 2009; Goel *et al.*, 2009) with rare progression to melanoma (Dhomen *et al.*, 2009; Goel *et al.*, 2009).

While activation of the RAS-RAF signaling cascade has been recognized as an obligate event in melanocyte transformation, Khavari and colleagues have demonstrated that activation of BRAF – MAPK requires concomitant AKT activation to effect melanoma development (Chudnovsky *et al.*, 2005). Consistent with importance of the PI3K-AKT pathway, over 60% of human melanomas exhibit activated AKT (Dhawan *et al.*, 2002), and mutational inactivation and/or deletion of the PI3K negative regulator, PTEN, occurs in 5-15% of uncultured melanoma specimens and metastasis, 17% of melanoma short-term cultures, and 30-40% of established melanoma cell lines (Birck *et al.*, 2000; Guldberg *et al.*, 1997; Lin *et al.*, 2008; Tsao *et al.*, 2004). Mouse modeling has also demonstrated that $Pten^{+/-}Ink4a/Arf^{+/-}$ mice do succumb to melanoma at low frequency (You *et al.*, 2002) and robust melanoma formation and metastases can occur upon combination with activated BRAF (Dankort *et al.*, 2009).

The PI3K-AKT signaling pathway can be activated by receptor tyrosine kinases. Active PI3K phosphorylates and converts the lipid phosphatidylinositol (4,5) bisphosphate (PIP_2) into PIP_3, which in turn activates AKT through PDK1 mediated phosphorylation. PTEN negatively regulates PI3K signaling by dephosphorylating PIP_3, converting it back to PIP_2. Therefore, deletion or inactivation of PTEN results in constitutive AKT activation (Cully *et al.*, 2006; Salmena *et al.*, 2008). On the other hand, RAS proteins can positively regulate the

PI3K–AKT pathway by direct binding of RAS to the p110 catalytic subunit (Gupta *et al.*, 2007) or by activating an autocrine signaling pathway involving EGFR family ligands (Bardeesy *et al.*, 2005). Since PTEN inactivation and RAS activating mutation can both target the PI3K pathway to drive constitutive AKT activation, it has been proposed that RAS mutation and PTEN inactivation are redundant events in tumorigenesis, as suggested by a reciprocal trend of their mutations in human melanoma and endometrial carcinoma (Ikeda *et al.*, 2000; Tsao *et al.*, 2000). However, constitutive AKT1 activation in mouse has resulted in a milder cancer phenotype than complete PTEN inactivation (Ma *et al.*, 2005; Majumder *et al.*, 2003; Stambolic *et al.*, 2000; Trotman *et al.*, 2003; Wang *et al.*, 2003), pointing to AKT independent activities of PTEN in tumorigenesis (Blanco-Aparicio *et al.*, 2007). Similarly, while expression of oncogenic K-RAS or conditional PTEN deletion in the ovarian epithelium gives rise to preneoplastic ovarian lesions, the combined effect of these two mutations in the ovary leads to invasive and widely metastatic endometrioid ovarian adenocarcinomas with complete penetrance and a disease latency of only 7 weeks (Dinulescu *et al.*, 2005). In this study, we addressed the potential collaborative interactions between RAS activation and PTEN inactivation on the genetic level.

Results

Pten inactivation cooperates with HRAS^{V12G} *in melanoma genesis* in vivo

We had previously shown that *Tyr-HRAS*^{V12G}*Ink4a/Arf*$^{-/-}$ compound mutant mice (hereafter "*RAS-Ink4a/Arf*") developed non-metastatic cutaneous melanomas with short latency and high penetrance (Chin *et al.*, 1997; Chin *et al.*, 1999; Kim *et al.*, 2006). The occurrence of PTEN loss in advanced melanoma prompted us to determine whether Pten inactivation can cooperate with HRAS activation to drive melanoma progression. Given that *Pten* nullizygosity is embryonic lethal (Di Cristofano *et al.*, 1998; Suzuki *et al.*, 1998), we studied the melanoma phenotype of the *RAS-Ink4a/Arf* model on *Pten*+/+ and *Pten*+/- background.

Inactivation of one copy of *Pten* in *RAS-Ink4a/Arf* mice led to an earlier onset of melanoma and decreased overall melanoma-free survival (Figure 1A and Table 1; p=0.0002). Although median survival was not notably different (median survival 21.6 vs. 18.9 wks, Supplemental Figure 1), melanoma-free survival was significantly decreased by Pten heterozygosity (29.6 vs. 18.9 wks comparing *RAS-Ink/Arf* mice with *Pten* +/- vs. *Pten*+/+ genotypes). In the absence of melanocyte-specific expression of activated RAS, the *Pten*+/-*Ink/Arf*-/- mice showed a median survival of 19.4 weeks, and did not develop melanomas (Supplemental Figure 1). In the period prior to the appearance of non-melanoma tumors, 75% of the *Pten* +/- *RAS-Ink4a/Arf* mice developed melanoma compared with 35.7% of mice with WT Pten (Table 1). Histopathologically, these primarily spindle cell tumors are similar to those observed in the melanomas from the *RAS-Ink4a/Arf* model (Chin *et al.*, 1997; Chin *et al.*, 1999). Melanocytic lineage of *Pten*+/-*RAS-Ink4a/Arf* cutaneous tumors was confirmed by expression of melanocytic markers on protein and RNA level (Figure 1B and 1C). These findings clearly establish that activated RAS and loss of Pten cooperate to derive the genesis of melanoma on the *Ink4a/Arf* null background.

Extensive experience with the *RAS-Ink4a/Arf* model has established that metastasis does not occur in this model (Bardeesy *et al.*, 2001; Bardeesy *et al.*, 2005; Chin *et al.*, 1997; Chin *et*

al., 1999). Among the 21 tumor-bearing *RAS-Ink4a/Arf* mice heterozygous for Pten, full histological surveys uncovered one case of melanoma metastasis to the lung and one case with metastsis in a draining lymph node, although tissue availability only enabled melanocyte marker confirmation in the former (Tyrosinase and Dct/TRP2 positive; Figure 1B). Although sample size is small, these observations prompted an examination for a potential role of Pten in suppression of melanoma metastasis.

Loss of PTEN enhances invasion of primary and transformed melanocytes

We next assessed the impact of Pten status on the migration and invasion activity of *RAS-Ink4a/Arf* melanoma cells *in vitro* (Figure 2). First, we asked whether Pten protein levels in early-passage melanoma cultures derived from spontaneously arising tumors track with migratory and invasive activity. We selected three tumors with high, intermediate or absent Pten protein levels (AL4, CN41 and CN44, respectively) (Figure 2A); early passage CN44 cells show absence of both Pten mRNA and protein with retention of the Pten gene consistent with epigenetic silencing (Figure 2B). Using the modified Boyden chamber assay, the level of Pten expression negatively correlated with invasion through the Matrigel (Figure 2C). Second, we observed an overall lower level of invasive activity across 4 independent *RAS-Ink4a/Arf Pten*+/+ melanoma cell lines when compared with 5 independent *RAS-Ink4a/Arf Pten*+/- melanoma cell lines (Figure 2D). Third, these correlations in tumors aligned well with Pten knockdown and reconstitution experiments *in vitro* and *in vivo*. Specifically, RNAi-mediated knockdown of *Pten* in a *Pten*+/+ *RAS-Ink4a/Arf* melanocyte and melanoma cell lines using two independent shRNAs (sh4 and sh11 with documented efficient knockdown of Pten protein levels) showed increased invasion in non-transformed melanocyte culture (C140) (Figure 3A) as well as in melanoma cells CN116 (Figure 3B), both were derived from *RAS-Ink4a/Arf* mice with intact *Pten*. Similar results were obtained with AL4 *RAS-Ink4a/Arf Pten*+/+ melanoma cell line (Supplemental Figure 2), demonstrating increased invasion in Boyden chamber *in vitro* compared to the control which was transduced with empty vector (EV). While we noted an increase in proliferation by Pten loss (Figure 3C), the level of invasion was still markedly increased when normalized for proliferation rates (see material and method). *In vivo*, Pten knockdown resulted in reduced melanoma-free survival upon subcutaneous implantation of these melanoma cells in severe combined immunodeficient (SCID) mice (Figure 3D). Finally, Adenoviral-mediated Pten reconstitution in CN44 melanoma cells decreased invasion relative to GFP-expressing adenovirus control cells (Figure 3E, $p=0.02$). Together, these data show that Pten levels can influence the invasive potential of primary and transformed melanocytes.

PTEN inactivation correlates with downregulation of E-cadherin

Many PTEN biological functions have been linked to its lipid phosphatase activity through which PIP_3 is converted to PIP_2 resulting in inactivation of AKT. At the same time, it is clear that PTEN exerts functions that are apparently mediated via AKT-independent pathways. Of relevance to the current study, PTEN has been shown to modulate actin cytoskeletal organization, focal contacts, and directional cell motility via focal adhesion kinase (FAK) (Tamura *et al.*, 1999) and can physically interact with E-cadherin at junctional complexes to stabilize cell-cell contacts, thereby suppressing invasiveness (Kotelevets *et al.*,

2005; Vogelmann *et al.*, 2005). In our model system, we sought to better understand the molecular action of Pten by assessment of known targets of Pten pathway (Figure 4).

Early passage *RAS-Ink4a/Arf Pten+/-* melanoma cells showed consistent E-cadherin downregulation relative to *RAS-Ink4a/Arf Pten+/+* controls; in contrast, the levels of p-AKT, p-FAK, and p-ERK did not track consistently with Pten genotype (Figure 4A). While increased p-STAT3 was observed in *RAS-Ink4a/Arf Pten+/-* cell lines relative to *RAS-Ink4a/Arf Pten+/+* controls, Pten knockdown in CN116 and AL4 *RAS-Ink/Arf Pten+/+* melanoma cells failed to show any enhancement in p-STAT3 as well as p-AKT and p-FAK (Figure 4B; Supplemental figure 2); whereas, Pten knockdown in primary *RAS-Ink4a/Arf-/-* melanocytes resulted in increased phosphorylation of these molecules (Figure 4C). In contrast, Pten knockdown results in consistent down-regulation of E-cadherin expression in both melanocyte and melanoma cell lines. Conversely, Pten reconstitution was associated with no change in p-STAT3, p-FAK, and p-ERK, a modest decrease in p-AKT, and robust increase in E-Cadherin (Figure 4D). In both CN116 and AL4 cells, Pten knockdown correlated well with loss of E-cadherin protein and mRNA expression (Figure 4B&E and Supplemental Figure 2B&C). Since loss of E-Cadherin has been linked to epithelial to mesenchymal transition and progression, our data raised the possibility that PTEN-mediated modulation of E-cadherin expression may be integral to the impact of PTEN on the invasive phenotype of melanoma. Indeed, RNAi-mediated knockdown of E-Cadherin resulted in increased invasion of CN116 cells (Figure 4F, p=0.04).

PTEN inactivation enhances invasion of human melanoma cells

To establish the human relevance of our findings, we next assessed the effect of PTEN loss on invasion as well as on p-AKT in human melanoma cell lines. In line with our murine studies, siRNA-mediate knockdown of PTEN in WM1366, human melanoma cells harboring NRAS Q61I mutation, resulted in increased invasion without a significant increase of AKT phosphorylation compared to siNT (non-targeting) control (1.34 fold, p=0.00008) (Figure 5A). In addition, we investigated the effect of PTEN reconstitution in human melanoma cells WM793A and 1205Lu harboring BRAF V600E mutation and PTEN loss. Previously, Stahl et al have shown that reconstitution of PTEN expression in PTEN null melanoma cell lines leads to significant decrease in AKT phosphorylation and apoptosis (Stahl *et al.*, 2003). Here, to assess the impact of PTEN on invasion, PTEN level was titrated to avoid activation of cell death associated with high level expression (Figure 5B). At this level of ectopic PTEN expression in WM793A and 1205Lu cells, PTEN reduced invasion compared to control without significant decrease of AKT phosphorylation (WM793A: 49%; p=0.003 and 1205Lu: 24%; p=0.048) (Figure 5B and data not shown). This is consistent with the study reported by Dankort et al showing the collaboration between Braf and Pten in melanoma progression (Dankort *et al.*, 2009). Together these studies support the view that PTEN inactivation enhances invasion of mouse and human melanoma cells harboring RAS/RAF/MAPK pathway activation without significant changes in total AKT phosphorylation.

PTEN inactivation preferentially activates AKT2 among AKT isoforms

Intriguingly, treatment with PI3K inhibitor, LY294002, in *RAS-Ink4a/Arf* melanoma cells led to a decrease in total p-AKT and abrogation of the increased invasion brought about by Pten knockdown (Figure 6A), suggesting that PI3K-AKT signaling does contribute to the invasion phenotype we observed. Although modulation of PTEN does not track well with total p-AKT levels in *RAS-Ink/Arf* melanoma cells (Figures 3 and 4), the fact that all three isoforms of AKT (AKT1, 2, and 3) are phosphorylated in *RAS-Ink/Arf* melanoma cells (data not shown and Supplemental figure 3) raised the possibility that Pten may regulate invasion through switching among activated AKT isoforms (AKT1, 2, and 3) without substantially affecting the total amount of active AKT.

To explore further the thesis that specific AKT isoform confers differential activity in melanoma cells, we first examined AKT3 activation since AKT3 is reported to be the predominant AKT isoform in human melanoma (Stahl *et al.*, 2004). Here, we ectopically expressed a constitutively active form of AKT3 (myristoylated AKT3) in CN116A cells. While it dramatically elevated AKT3 phosphorylation at Ser 473 without affecting p-FAK and p-STAT3, myr-AKT3 expression actually inhibited invasion (Supplemental figure 4A). Of note, this result is consistent with the anti-invasive role of myristoylated AKT1 in breast cancer cells reported by Bissell and colleagues (Liu *et al.*, 2006). On the other hand, a dominant negative form of AKT2 expression (Yuan *et al.*, 2003)in invasive CN44 melanoma cells led to inhibition of invasion (Supplemental figure 4B), supporting a pro-invasive role for AKT2. Furthermore, in line with the thesis that PTEN loss results in switching of AKT isoform, siRNA-mediated knockdown of PTEN in the human melanoma cell line WM1366 enhanced level of p-AKT2 without significantly impacting on total pan-isoform p-AKT when compared to siNT control (Figure 6C). This can be further corroborated by ELISA measurement, where p-AKT2 levels were found to be significantly higher in PTEN knockdown cells compared to non-targeting siRNA controls (Figure 6D, 15ug; 1.26 fold ($p=0.024$) and 40ug; 1.15 fold ($p=0.023$)). Accordingly, in the mouse, loss of Pten in CN 116 and AL4 melanoma cells was associated with increased p-AKT2 without significant change on total p-AKT (Figure 6B and Supplemental Figure 4). Together, these data point to PTEN inactivation in RAS activated melanomas promotes invasion through preferential activation of AKT2.

Discussion

Utilizing genetically engineered models, we investigated the interactions of three melanoma prone genetic elements, namely RAS activation, Pten loss, and Ink/Arf deficiency. Previously, RAS activation in mouse model has been shown to cooperate with Ink/Arf inactivation to drive non-metastatic melanomas (Chin *et al.*, 1997) and loss of Pten when combined with loss of Ink/Arf function contributes to melanoma tumorigenesis at low frequency (You *et al.*, 2002). However, there still exists considerable uncertainty surrounding the genetic interactions of these lesions. Here, using genetically engineered mouse models *in vivo* and mouse and human cell systems *in vitro*, we demonstrated synergistic interaction of these three components in melanoma genesis and progression, manifested as accelerated melanoma development and enhanced migration and invasion of

melanoma cells from *Pten+/-RAS-Ink/Arf* mice compared to *Pten+/+Ras-Ink/Arf* mice. In addition, we have further elucidated the key molecular events acting as points of convergence and distinction of their downstream signaling pathways. Firstly, we observed E-Cadherin downregulation associated with PTEN loss. Secondly, we have shown differential regulation of AKT isoforms by PTEN loss in RAS mutated melanomas. In particular, we observed preferential increase of phospho-AKT2, much less appreciated isoform in melanoma tumorigenesis.

Previous work has proposed that RAS activation and PTEN extinction are functionally and genetically redundant based on the relative reciprocity of NRAS and PTEN alterations observed in human melanomas and cell lines (Tsao *et al.*, 2000). However, in human melanoma, we found that 14% (2/14) of the NRAS mutated tumor samples harbor Pten loss or mutation (data not shown). Conversely, 17% (2/12) of the melanoma samples with PTEN loss or mutation also contain NRAS mutation (L. Chin, unpublished data). Thus, our observations in the genetically engineered mouse models are relevant for the human disease, as further supported by our work in human cell lines.

PTEN is a major tumor suppressor located on chromosome 10q23-24, the frequently deleted region in multiple tumor types (Li *et al.*, 1997; Steck *et al.*, 1997). In human melanoma, PTEN is deregulated via genetic mechanisms (mutation and allelic loss) (Guldberg *et al.*, 1997; Tsao *et al.*, 1998)) as well as via epigenetic silencing such as methylation (Mirmohammadsadegh *et al.*, 2006) or altered subcellular localization (Trotman *et al.*, 2007). PTEN methylation status serves as a prognostic marker for poor survival (Lahtz *et al.*). In our study, murine melanomas derived from from *Pten+/-RAS-Ink4a/Arf* mice commonly exhibit loss of Pten protein expression, consistent with the observation in human.

AKT kinase family consists of 3 highly homologous isoforms: AKT1/PKB-alpha, AKT2/PKB-beta, and AKT3/PKB-gamma. Although structurally similar, each isoforms of AKT have non-overlapping functions in cancer (Gonzalez and McGraw, 2009). Phenotypic analyses of polyoma middle T (PyMT) and ErbB2/Neu-driven mammary adenocarcinoma mouse models suggest that Akt1 may inhibit and Akt2 may enhance the invasion (Maroulakou *et al.*, 2007). This is consistent to previous reports that AKT2 expression in breast and ovarian cancer cells increased adhesion, invasion, and metastasis, associated with β1 integrin upregulation (Arboleda *et al.*, 2003). Several mechanisms have been proposed to achieve isoform specific signaling such as distinct tissue distribution, differential activation by extracellular stimuli, distinct intrinsic catalytic activity and substrage specificity, and differential subcellular localization of the AKT isoforms (Gonzalez and McGraw, 2009). Although the role of PTEN loss in AKT activation is well established, the effect of PTEN loss on the activity of each AKT isoform has not been addressed in detail.

Among the 3 isoforms, AKT3 is considered to be the predominant isoform activated in melanomas by amplification and somatic activating mutations (Davies *et al.*, 2008; Stahl *et al.*, 2004). Here, in our mouse melanomas with activated RAS signaling, Pten loss is associated with enhanced invasion accompanied by a shift to increase phosphorylation of AKT2 isoform, which has been linked to invasiveness, without significant change in total amount of AKT activation (Figure 6). Interestingly, Tsichils and colleagues recently

reported that it is the balance between AKT1 and AKT2 rather than the amount of total AKT activity that differentially regulates microRNA expression, whereby AKT2 induces miR-200 microRNA family, which in turn decreases E-Cadherin expression (Iliopoulos *et al.*, 2009). In this study, we report an increase on pAKT2 and decrease of E-Cadherin protein levels upon Pten inactivation. It will be of interest to asses if mir-200 is induced in our model and if it is responsible for the observed downregulation of E-Cadherin. In this regard, it is of interest to note that, although real time quantitative PCR analysis revealed that *E-cadherin* was downregulated at the transcriptional level following downregulation of Pten expression in *RAS Ink/Arf* melanoma cells, upregulation of its known transcriptional regulators including Slug, Snail, and Twist was not observed (data not shown). Zeb1 showed small nonsignificant upregulation by Pten loss (1.23 fold for sh4, 1.38 fold for sh11 vs. EV, data not shown).

In conclusion, we provide genetic evidence *in vivo* that RAS activation and Pten loss cooperate to drive melanoma genesis and progression in the mouse. Our data encourages more systematic and comprehensive characterization for the activity of each AKT isoforms at distinct stages of melanoma tumorigenesis and in clinical trials when addressing the effect of AKT inhibitors.

Material and Methods

Mouse tumor cohorts

Tyrosinase enhancer-promoter-driven *H-RAS*^{V12G} transgenic mice in FVB (N10) (Chin *et al.*, 1997) were crossed onto the *Pten* mutant background in mixed FVB/C57Bl/6 strain (Podsypanina *et al.*, 1999) and the *Ink4a/Arf*$^{-/-}$ background in FVB (N10) (Serrano *et al.*, 1996) to generate the compound mice *Tyr-H-RAS*^{V12G}*Ink4a/Arf*$^{-/-}$*Pten*$^{+/+}$ *and Tyr-H-RAS*^{V12G}*; INK4a/Arf*$^{-/-}$*Pten*$^{+/-}$. Mice were observed biweekly for development of tumors or appearance of ill health. Premoribund animals or animals with significant tumor burdens were sacrificed, and detailed autopsies were performed. Tumor specimens were fixed in 10% formalin and embedded in paraffin for histological analysis as previously described (Chin *et al.*, 1997). In cases in which sufficient specimens were available, primary tumors were adapted to culture to establish derivative cell lines and/or flash-frozen for subsequent analyses.

Melanocyte and melanoma cell culture

Melanoma cell lines were derived from mouse tumors by digestion with collagenase and Hyaluronidase (2 mg/ml each; Sigma-Aldrich, St Louis,MO, USA) for 2 hours followed by cultivation with RPMI 1640 media (Gibco BRL, Gaithersburg, MD, USA) containing 10% FBS and 1% penicillin/streptomycin. Melanocyte cultures were generated from newborn mouse epidermis as described (Sviderskaya *et al.*, 1997) and maintained in RPMI 1640 containing 5% FBS, 1% penicillin/streptomycin, 200 pM cholera toxin, 200 nM 12-*O*tetradecanoylphorbol- 13-acetate (TPA).

RNA analysis

For analyses of gene expression, total RNA was isolated from primary cutaneous melanomas or from cultured cells using Trizol (Gibco BRL, Gaithersburg, MD, USA) according to manufacturer's protocol. Total RNA was treated with RQ1 DNAse (Promega, Madison, WI, USA) and 1ug total RNA was used for reverse transcription reaction using Superscript II polymerase (Invitrogen, Carlsbad, CA, USA) primed with oligo(dT). Coding regions were amplified by PCR or quantitative real time PCR using SYBR Green (Applied Biosystems, Foster City, CA, USA) on an Mx3000P real-time PCR system (Stratagene, La Jolla, CA, USA). Ribosomal protein R15 was used as an internal expression control. Primer sequences are as follows: Tyr: F-ccagaagccaatgcacctat and R-agcaataacagctcccacca; Trp1: F-attctggcctccagttacca and R-ggcttcattcttggtgcttc; Dct: F-aacaacccttccacagatgc and R-tctccattaagggcgcatag; RAS transgene: F- cagatcaaacgggtgaagg and R- cacttgcagctcatgcag; R15: F-cttccgcaagttcacctacc and R-tacttgaggggatgaatcg; Pten: F- ttgcaatcctcagtttgtgg and R- tggctgagggaactcaaagt; E-Cadherin: F- ctttaagcccagcactcagg and R- cctgcttcctgagaaaatgc

Protein analyses

For immunoblots, lysates were resolved on 4-12% Nu-Page minigels (Invitrogen). Western blots were probed with antibodies against AKT, phospho-AKT (Ser473), PTEN, p44/42 and phospho-p44/42 Map Kinase (Thr202/Tyr204), phospho-STAT3 (Ser727) from Cell Signaling Technology (Danvers, MA). Anti-FAK [pY397] antibody was obtained from Invitrogen Biosource, E-CAD and N-CAD from BD Transduction Laboratories (San Jose, California), and p-AKT2 (Ser474) from Abcam (Cambridge, MA). Beta-Actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), beta-Tubulin (Sigma), and GAPDH (Sigma) protein expression served as loading control. All antibodies were used at 1:1000 dilutions. p-AKT2 level was measured with PathScan p-AKT2 (Ser 474) Sandwitch ELISA Kit (Cell Signaling Technology) following manufacturer's protocol. Immunohistochemical staining of Tyrosinase (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and DCT (TRP2) (provided by Dr. Glenn Merlino (National Cancer Institute) was performed on formalin-fixed paraffin-embedded tissues with heat induced epitope retrieval (0.01M citrate buffer, pH6.0).

siRNAs, Plasmids, and viral infection

In order to knock-down *Pten* using RNAi, *Pten* shRNA oligos were designed using iRNAi software (http://www.mekentosj.com/irnai). *Pten* shRNA oligos were cloned into the pRetroSuper retroviral vector (Brummelkamp *et al.*, 2002). Viral supernatants were produced using standard protocols. Retroviral vectors were transfected into 293T cells using the pCL-Eco helper plasmid. Retroviral supernatants isolated at 36 and 48h after transfection were diluted 1:1 in culture medium and used to infect melanoma cell lines in the presence of 4 μg/ml Polybrene. At 24 h postinfection, the cells were selected for 2 days in growth medium containing 2.5 μg/ml puromycin. Western blot analysis of cell extracts from cells infected with the *Pten* shRNA containing retroviral vectors showed that two *Pten* shRNA oligos (*Pten* sh4: 5′ GGCACAAGAGGCCCTAGAT 3′ and *Pten* sh11: 5′AGACAAGGCCAACCGATAC 3′) show efficient Pten knock-down. Adenoviruses for PTEN was provided by Dr. Jin Q. Cheng (Moffitt Cancer Center). The siRNAs targeting

human PTEN was purchased from Cell Signaling Technology and mouse E-Cadherin from Dharmacon (Lafayette, CO, USA).

Boyden Chamber

Invasion assay was performed in Matrigel Invasion Chambers (24 well plate format from BD BioCoat™ San Jose, CA, USA) or in Chemotaxis Chambers (96 well format from NeuroProbe (Gaithersburg, MD, USA) following coating with Matrigel (BD). Briefly, cells were trypsinized, rinsed twice with PBS, resuspended in serum-free RPMI 1640 media, and were loaded on upper chamber and allowed to invade through the Matrigel towards 10% FBS serum for 20 hours. Non-invasive cells were removed and remaining cells were fixed and stained with Cristal Violet stain. Invasive cells were either counted under a microscope (5 fields per well) or quantitated via Pixel quantification (Richard Rosenman V1.2), or Cristal Violet dye was extracted with 10% Acetic Acid and cells were quantified at OD 560nm. For loading control and to normalize for differences in cell proliferation, cells were seeded in a regular 24 well plate and allowed to grow for the same time as the incubation in the Boyden chambers after which cells were stained with Cristal Violet. Cells were quantified at OD 560nm after dye extraction and this number was used to normalize the invasion value. Statistical analysis was done by t-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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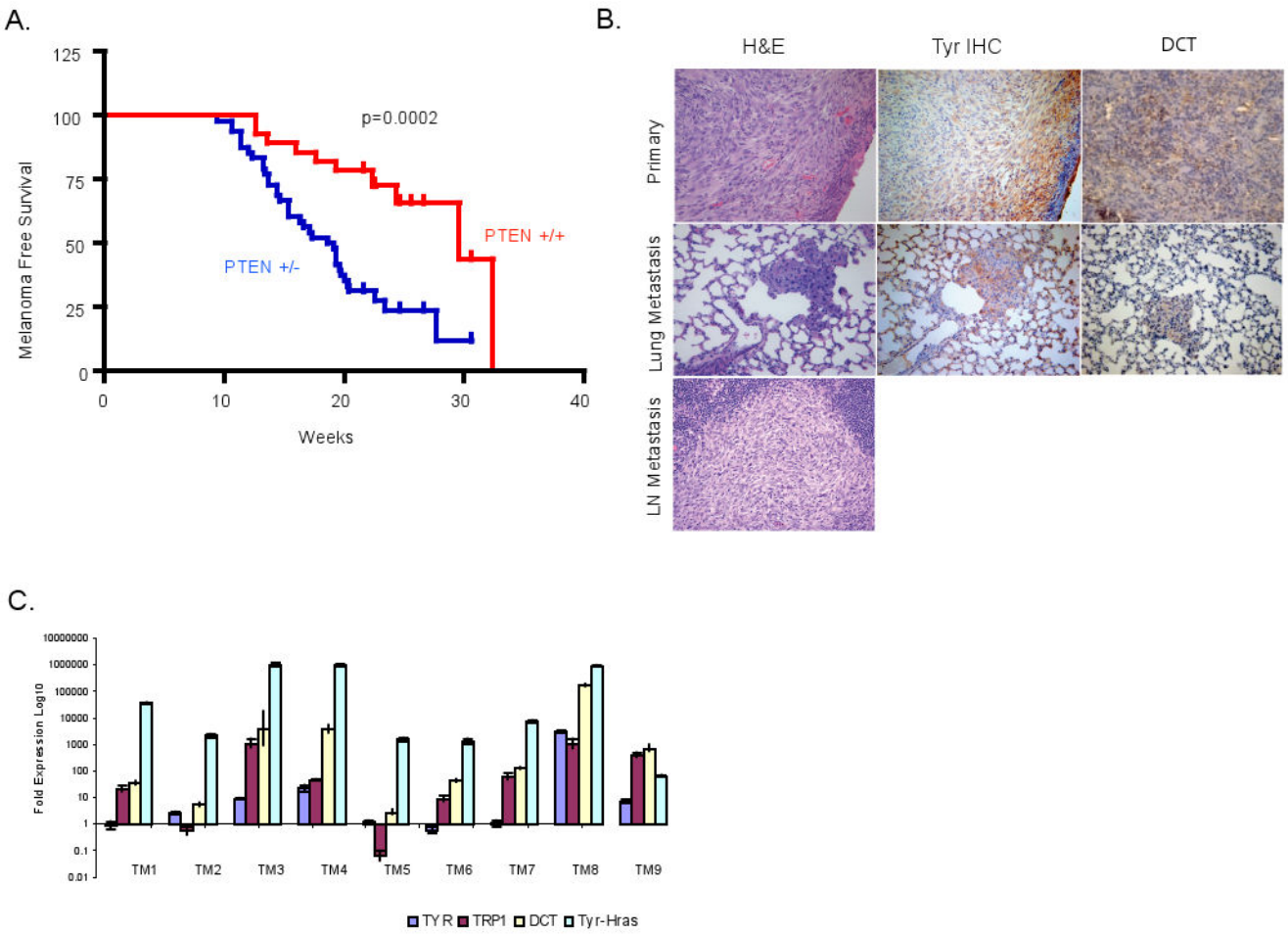

Figure 1. Collaboration of PTEN loss and HRAS activation for the pathogenesis of cutaneous melanoma

(A) Kaplan-Meier melanoma free survival analysis of *Ras-Ink4a/Arf* mice with wild type (n=28) or heterozygous (n=48) *Pten* allele. Non-melanoma tumors were censored from this analysis. Statistically significant differences were detected between the two cohorts (p=0.0002) **(B)** H&E (left), Tyrosinase (middle) and Dct (right) staining of primary melanoma (top panels) and lung metastasis (middle panels). Bottom panel shows H&E of a probable melanoma metastasis on a lymph node, based on cell morphology. **(C)** Real time reverse transcription PCR analysis for mRNA level of *Tyr-Hras* transgene and *Tyr*, *Trp1* and *Dct* melanocytic markers in cutaneous melanomas from *Ras-Ink4a/Arf* animals that were wild type (TM1-4) or heterozygous for *Pten* allele (TM5-9) relative to the ones from non-transformed *INK4a-/-* melanocytes.

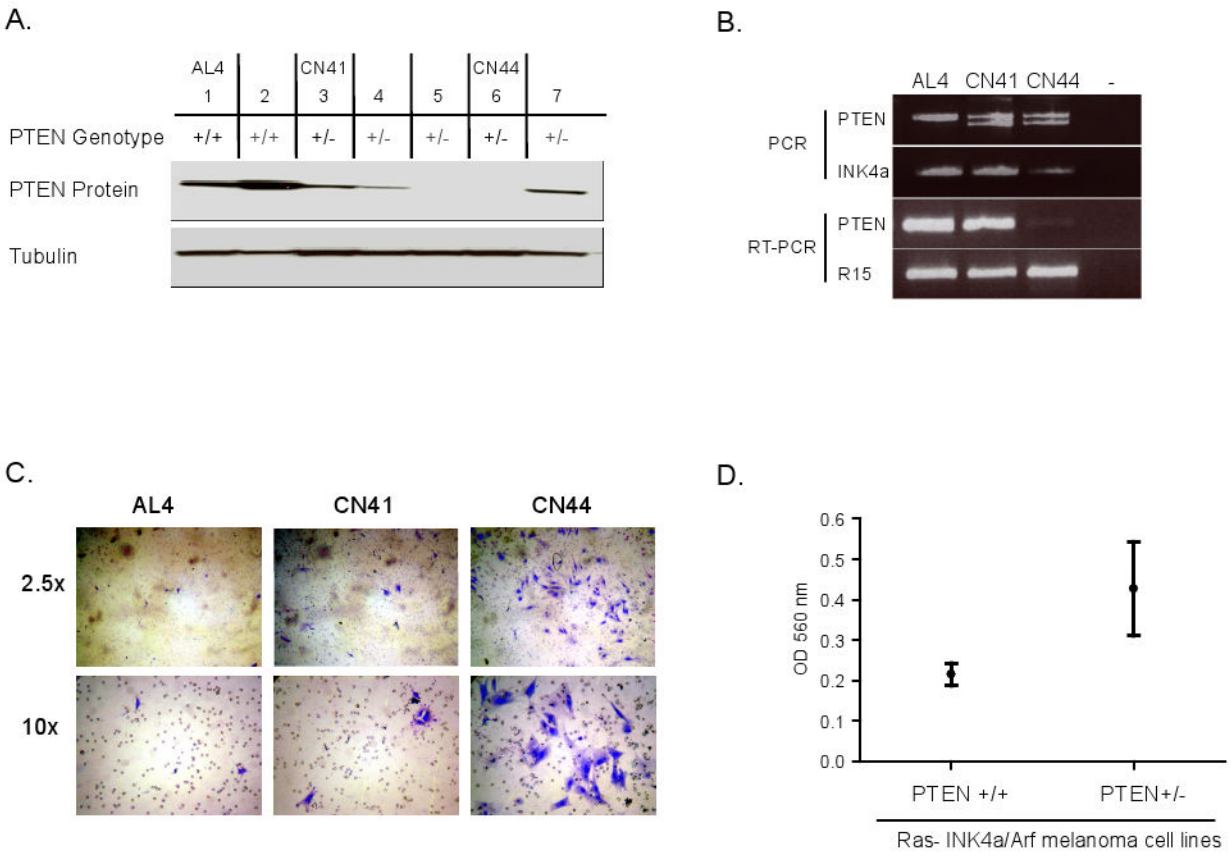

Figure 2. Migration and invasion capability of Ras-Ink4a/Arf melanoma cells inversely correlates to the PTEN protein level

(A) Immunoblot analyses of PTEN in cell lysates from early-passage *Ras-Ink4a/Arf* melanoma. The corresponding *Pten* genotype is shown on top of the blot. Note the different level of Pten in AL4 (high), CN41 (intermediate), and CN44 (loss), which were used in B and C. **(B)** (Top) PCR analysis on genomic DNAs of early passage AL4, CN41, and CN44 melanoma cells of Pten (exon 5) and Ink4a allele. (Bottom) Semi-quantitative reverse transcription PCR analysis for Pten mRNA expression (20 cycles). Ribosomal protein R15 mRNA expression serves as an internal control (19 cycles). **(C)** Boyden Chamber assay. Photographs of a representative field of the stained membrane show highest level of invasion through the Matrigel by CN44 melanoma cells. **(D)** Optical quantitation of Boyden Chamber assay on *Ras-Ink4a/Arf-Pten*+/+ (n=4) and *Ras-Ink4a/Arf-Pten*+/- (n=5) early passage melanoma cell lines. *Pten* wt cells showed overall lower invasive capacity (OD560 mean= 0.2152 ± 0.02781, N=4) when compared to *Pten* heterozygous cells (OD560 mean= 0.4268 ± 0.1160, N=5).

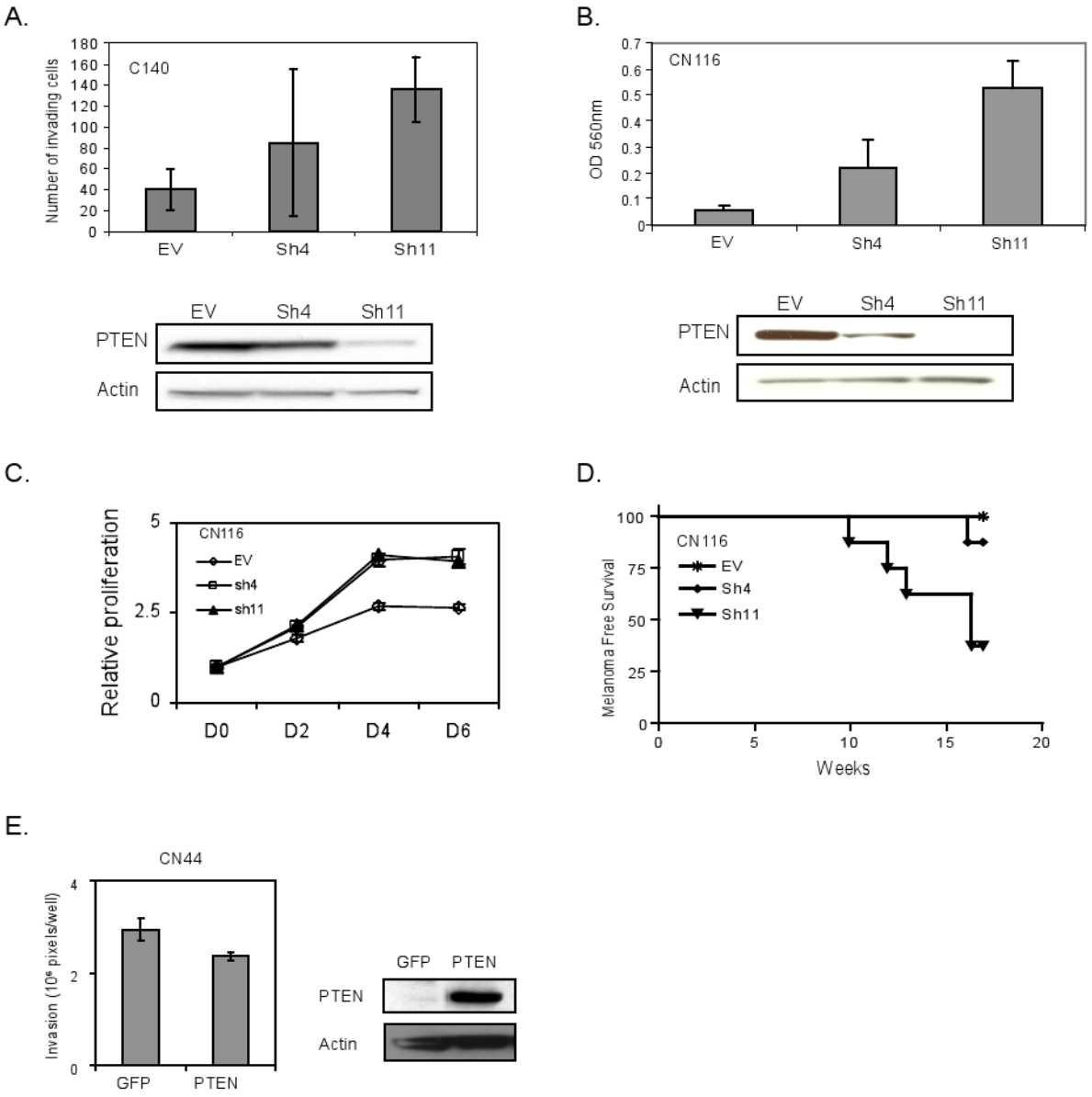

Figure 3. RNAi mediated Pten inactivation accelerates tumorigenesis and promotes invasion of Ras-Ink4a/Arf melanoma and melanocyte cell lines

C140 (*Ras-Ink4a/Arf-Pten+/+*) non-transformed melanocytes (A) and CN116 (*Ras-Ink4a/Arf-Pten+/+*) melanoma cell line (B-D) were infected with pSuper.Retro empty vector (EV) or two independent shRNAs targeting *Pten*- Sh4 and Sh11. CN44 (*Pten+/-Ras-Ink4a/Arf-*) melanoma cell line with Pten protein loss (E) was transduced with adenovirus expression GFP or PTEN. Western blots in A, B, and E show Pten level. Actin serves as a loading control).

(A, B &E) Boyden Chamber assay. Cell invasion through a Matrigel was quantified by counting cristal violet stained cells under a microscope (5 fields per well) (A), by measuring absorbance at O.D. 560nm after dye extraction (B), or by pixel quantification (E). Results reflect normalization for differences in proliferation (see Material and Methods). **(C)** Cell proliferation curve. The effect of Pten loss on growth of CN116 melanoma cells in 1%

serum containing media was measured over 6 days by quantification of crystal violet staining. **(D)** Kaplan-Meier melanoma free survival analysis. SCID mice were subcutaneously injected into the flanks with half million cells of CN116 *Ras-Ink4a/Arf* melanoma cells infected with pSuper.Retro Sh4 (N=8), Sh11 (N=8) or empty vector (N=8). Inactivation of *Pten* by Sh11 resulted in earlier melanoma onset (P value=0.0081).

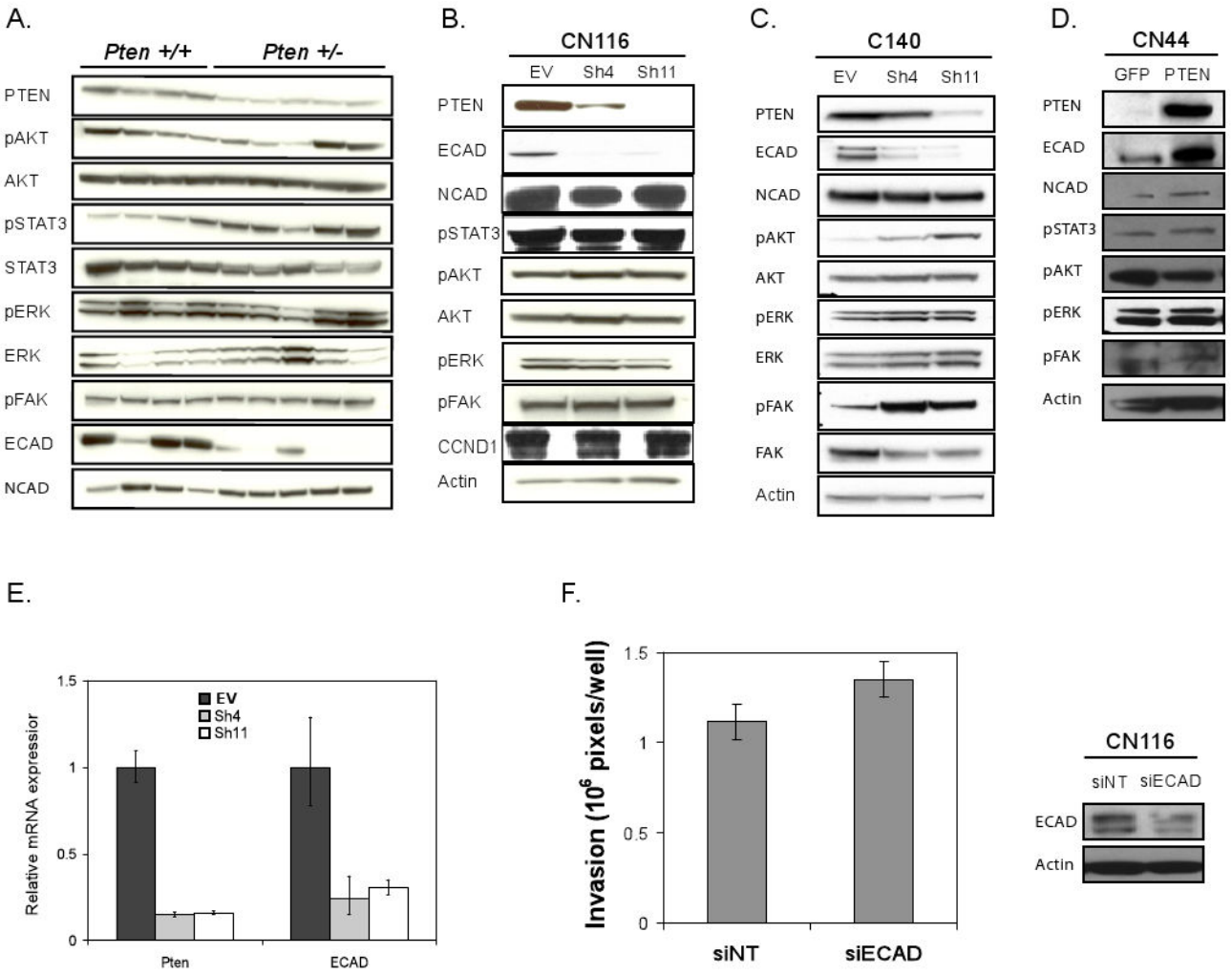

Figure 4. E-cadherin protein levels are correlated to Pten level

(A) Molecular profile comparison of *Ras-Ink4a/Arf Pten+/+* and *Pten +/-* early passage melanoma cell lysates by immunoblotting with antibodies labeled on left. The corresponding *Pten* genotype is shown on top of the blot. E-cadherin protein levels are lower on *Pten +/-* melanoma cells compared to on *Pten +/+* cells. **(B-D)** Immunoblot analysis CN116 (B) and C140 (C) with EV, sh4, or sh11, and CN44 (D) cells with GFP or PTEN expression with antibodies indicated. **(E)** Real time rt-PCR analysis of Pten and E-Cadherin expression in CN116 cells with EV, Sh4, or Sh11. **(F)** Boyden chamber assay (left) of CN116 cells following siRNA-mediated E-Cadherin downregulation (siECAD) compared to non-targeting control (siNT) and western blot analysis (right).

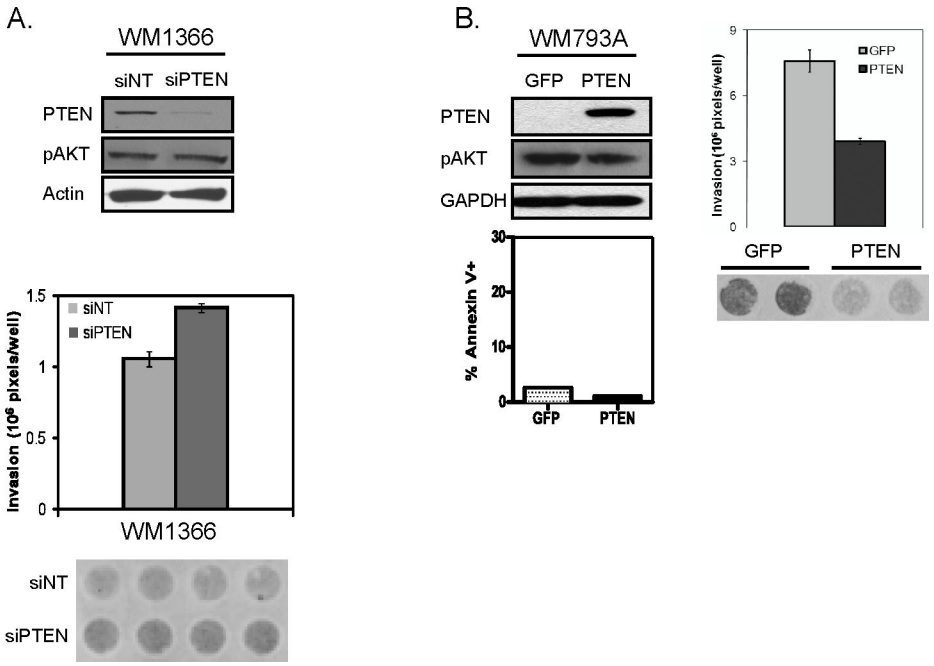

Figure 5. PTEN inhibits invasion of human melanoma cells with RAS/RAF/MAPK activation

(A) Western blot analysis with indicated antibodies (top) and invasion chamber analysis (bottom) of NRAS mutated human melanoma cell WM1346 with siNT (non-targeting) control or with siRNA targeting PTEN (siPTEN).

(B) WM793A human melanoma cell lines harboring BRAF mutation and loss of PTEN expression were transduced with adenovirus encoding control GFP or with PTEN. Immunoblot (top left), Annexin V/PI apoptosis (bottom left), and invasion chamber analysis (right) are shown.

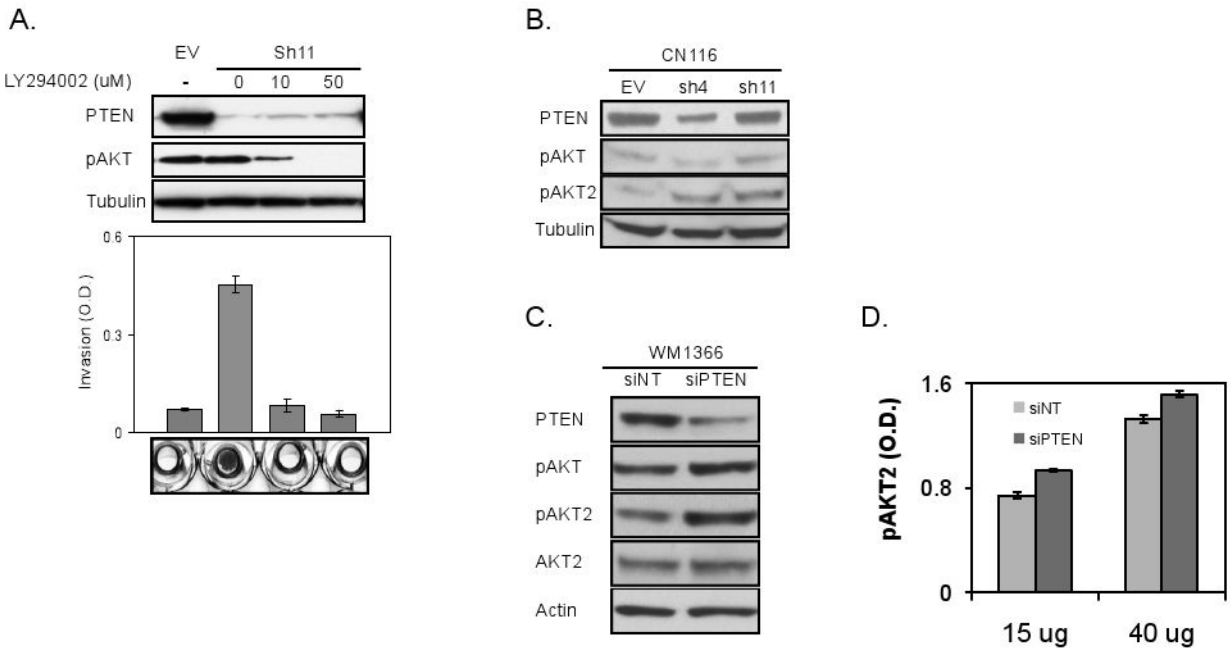

Figure 6. PTEN loss has more impact on pAKT2 among AKT isoforms

(A) LY294002 treatment decreased p-AKT level of CN116 cells with Pten loss (sh11) (*top*, immunoblots with indicated antibodies), which correlates with invasive capability (*bottom*, Boyden chamber assay). Invasion was quantitated by measuring absorbance of crystal violet dye. Representative images of invasion chamber are shown.

(B) Immunoblot analysis of CN116 *RAS Ink/Arf* melanoma cells with EV, Sh4, or Sh11. Note much higher increase of pAKT2 compared to total pAKT by loss of Pten protein expression.

(C&D) PTEN expression was knocked down with siRNA targeting PTEN (siPTEN) in WM1366 and its effect on AKT phosphorylation (total and AKT2) was measured by immunoblotting (C). p-AKT2 level was analyzed by ELISA (D).

Table 1

Incidence of melanoma and median survival of *RAS-Ink4a/Arf* animals on *Pten* wild type and heterozygous background

RAS Ink4a/Arf	***Pten* +/+**	***Pten* +/-**
Melanoma	10	36
Non-Melanoma	18	12
Median survival (weeks)	30	19