

Deregulated Akt3 Activity Promotes Development of Malignant Melanoma

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ABSTRACT

Malignant melanoma is the skin cancer with the most significant impact on man, carrying the highest risk of death from metastasis. Both incidence and mortality rates continue to rise each year, with no effective long-term treatment on the horizon. In part, this reflects lack of identification of critical genes involved and specific therapies targeted to correct these defects. We report that selective activation of the Akt3 protein promotes cell survival and tumor development in 43 to 60% of nonfamilial melanomas. The predominant Akt isoform active in melanomas was identified by showing that small interfering RNA (siRNA) against only Akt3, and not Akt1 or Akt2, lowered the amount of phosphorylated (active) Akt in melanoma cells. The amount of active Akt3 increased progressively during melanoma tumor progression with highest levels present in advanced-stage metastatic melanomas. Mechanisms of Akt3 deregulation occurred through a combination of overexpression of Akt3 accompanying copy number increases of the gene and decreased PTEN protein function occurring through loss or haploinsufficiency of the *PTEN* gene. Targeted reduction of Akt3 activity with siRNA or by expressing active PTEN protein stimulated apoptotic signaling, which reduced cell survival by increasing apoptosis rates thereby inhibiting melanoma tumor development. Identifying Akt3 as a selective target in melanoma cells provides new therapeutic opportunities for patients in the advanced stages of this disease.

INTRODUCTION

Of the three major forms of skin cancer, malignant melanoma carries the highest risk of mortality from metastasis (1–3). The prognosis for patients in the late stages of this disease remains very poor with average survival from 6 to 10 months (3, 4). Currently, there is no effective long-term treatment for patients suffering from the advanced stages of this cancer despite many clinical trials testing the efficacy of a wide variety of therapeutics ranging from surgery to immuno-, radio-, and chemotherapy (4–9). The lack of effective therapeutic regimens is due, in part, to a lack of information about the predominant genes altered during melanoma development and therapies specifically targeted to correct these defects (10, 11).

To identify key genes important in melanoma development, we have created melanoma tumor progression models to identify proteins that may be potential therapeutic targets in particular signaling pathways. Recently, we found that the phosphatidylinositol 3'-kinase (PI3k)/Akt signaling pathway plays a critical role in melanoma tumorigenesis (12). Deregulated Akt activity through loss of the phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*) phosphatase, a negative regulator of PI3k/Akt signaling, was found to decrease the apoptotic capacity of melanoma cells and thereby regulate melanoma tumorigenesis (12). This study provided direct support

for elevated Akt activity playing a central role in melanomas, but the specific Akt isoforms altered and the mechanisms leading to deregulation of these proteins in melanomas are currently unknown.

The Akt protein kinase family consists of three members, Akt1/PKB α , Akt2/PKB β and Akt3/PKB γ , which share a high degree of structural similarity (13, 14). Although all isoforms may be expressed in a particular cell type, only certain isoforms may be active. It also appears that each isoform can perform unique as well as common functions in cells (13–16). Knockout mice lacking Akt1 are growth retarded and have increased rates of spontaneous apoptosis in the testis and thymus (15, 17, 18). In contrast, Akt2 knockout mice have impaired insulin regulation and consequently a defective capability of lowering blood glucose levels due to defects in the action of insulin on liver and skeletal muscle (16, 18). Currently, there is no published report describing the phenotype associated with an Akt3 knockout mouse; thus, very little is known about the specific functions of Akt3 or its role in human cancer.

Genetic amplifications that increase the expression of Akt1 or Akt2 have been reported in cancers of the stomach, ovary, pancreas, and breast (19–24). Although no activating mutations of Akt have been identified in melanomas (25, 26), blocking total Akt function by targeting PI3k (with the PI3k inhibitors Wortmannin or LY-294002) inhibits cell proliferation and reduces the sensitivity of melanoma cells to ultraviolet radiation (27). Total Akt activity has also been measured in melanomas using immunohistochemistry to demonstrate increased levels of total phosphorylated Akt in severely dysplastic nevi and metastatic melanomas compared with normal or mildly dysplastic nevi (28). However, the roles played by individual Akt isoforms and mechanisms leading to deregulation of particular Akt isoforms in melanoma is unknown.

Here, we identify that selective activation of Akt3 occurs in 43 to 60% of sporadic melanomas, occurring as a result of a combination of increased Akt3 expression accompanying copy number increases of the *Akt3* gene and decreased *PTEN* protein activity due to loss or haploinsufficiency of the *PTEN* gene. Furthermore, targeted decrease of Akt3 activity using siRNA or *PTEN* protein expression stimulated apoptotic signaling, which reduced cell survival and inhibited melanoma tumor development. Thus, these studies identify Akt3 as a selective target in melanoma tumor progression and provide a mechanistic basis for increased Akt3 activity during the genesis of sporadic melanomas.

MATERIALS AND METHODS

siRNA Mediated Down-regulation of the Akt Isoforms. To demonstrate the specificity of siRNA against Akt1, Akt2, and Akt3 (Dharmacon, Lafayette, CO) in UACC 903 cells, hemagglutinin A-tagged Akt1, Akt2, or Akt3 constructs were nucleofected together with each respective siRNA. The generation of the Akt constructs used for these studies and use in prior studies are detailed by Sun *et al.* (29), Mitsuuchi *et al.* (30), and Brodbeck *et al.* (31). Each construct (5 μ g), either alone or in combination with 100 or 200 pmol of each respective siRNA pool, was introduced into 7×10^5 UACC 903 cells via nucleofection using an Amaxa Nucleofector. The resultant transfection efficiency using constructs expressing GFP was >60%. Protein lysates were harvested 72 hours later, and Western blot analysis was performed as described previously (12). Nucleofection with siRNA was also used to knockdown endogenous expression of the Akt isoforms and/or *PTEN* (Dharmacon) in

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Note: J. Stahl and A. Sharma contributed equally to this work.

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melanocytes and in the melanoma cell lines UACC 903, SK-MEL-24, WM115, and WM35. A scrambled siRNA pool (Dharmacon) was used as a negative control. The Amaxa nucleofection reagents and protocol for melanocytes were also used with WM35 cells, whereas the other cell lines were nucleofected using Amaxa Solution R/program K-17. The growth conditions for these cell lines have been described previously (12, 32).

Western Blotting, Immunoprecipitation, and Kinase Assays. The Western blot procedure and antibodies used, except for Akt1 (Cell Signaling Technologies, Beverly, MA), Akt2 (Santa Cruz Biotechnology, Santa Cruz, CA), and Akt3 (Upstate Biotechnology, Lake Placid, NY), have been reported previously (12). An anti-phospho-Akt (Ser-473) antibody was used for these studies (Cell Signaling Technologies; ref. 12). For immunoprecipitation, protein was collected after addition of protein lysis buffer [50 mmol/L Tris-HCl (pH 7.5), 0.1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 50 mmol/L NaCl, 10 mmol/L sodium β -glycerol phosphate, 5 mmol/L sodium inorganic pyrophosphate, 1 mmol/L sodium orthovanadate, 0.1% 2-mercaptoethanol, and 0.5% protease inhibitor mixture (Sigma, St. Louis, MO)] to plates of cells followed by snap freezing in liquid nitrogen. Cellular debris was pelleted by centrifugation ($\geq 10,000 \times g$) of lysates, and protein concentration was quantitated using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Protein for immunoprecipitation (100 μ g) was incubated with 2 μ g of Akt1 or Akt2 or 5 μ L of Akt3 antibody overnight at 4°C with constant mixing. A no antigen negative control was prepared by adding antibody to the lysis buffer only. Next, 15 μ L of equilibrated GammaBind G Sepharose beads (Amersham Biosciences, Piscataway, NJ) were added to each tube and incubated for 2 hours (4°C) with constant mixing. Pelleted beads were washed twice with lysis buffer to remove unbound antibody and protein. Samples were then electrophoresed under reducing conditions according to the protocol provided by Invitrogen Life Technologies, Inc. (Carlsbad, CA) with the NuPage Gel System. Western blots were probed with an anti-phospho-Akt (Ser-473) antibody and quantitated by densitometry as described previously (12).

For each Akt kinase assay, 15 μ L of equilibrated GammaBind G Sepharose beads were incubated with 2 μ g of Akt1 or Akt2 antibody, or 5 μ L of Akt3 antibody in a volume of 350 μ L of lysis buffer at 4°C with constant mixing for ≥ 2 hours. Microcystin (1 μ mol/L) from MP Biomedicals (Irvine, CA) was added to the lysis buffer to ensure complete inactivation of cellular PP1 and PP2 phosphatases. The antibody/Sepharose complex was washed twice with 750 μ L of lysis buffer and then incubated with 100 μ g of protein in a volume of 350 μ L overnight at 4°C with constant mixing. This complex was washed with 500 μ L of lysis buffer (3 \times) and then once with 500 μ L of assay dilution buffer [20 mmol/L 4-morpholinepropanesulfonic acid (pH 7.2), 25 mmol/L β -glycerol phosphate, 1 mmol/L sodium orthovanadate, and 1 mmol/L dithiothreitol]. Protein kinase A (PKA) inhibitor peptide (10 μ mol/L) from Santa Cruz Biotechnology (Santa Cruz, CA), 37.5 μ mol/L ATP, 17 mmol/L MgCl₂, 0.25 μ Ci/ μ L [γ -³²P]ATP, and 90 μ mol/L Akt-specific substrate Crosstide from Upstate Biotechnology (Lake Placid, NY) were added to the tubes in assay dilution buffer and incubated at 35°C for 10 minutes with continuous mixing. Next, 20 μ L of liquid were transferred to phosphocellulose paper, which was washed three times for 5 minutes with 40 mL of 0.75% phosphoric acid. After a 5-minute acetone wash, the phosphocellulose was allowed to dry and transferred to a scintillation vial with 5 mL of Amersham Biosciences scintillation fluid, and counts per minute were measured in a Beckman Coulter LS 3801 Liquid Scintillation System (Fullerton, CA).

Studies Using Human Tumor Material. Collection of melanoma tumors from human patients was performed according to protocols approved by the Penn State Human Subjects Protection Office, the Dana-Farber Cancer Institute Protocol Administration Office, and Cooperative Human Tissue Network. Formalin-fixed paraffin-embedded archival melanoma specimens were used for immunohistochemistry to measure phosphorylated Akt. Sixty-three archival specimens of melanocytic lesions were used for immunohistochemistry experiments with the phospho-Akt (Ser-473) monoclonal antibody (Cell Signaling Technologies) at a 1:50 titer according to the manufacturer's recommended protocol. Specificity and intensity of staining were determined through qualitative comparison with internal blood vessel endothelium, squamous epithelium, or smooth muscle controls present in each specimen.

Tumor protein for Western blotting or immunoprecipitation was collected by using a mortar and pestle chilled in liquid nitrogen to pulverize tumor material flash frozen in liquid nitrogen, which consisted of $>60\%$ tumor material. One mL of protein lysis buffer was added for every 200 mg of tissue

powder and sonicated for 2 minutes (15-second intervals) in an ice-filled sonicator bath. The samples were centrifuged ($\sim 12,000 \times g$) at 4°C for 10 minutes. The supernatant was transferred to a clean tube and quantitated using the Bio-Rad Protein Assay.

Animal Studies and Apoptosis Measurements. Animal experimentation was performed according to protocols approved by the Institutional Animal Care and Use Committee at the Pennsylvania State College of Medicine. Athymic female nude mice were purchased from Harlan Sprague Dawley (Indianapolis, IN), and tumor kinetics were measured by s.c. injection of 1×10^6 cells in 0.2 mL of DMEM containing 10% fetal bovine serum above the left and right rib cages of 4- to 6-week-old nude mice. For animal experimentation involving siRNA, 1×10^6 UACC 903 cells were nucleofected with siRNA to Akt isoforms, and 48 hours later, cells in 0.2 mL of DMEM containing 10% fetal bovine serum were s.c. injected above the left and right rib cages of nude mice. The dimensions of the developing tumors were measured on alternating days using calipers. When measuring apoptosis, 5×10^6 cells were injected per site, and four to six tumors were harvested 4 days later. Apoptosis measurements were performed on formalin-fixed, paraffin-embedded tumor sections using the Roche terminal deoxynucleotidyl-transferase-mediated nick end labeling (TUNEL) TMR Red Apoptosis kit (Mannheim, Germany) as described previously (12). A minimum of eight fields were counted from three or four different tumor sections, and the number of TUNEL-positive cells was expressed as the percentage of apoptotic cells.

Statistics. For statistical analyses, the one-way analysis of variance (ANOVA) or Kruskal-Wallis ANOVA on ranks was used for groupwise comparisons, followed by the appropriate *post hoc* tests (Dunnett's or Dunn's). Results were considered significant at $P < 0.05$.

RESULTS

Isoform-Specific siRNA Identify Akt3 Involvement in Melanoma. In a recently described experimental genetic melanoma model (12), we found that deregulated Akt activity (through PTEN loss) plays a critical role in melanoma tumorigenesis by decreasing the apoptotic capacity of melanoma cells (12). The model was established using the melanoma cell line UACC 903, which has elevated levels of phosphorylated Ser-473 Akt (a measure of activity) due to loss of active PTEN protein (Fig. 1A; ref. 12). Expression of PTEN protein in UACC 903 cells resulted in diminished Akt activity in three independently derived cell lines (36A, 29A, and 37A), which was reversed in revertant cell lines that lost functional PTEN activity (36A revertants) during tumorigenesis. Therefore, we reasoned that because this model reflects the importance of Akt in melanoma tumorigenesis, it could be used to identify the specific Akt isoforms whose deregulated activity controls melanoma tumor development.

To identify the predominant Akt isoform active in melanomas, we used siRNA specific to each Akt isoform to determine the extent to which each isoform reduced the amount of phosphorylated (active) Akt in the parental UACC 903 (–PTEN) cell line. The specificity of knocking down expression for each Akt isoform in UACC 903 cells was determined by conucleofecting constructs expressing tagged hemagglutinin A-Akt1, hemagglutinin A-Akt2, or hemagglutinin A-Akt3 together with siRNA specific for each isoform. Attesting to specificity, each Akt siRNA was found to only reduce expression of the endogenous or ectopically expressed Akt isoform against which it was made (Fig. 1B). The specificity of the antibodies used throughout this study was measured by Western blot analysis of immunoprecipitated ectopically expressed hemagglutinin A-tagged Akt1, Akt2, or Akt3. Attesting to the specificity of the antibodies, each only recognizes the specific isoform against which it was made (Fig. 1C). A no antigen control lane for the immunoprecipitates was used to differentiate between nonspecific background bands in the immunoprecipitate and specific interaction between antigen and antibody. Next, siRNA to each Akt isoform was nucleofected into the UACC 903 cell line as well as two additional independently derived melanoma cell lines (WM115 and SK-MEL-24) to determine which

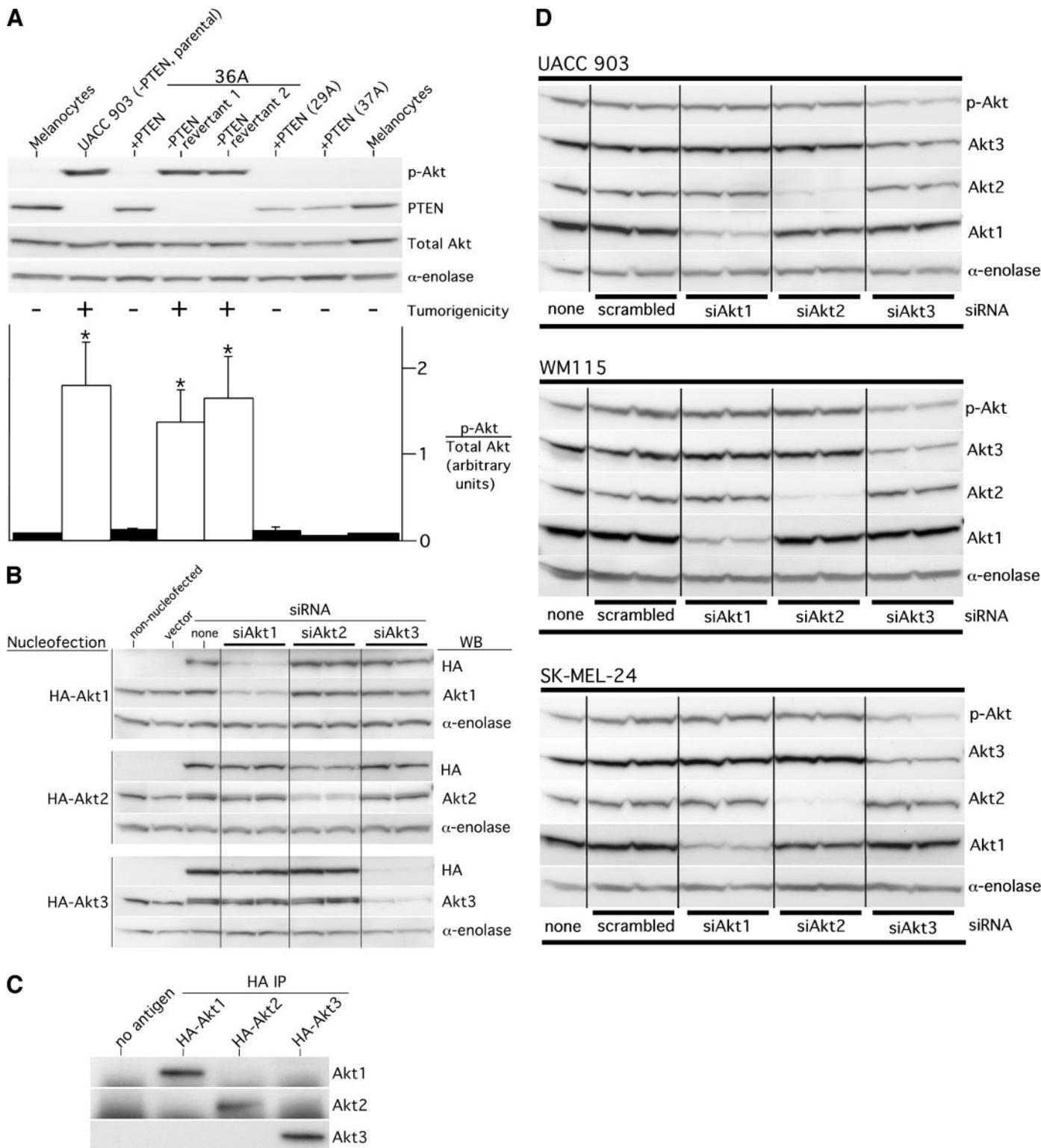


Fig. 1. Identification of Akt3 involvement in malignant melanoma. **A**, Akt activity in the melanoma cell line UACC 903 is regulated by PTEN. Western blot analysis showing expression of phosphorylated-Akt (Ser-473), total Akt, PTEN, and α-enolase (loading control). The 36A, 29A, and 37A cell lines are genetically related cell lines created from the UACC 903 parental cell line that express PTEN. Tumorigenic revertant cell lines derived from the 36A cell line are considered isogenic, differing only in PTEN expression. Melanocytes serve as a control for normal cells. The graph represents densitometric scans from three separate Western blots to quantitatively demonstrate the level of phosphorylated to total Akt in each cell line; bars, ±SEM; statistics, one-way ANOVA followed by Dunnet's multiple comparisons versus the melanocyte control; *, $P < 0.05$. **B**, siRNA for each of the Akt isoforms demonstrates specific knockdown of endogenous and/or each ectopically expressed Akt isoform in the UACC 903 cell line. Constructs expressing tagged hemagglutinin A-Akt1 (HA-Akt1), hemagglutinin A-Akt2 (HA-Akt2), or hemagglutinin A-Akt3 (HA-Akt3) were conucleofected together with siRNA specific to Akt1, Akt2, or Akt3 into UACC 903 cells. Cells were nucleofected with 100 (left) or 200 pmol (right) of each respective siRNA. Controls were non-nucleofected or vector-only nucleofected cells. Western blots (WB) were probed with antibodies to hemagglutinin A (HA) to detect the ectopically expressed protein as well as with antibodies to Akt1, Akt2, and Akt3 to measure changes in endogenous and ectopic protein levels. α-Enolase served as a loading control. **C**, Antibodies recognize only the specific Akt isoform against which each individual one was made. Western blot analysis of immunoprecipitated ectopically expressed hemagglutinin A-tagged Akt1, Akt2, or Akt3 (HA-Akt1, HA-Akt2, and HA-Akt3). Control was immunoprecipitated with no antigen added. **D**, siRNA-mediated knockdown of Akt3, but not Akt1 or Akt2, alters level of phosphorylated Akt (activity) in the melanoma cell lines UACC 903, WM115, and SK-MEL-24. Western blot analysis showing expression of phosphorylated-Akt (Ser-473), Akt3, Akt2, Akt1, and α-enolase after nucleofection with 50 (left) or 100 pmol (right) for each respective siRNA. Controls were non-nucleofected or cells nucleofected with scrambled siRNA. Data are representative of a minimum of two separate experiments. The loading control for these

siRNA lowered the level of phosphorylated (active) Akt in these cells (Fig. 1D). Whereas siRNA to Akt1 or Akt2 had only a negligible, nonsignificant effect, siRNA to Akt3 significantly reduced the levels of phosphorylated total Akt, suggesting that Akt3 was the isoform regulating tumor development in the UACC 903 (PTEN) model (12). Because all three independently derived melanoma cell lines indicated that Akt3 was the predominant active isoform in melanomas, subsequent experiments primarily focused on examining Akt3 deregulation and used Akt2 and/or Akt1 as a control for comparison because these isoforms have been reported to be activated or amplified in multiple types of cancers (20–24).

To further confirm that Akt3 was the predominant isoform whose activity was specifically reduced by PTEN in the UACC 903 (PTEN) tumorigenesis model, Akt3 activity was measured by immunoprecipitating total Akt3 or Akt2 from cell lysates followed by Western blotting to estimate the amount of phosphorylated (active) Akt in the immunoprecipitate (Fig. 1E). Phosphorylated Akt3, but not Akt2, levels were elevated in the parental UACC 903 cell line as well as the two tumorigenic revertant 36A cell lines that lack PTEN. In contrast, barely detectable levels of phosphorylated Akt3 were observed in the 36A, 29A, or 37A cell lines, which had low levels of Akt activity, ostensibly due to PTEN expression (12). To verify that the phosphorylated levels of Akt3 reflected active Akt, immunoprecipitated Akt3 and Akt2 were assayed in an *in vitro* kinase assay, and the results for Akt3 are shown in Fig. 1F. A

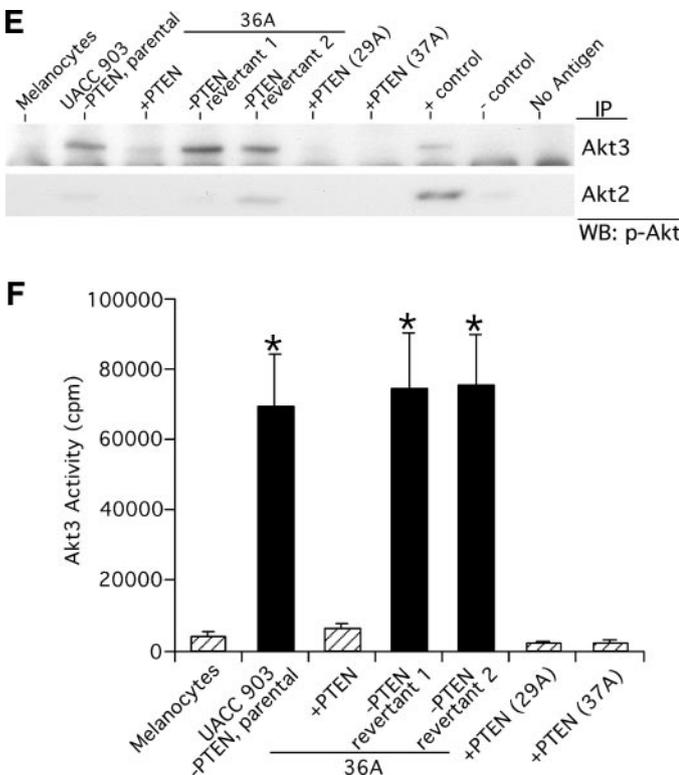


Fig. 1. Continued.

experiments was α -enolase. E, Phosphorylated Akt3 is reduced when PTEN protein is present in the UACC 903 (PTEN) tumorigenic model. Akt3 and Akt2 were immunoprecipitated from cell lines in the UACC 903 (PTEN) tumorigenic model and analyzed by Western blotting with an antibody recognizing phosphorylated Akt. The PTEN-expressing 36A, 29A, and 37A cell lines were derived from the UACC 903 parental cell line that lacks PTEN protein. The two tumorigenic revertant cell lines were derived from the 36A cell line and no longer express PTEN. A negative antigen control is shown together with positive and negative controls for Akt3 and Akt2. Controls for Akt3 and Akt2 were HEK 293T or LNCaP cells, respectively, untreated (positive) or treated with LY-294002 (negative), an inhibitor of PI3k. F, Akt3 activity is reduced in the presence of PTEN in the UACC 903 (PTEN) tumorigenic model. Immunoprecipitated Akt3 was used in an *in vitro* kinase assay in which Crosstide was phosphorylated by Akt3 to estimate activity. Plot shows activity after subtraction of the no antigen negative control; bars, \pm SEM; statistics, one-way ANOVA followed by Dunnett's multiple comparisons versus the melanocyte control; *, $P < 0.05$.

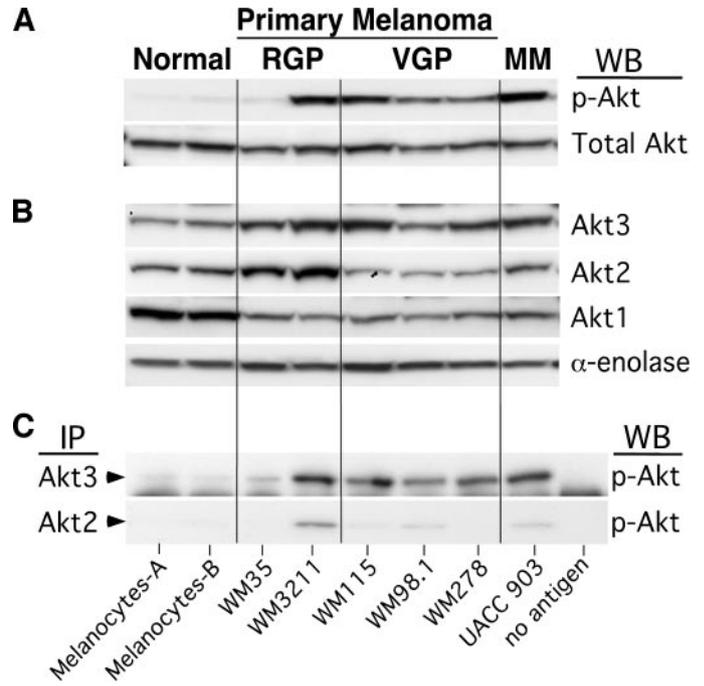


Fig. 2. Increased Akt3 expression and activity occur during melanoma tumor progression. A, An increase in the level of phosphorylated (active) Akt (*p-Akt*) occurs during the radial growth phase (RGP) in the melanoma tumor progression model. Western blot (WB) comparing the amount of phosphorylated Akt in melanocytes with low-passage melanoma cell lines established from primary tumors at the radial (WM35 and WM3211) and vertical (VGP; WM115, WM98.1, and WM278) stages of growth. Total Akt is shown for comparison. B, Comparison of Akt3 versus Akt2 and Akt1 expression in the melanoma tumor progression model. Western blots showing the levels of expression of Akt3, Akt2, and Akt1 are shown together with α -enolase as a loading control. C, Akt3 is preferentially activated in cell lines of the melanoma tumor progression model compared with Akt2. Akt3 or Akt2 was immunoprecipitated from each cell line and subjected to Western blot analysis to measure the amount of phosphorylated Akt in the immunoprecipitate. An increase in the level of phosphorylated (active) Akt3 occurs during the radial growth phase in the melanoma tumor progression model.

statistically significant difference in Akt3 (Fig. 1F), but not Akt2 activity (data not shown), was identified in revertants (–PTEN) compared with PTEN-expressing cells ($P < 0.05$). Collectively, these results indicate that Akt3 activity was specifically regulated by PTEN in the UACC 903 (PTEN) tumorigenesis model.

Increased Akt3 Activity Occurs Early during Melanoma Tumor Progression. Melanocytes are thought to be capable of transforming directly into a melanoma (33). Alternatively, melanocytes can follow a model of tumor progression in which they evolve in a stepwise fashion from common nevi, to atypical nevi, to melanoma *in situ* (radial and vertical growth phases), and finally to metastatic melanomas (33). Regardless of the process, the evolution of more aggressive tumor cells requires the accumulation of alterations affecting tumor suppressor genes and oncogenes. These, in turn, result in subpopulations of cells that have ever-increasing selective growth or survival advantages that promote the tumorigenic process.

To provide evidence for the selective involvement of Akt3 during melanoma tumor progression, expression and activity of the Akt isoforms were measured in a melanoma tumor progression model (generously provided by Dr. Meenhard Herlyn; refs. 32 and 33). In this progression model, melanocytes are compared with low-passage cell lines established from primary melanoma tumors at the radial (WM35 and WM3211) and vertical (WM115, WM98.1, and WM278) stages of growth. In comparison with melanocytes, Fig. 2A shows that one of the two radial growth phase and all three of the vertical growth phase cell lines had elevated phosphorylated Akt, which suggested that Akt activity increased early during primary melanoma develop-

ment in the radial growth phase. Next, expression of the Akt3 isoform was examined by Western blotting and compared with Akt1 and Akt2 expression in these cell lines (Fig. 2B). Akt3 expression was found to be elevated in all except the WM98.1 radial growth phase cell line when compared with melanocytes. Because expression does not necessarily reflect activity, the amount of active Akt3 was examined by immunoprecipitation of Akt3, Akt2, or Akt1 followed by Western blot analysis to measure the level of phosphorylated Akt in the immunoprecipitate. In comparison with melanocytes, Akt3 activity was elevated in all except the WM35 radial growth phase cell line (Fig. 2C). Note that even though Akt3 protein expression in the WM98.1 vertical growth phase cell line was similar to that observed in melanocytes, Akt3 activity was significantly higher. In contrast to the Akt3 results, Akt2 expression was elevated only in the radial growth phase cell lines in comparison with melanocytes (Fig. 2C). Only the WM3211 radial growth phase cell line had a corresponding increase in Akt2 activity, however, these cells also had elevated Akt3 activity when compared with melanocytes. Akt1 expression for all of the cells lines was lower than that observed in melanocytes (Fig. 2B). These data suggest that Akt3 was the predominant Akt isoform active in the melanoma tumor progression model.

Frequency of Akt3 Deregulation in Tumors from Melanoma Patients. Because the foregoing experiments identified Akt3 as the predominantly active Akt isoform in both the UACC 903 (PTEN) tu-

morigenesis and melanoma tumor progression models, subsequent *in vivo* studies focused on establishing the frequency of Akt3 deregulation in tumors from melanoma patients. The relative intensity of total phosphorylated Akt was initially assessed in melanocytic lesions by immunohistochemical analysis of common nevi, dysplastic nevi, primary melanomas, and metastases from melanoma patients to determine the frequency of Akt activation. Examples of the staining results are shown in Fig. 3A. Whereas weak-to-moderate levels of staining were detected in 100% of common nevi, strong staining was observed in 12% of dysplastic nevi, 53% of primary melanomas, and 67% of metastatic melanomas (Fig. 3B). These results suggest that whereas Akt activity may serve some unidentified role in nevi development, deregulated Akt activity is indicative of a more advanced tumor stage.

Analyses of the genomic regions containing the *Akt1*, *Akt2*, and *Akt3* genes, from a published report (34), has not found amplification in melanoma. However, the 1q43-44 region containing Akt3 does undergo copy number increases (34-36), which suggest overexpression as a mechanism contributing to increased Akt3 activity in melanomas. In contrast, the 14q32 region containing Akt1 and the 19q13 region containing Akt2 remain unchanged or tend to undergo loss (34-36). To establish whether increased Akt3 expression could be a selective mechanism leading to increased activity, protein lysates from 30 metastatic melanoma patients' tumors were extracted to compare the level of expression and activity of the three Akt isoforms in the tumor material.

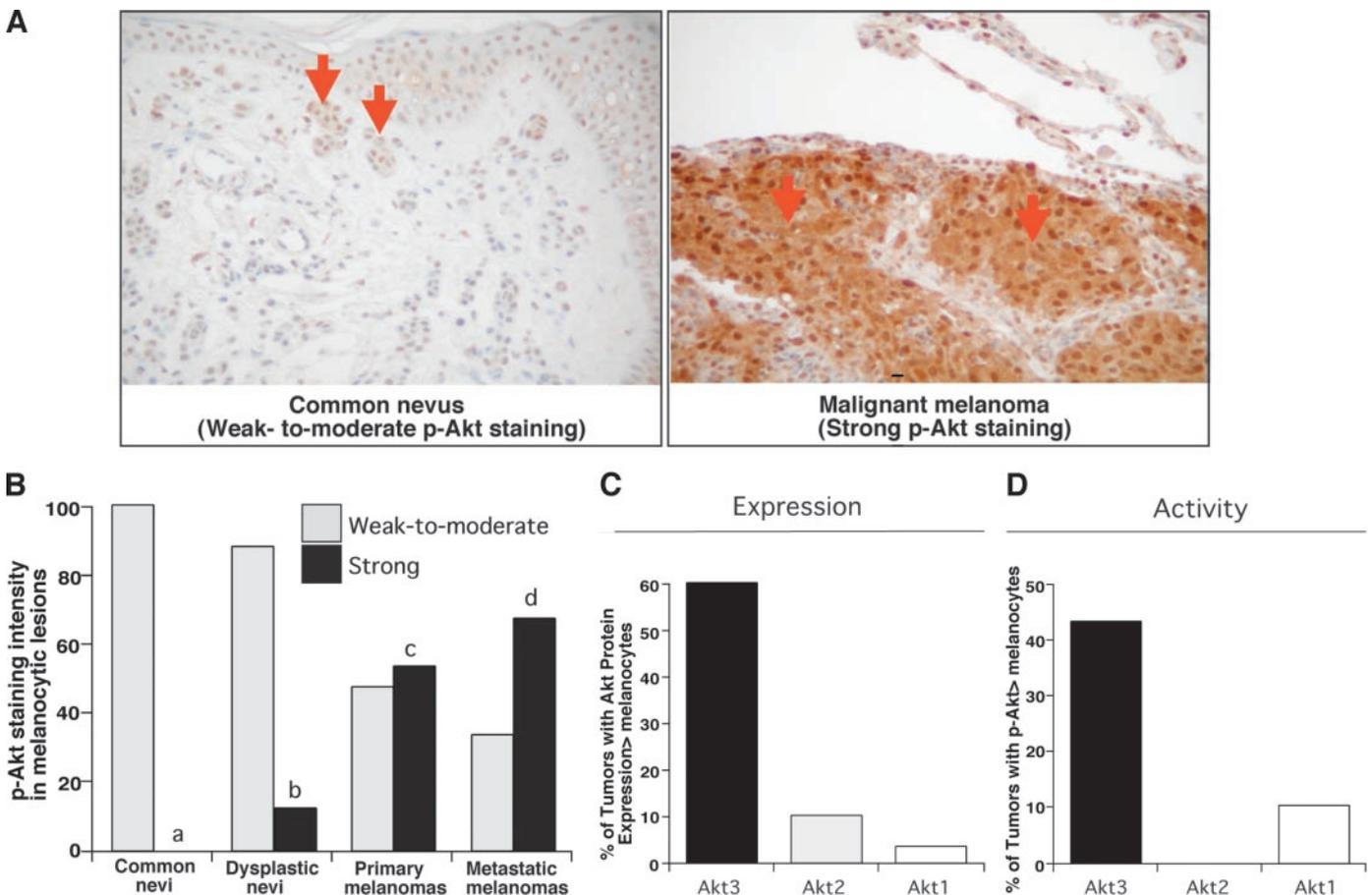


Fig. 3. Akt3 activity is increased in tumors from melanoma patients. *A* and *B*, relative intensity of phosphorylated Akt (*p-Akt*) staining in common nevi, dysplastic nevi, primary melanomas, and metastases from melanoma patients. *A*, Staining intensity was scored as weak-to-moderate or strong. Weak-to-moderate staining indicates that tumor cells were stained to an intensity similar to that in pericytes adjacent to blood vessels in the same section. In contrast, strong staining indicates staining to a greater intensity than in pericytes. *Arrows* point to examples of each type of staining. *B*, percentage of common nevi, dysplastic nevi, primary melanomas, and metastases from melanoma patients staining weak-to-moderate or strongly for p-Akt. Statistics, $P < 0.05$ for *a* versus *c*, *d* and *b* versus *d*. *C*, Akt3 is preferentially overexpressed in metastatic melanomas from human patients compared with melanocytes. Akt3, Akt2, and Akt1 expression were measured from metastatic melanomas derived from 30 tumors. Expression of each isoform was normalized to α -enolase expression. The graph quantitatively compares the level of expression of each Akt isoform. Only tumors in which expression is >2 -fold that occurring in melanocytes were scored. *D*, Activity of Akt3, but not Akt2 or Akt1, increases in tumors from melanoma patients compared with melanocytes. Activity was determined by immunoprecipitation of Akt3, Akt2, or Akt1 followed by Western blot analysis with an antibody recognizing phosphorylated Akt.

Three independent Western blots were used to quantitate expression in each sample, which was then compared with expression in melanocytes (Fig. 3C). Overall, 60% (18 of 30) of the tumors had elevated Akt3 protein expression, ranging from an ~2- to 9-fold increase over the expression observed in melanocytes. In comparison, Akt1 and Akt2 protein expression was elevated in 10% (3 of 30) and 3% (1 of 30) of tumors respectively, ranging from a 2- to 3-fold increase over the expression observed in melanocytes. These results are consistent with the type of copy number increases of the region of chromosome 1q43-44 containing the *Akt3* gene reported in the literature to occur in melanoma tumors (34–36). Next, levels of activity were measured by quantifying phosphorylated Akt in Akt3, Akt2, or Akt1 immunoprecipitates. Strikingly, levels of phosphorylated (active) Akt3 greater than that observed in melanocytes were detected in 43% of the samples (Fig. 3D). In contrast, no phosphorylated Akt2 (except for the positive control) and 10% phosphorylated Akt1 was detected in these tumors. These data confirm the involvement of Akt3 deregulation in 43 to 60% of tumors from advanced-stage melanoma patients and suggest that increased expression is one of the mechanisms contributing to deregulated Akt3 activity in melanomas.

Mechanisms Underlying Akt3 Deregulation in Melanomas. The foregoing experiments identified Akt3 as the predominantly active isoform *in vitro* in cell culture models and *in vivo* in patient tumors. Therefore, we next focused on determining the mechanisms leading to deregulated Akt3 activity in melanomas. Because the initial UACC 903 (PTEN) tumorigenesis model suggested that PTEN played a significant role regulating Akt activity in melanomas, we examined whether decreased expression of PTEN directly and specifically increased Akt3 activity. To accomplish this objective, PTEN expression (activity) was knocked down by siRNA in melanocytes and radial growth phase primary melanoma cells (WM35) to measure the effect on the level of phosphorylated Akt. The WM35 cell line was chosen because these cells have negligible basal Akt3 activity (see Fig. 2C) and express PTEN protein. As predicted, Fig. 4A and B show that siRNA-mediated down-regulation of PTEN led to an increase in total phosphorylated Akt (Fig. 4A and B, Lanes 4 and 11), whereas a scrambled siRNA control exerted a negligible, nonsignificant effect (Fig. 4A and B, Lanes 2 and 9). The predominant Akt isoform activated after PTEN down-regulation was determined by conucleofection of siRNA against PTEN together with siRNA to Akt1 (Fig. 4A and B, Lanes 5 and 12), Akt2 (Fig. 4A and B, Lanes 6 and 13), or Akt3 (Fig. 4A and B, Lanes 7 and 14). Only siRNA directed against Akt3 (Fig. 4A and B, Lanes 7 and 14) lowered the level of phosphorylated Akt to that observed in non-nucleofected cells (Fig. 4A and B, Lanes 1 and 8) or cells nucleofected with scrambled siRNA only (Fig. 4A and B, Lanes 2 and 9). In contrast, reduction of Akt1 or Akt2 protein levels did not reduce the amount of phosphorylated Akt, again attesting to the selectivity of the Akt3 deregulation. Hence, selective regulation of Akt3 activity by PTEN is a significant mechanism for activating Akt3 in melanomas, because PTEN loss increases Akt3 activity without overexpression. Thus, a reduction in PTEN could, in turn, lead to an increase in the cellular phosphatidylinositol 3,4,5-trisphosphate concentration, which would be effective for specifically increasing Akt3 activity in melanomas. The mechanism underlying this specificity is currently unknown.

Studies involving tumor material from melanoma patients indicated that increased expression of Akt3 might also play a significant role augmenting Akt3 activity in melanomas. To investigate this possibility, Akt3 was overexpressed in melanocytes and WM35 cells (not shown) that express PTEN protein. Five μ g of hemagglutinin A-tagged wild-type Akt3, a kinase dead version of Akt3 T305A/S472A (inactive) or a myristoylated Akt3 (active) was overexpressed in melanocytes (Fig. 4C). Cells were then starved of growth factors for 24 hours and replenished with complete media, and then lysates were harvested 30 minutes later.

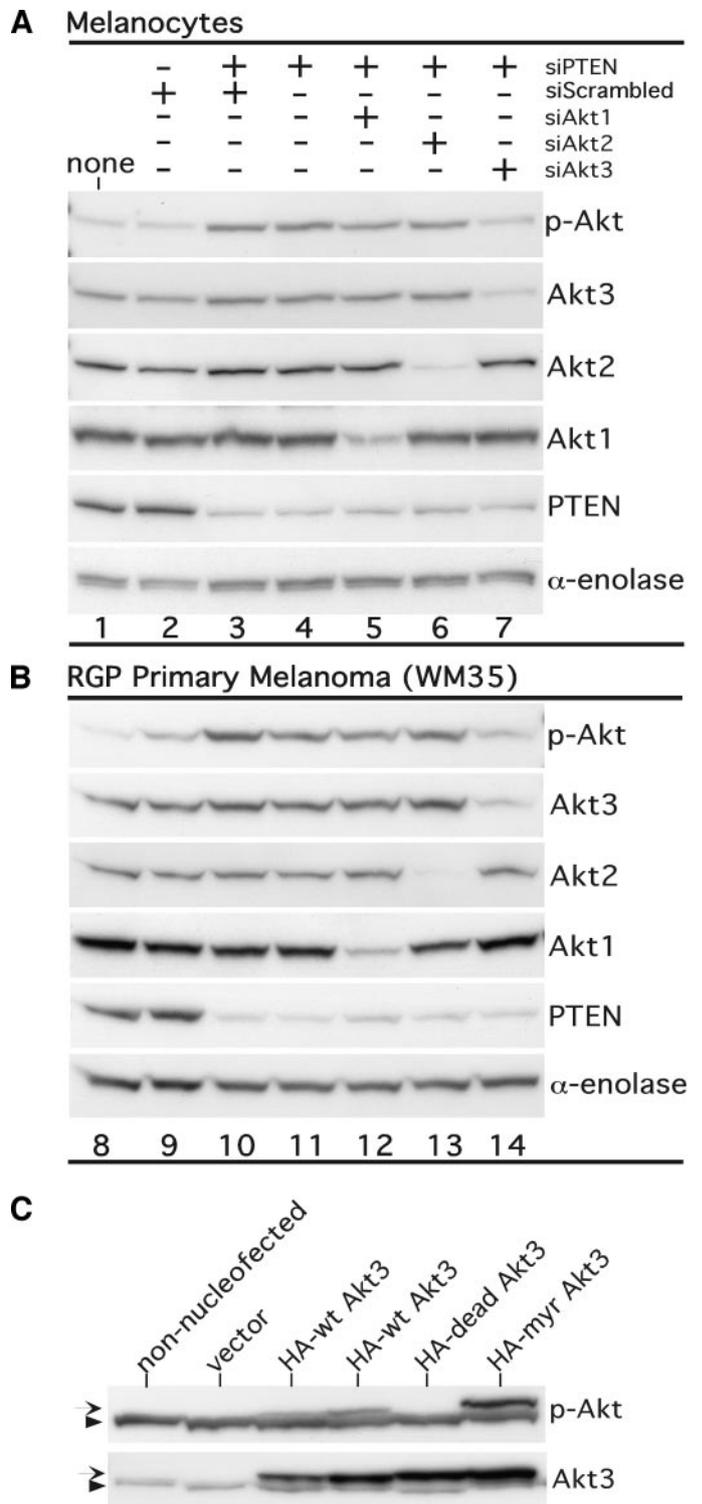


Fig. 4. Mechanism underlying deregulated Akt3 activity in malignant melanomas. Decreased PTEN expression (activity) specifically increases Akt3 activity in melanocytes (A) and WM35 [radial growth phase (RGP)] cells (B). siRNA-mediated reduction of PTEN is shown alone (control) or in combination with scrambled siRNA or with siRNA against Akt3, Akt2, or Akt1. Western blot analysis shows expression of phosphorylated Akt, Akt3, Akt2, Akt1, and PTEN. α -Enolase served as a loading control. C. Overexpression of Akt3 in human melanocytes increases the levels of phosphorylated Akt. Wild-type (*wt*) Akt3, dead Akt3 (inactive), or myristoylated (*myr*) Akt3 (active) was nucleofected into melanocytes. Akt phosphorylation (activity) was measured by Western blot analysis to measure levels of phosphorylated Akt (Ser-473). Western blot analysis of Akt3 shows both endogenous and ectopically expressed protein levels. Arrowhead shows location of endogenous Akt3, whereas arrow indicates ectopically expressed hemagglutinin A-tagged Akt3.

Overexpression of wild-type Akt3 and myristoylated Akt3 led to increased levels of phosphorylated total Akt in comparison with vector only or cells nucleofected with kinase dead Akt3. Furthermore, siRNA-mediated knockdown of PTEN together with Akt3 overexpression led to higher levels of phosphorylated Akt compared with wild-type Akt3 expression alone (data not shown). Thus, overexpression of Akt3 alone or in combination with PTEN loss is an additional mechanism contributing to elevated Akt3 activity in melanomas.

Increased Akt3 Activity Promotes Melanoma Tumorigenesis by Decreasing Apoptosis. Because deregulated Akt3 activity was observed consistently in melanoma tumors, subsequent studies focused on determining the mechanisms by which increased Akt3 activity promoted *in vivo* tumorigenesis. Cell lines from the UACC 903 (PTEN) tumorigenesis or UACC 903 (Akt) siRNA model were used to determine whether altered apoptosis was the mechanism by which elevated Akt3 activity promoted melanoma tumorigenesis in a nude mouse model. Five million cells were injected subcutaneously into nude mice, and temporally and spatially matched tumor masses developing in parallel from each cell type were then harvested 4 days later to compare the magnitude of apoptosis, assessed by TUNEL (Fig. 5; ref. 12). A significantly greater number of apoptotic cells were observed in 36A (+PTEN) tumor masses having low Akt3 activity than in tumors formed from the parental UACC 903 (–PTEN) or 36A revertant (–PTEN) cell lines, which have high Akt3 activity (Fig. 5A and C; $P < 0.05$). Similar results were observed in UACC 903 cells in which siRNA against Akt3 was used to lower Akt3 expression (activity). Cells nucleofected with buffer only or siRNA against Akt2 had approximately 5- to 7-fold fewer apoptotic cells than UACC 903 cells treated with siRNA against Akt3 (Fig. 5B and D; $P < 0.05$).

To measure the effect of the altered apoptotic response on tumor development, one million cells from the UACC 903 (PTEN) tumorigenesis or UACC 903 siRNA (Akt) model were injected beneath the skin of 4- to 6-week-old female nude mice, and the size of the tumor formed was measured 10 days later. Fig. 5E shows that 36A cells having reduced Akt3 activity were nontumorigenic in comparison with parental UACC 903 and revertant 36A cells having elevated Akt3 activity ($P < 0.05$). Although the tumorigenic potential of the 36A revertant cells increased significantly compared with 36A cells, tumor development remained delayed due to retention of a second melanoma suppressor gene on chromosome 10 that was used to create this model (37). In a similar fashion, siRNA-mediated reduction of Akt3 expression (activity) in UACC 903 cells significantly slowed tumor development in comparison with cells nucleofected with only buffer, scrambled siRNA, or siRNA against Akt2 or Akt1 ($P < 0.05$; Fig. 5F). Thus, either specifically reducing Akt3 activity using siRNA against Akt3 (Fig. 4B) or increasing PTEN expression (Fig. 4A) inhibited melanoma tumor development in nude mice. Thus, these results demonstrate that Akt3 activity preferentially regulates the extent of apoptosis, thereby aiding melanoma cell survival and promoting tumorigenesis.

DISCUSSION

In this study, we demonstrate that Akt3 is an important survival kinase, in part, responsible for melanoma development. The UACC 903 (PTEN) melanoma model reflected the importance of Akt in melanoma tumorigenesis and was used to identify Akt3 as the predominant isoform deregulated during melanoma tumorigenesis. The use of siRNA in this model also demonstrated that selective knockdown of Akt3, but not Akt1 or Akt2, decreased the level of total phosphorylated Akt and lowered the tumorigenic potential of melanoma cells. Similar results were found in two independently derived melanoma cell lines (WM115 and SK-MEL-24), further supporting the significance of this discovery. The clinical relevance of this

observation was validated by demonstrating that selective inhibition of Akt3 expression (by siRNA knockdown) or activity (by PTEN expression) significantly reduced melanoma tumor development.

Two distinct mechanisms leading to Akt3 activation in melanomas were identified in this study. The first mechanism is dependent upon overexpression of the structurally normal Akt3 protein. Analysis of advanced-stage melanomas from human patients showed increased expression in >60% of the cases. Overexpression of Akt3 in melanocytes and WM35 cells led to increased activity confirming the human tumor results. Overexpression of Akt is not unique to melanomas but has been documented in several human cancers with a number of studies reporting amplifications of the Akt isoforms. Amplification of *Akt1* has been reported in stomach cancer (19), whereas *Akt2* gene amplification has been found in cancers of the ovary, pancreas, stomach, and breast (20–24). Although no amplifications of the genomic regions containing the *Akt* genes have been reported in melanomas, several reports describe copy number increases of the long arm of chromosome 1 containing the *Akt3* gene (34–36). In contrast, the long arms of chromosome 14 and chromosome 19 containing the *Akt1* and *Akt2* genes, respectively, tend to be unchanged or undergo loss (34–36). Thus, copy number increases of the *Akt3* gene are one of the mechanisms contributing to increased expression and activity of Akt3 in melanoma development.

The second mechanism involved selective Akt3 activation in the UACC 903 (PTEN) model and was in part due to decreased PTEN activity. A related observation in melanocytes and primary melanoma cells that retain PTEN expression (WM35) showed that siRNA-mediated reduction of PTEN specifically increased Akt3 phosphorylation (activity), further reinforcing the significance of Akt3 involvement in melanoma development. Published studies that characterize the genetic changes occurring in tumor material obtained from melanoma patients provide additional support for decreased PTEN expression playing a significant role in early melanoma development (34–36, 38). Specifically, loss of one allele of PTEN, or PTEN haploinsufficiency, commonly occurs in early melanomas through loss of an entire copy of chromosome 10 (34–36, 38). Under this condition, it is predicted that loss of chromosome 10 reduces PTEN expression in a subpopulation of evolving melanoma cells, leading to increased Akt3 activation, providing these cells with a selective growth and survival advantage. Therefore, decreased expression due to haploinsufficiency or loss of activity of PTEN in melanoma plays an important role in melanoma tumor progression by specifically increasing Akt3 activity.

The underlying molecular basis for selective Akt3 activation, over Akt1 and Akt2, after decreased PTEN expression in melanomas is unknown. However, we speculate that the mechanism leading to this specificity involves preferential interaction of phosphatidylinositol 3,4,5-trisphosphate and/or accessory proteins with the pleckstrin homology domain. The NH₂-terminal pleckstrin homology domain mediates protein-protein and phosphatidylinositol 3,4,5-trisphosphate lipid-protein interactions. The pleckstrin homology domain of human Akt3 is ~104 amino acids long (National Center for Biotechnology Information accession no. NP_005456) and 84% and 78% identical to Akt1 and Akt2, respectively (13, 14). Furthermore, within the pleckstrin homology domain are phosphorylation sites that differ between the Akt isoforms and have as yet uncharacterized functions. Recently, a ceramide-induced, PKC ζ -dependent, phosphorylation site at threonine 34 (within the pleckstrin homology domain), has been shown to lead to inactivation of Akt1 by preventing binding to phosphatidylinositol 3,4,5-trisphosphate (39). On the other hand, Akt2 and Akt3 have a serine at this position, which may be phosphorylated and regulated differently. Our analysis of other potential phosphorylation sites within the pleckstrin homology domain of the three Akt isoforms identified three potential unique Akt3 sites. Residue 21 of Akt3 is an

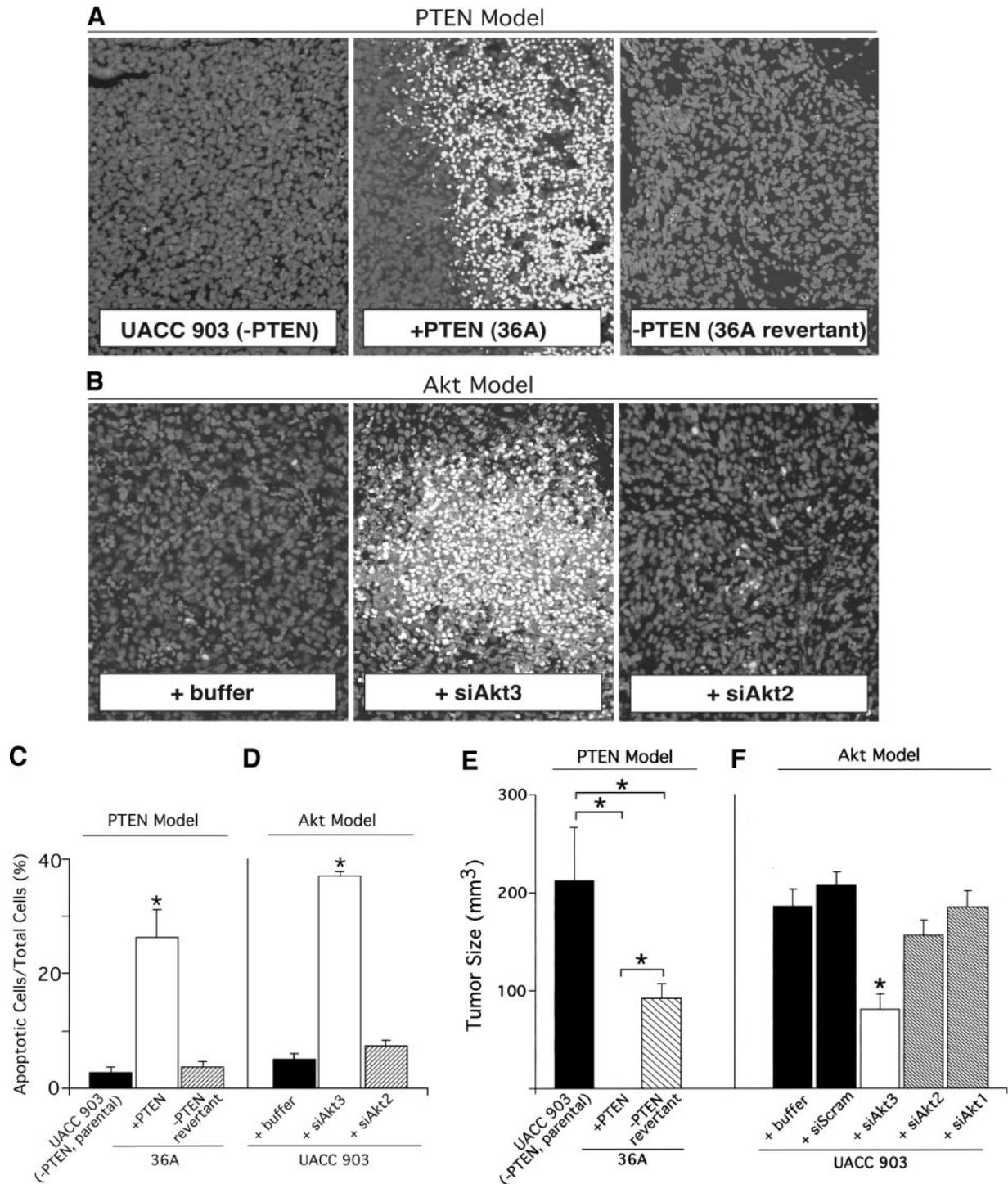


Fig. 5. Increased Akt3 activity promotes melanoma tumor development by reducing apoptosis rates. *A* through *D*, PTEN or siRNA-mediated reduction of Akt3 increases apoptosis in tumors growing in nude mice. Photographs (*A* and *B*) and quantification (*C* and *D*) of TUNEL-positive cells in tumor masses derived from UACC 903 cells expressing PTEN (36A) or nucleofected with siRNA to Akt3 and Akt2; bars, \pm SEM; statistics, Kruskal-Wallis followed by Dunnet's multiple comparisons versus UACC 903; *, $P < 0.05$. Tumors were analyzed 4 days after injection of cells into nude mice; magnification, $\times 200$. The controls were UACC 903 cells or UACC 903 cells nucleofected with buffer only. *White nuclei* represent cells undergoing apoptosis. *E*, PTEN-mediated reduction of Akt3 activity inhibits melanoma tumor development. Size of tumors formed by parental UACC 903 melanoma cells, the isogenic 36A (retaining PTEN), and revertant cell line (lacking PTEN) were measured 10 days after injection into nude mice. Values are means of a minimum of two experiments consisting of six injection sites in three mice per cell line; bars, \pm SEM; statistics, one-way ANOVA followed by Dunnet's multiple comparisons versus UACC 903; *, $P < 0.05$. *F*, siRNA-mediated down-regulation of Akt3 reduces the tumorigenic potential of UACC 903 melanoma cells. siRNA against Akt3, Akt2, and Akt1 were nucleofected into UACC 903 cells, and after 48 hours, cells were injected into nude mice. Size of tumors was measured 10 days later. Controls are UACC 903 cells nucleofected with buffer only or a scrambled siRNA. Values are means of a minimum of six injection sites in three mice per cell line; bars, \pm SEM; statistics, one-way ANOVA followed by Dunnet's multiple comparisons versus UACC 903; *, $P < 0.05$.

asparagine, whereas the equivalent sites on Akt1 and Akt2 are threonines. Furthermore, threonine 31 and tyrosine 49 of Akt3 were also found to differ from the other two Akt isoforms (asparagine 31 and

serine 31 of Akt1 and Akt2, respectively; alanine 50 and proline 50 of Akt1 and Akt2, respectively). Thus, differential regulation of putative phosphorylation sites within the phosphatidylinositol 3,4,5-trisphos-

phate lipid binding pleckstrin homology domain may offer a basis for the specificity of Akt3 activation in melanomas. It is also possible that unsuspected interactions between known oncogenes might be selectively regulating Akt isoform activation in melanomas. For example, TCL1 has been shown to selectively bind the Akt3 pleckstrin homology domain and promote hetero-oligomerization of Akt1 with Akt3 leading to transphosphorylation of the Akt molecules in leukemogenesis (40). TCL1 or other uncharacterized factors in melanoma cells may promote selective Akt3 activation in a similar manner.

Increased Akt3 activation also plays a significant role in the progression to more advanced aggressive tumors. Examination of Akt3 expression and activity in metastatic melanomas indicated that deregulated expression or activity occurs in 43 to 60% of advanced-stage metastatic melanomas. However, it is currently unknown whether the presence of elevated Akt3 activity can predict disease prognosis or the outcome of therapeutic regimes. Measurement of Akt3 activation in melanomas offers hope as a novel, more accurate prognostic indicator of disease outcome than the histopathologic measurements such as Breslow depth (*i.e.*, the distance measured in millimeters from the granular cell layer to the deepest tumor cell) and ulceration (*i.e.*, loss of the epidermis overlying the melanoma) that are currently used. A molecular-based test assessing the activation state of Akt3 in melanocytic lesions may be more sensitive and less subjective than histologic evaluation. This approach might also be useful for selecting appropriate patients for clinical trials using drugs that are designed to target activated Akt3 or other members of this signaling pathway.

This study has shown that use of siRNA or expression of PTEN to selectively lower Akt3 activity can effectively reduce the tumorigenic potential of melanoma cells by altering apoptotic sensitivity. Thus, melanoma cells having high levels of Akt3 activity are better suited for surviving in the *in vivo* tumor environment and inhibition of Akt3 activity, either directly or by interfering with its upstream regulators, is likely to represent an effective anticancer strategy for melanoma patients (4, 41). Indeed, because the vast majority of chemotherapeutic agents work by inducing apoptosis, one would predict that inhibition of Akt3 could lower the threshold doses of drugs or radiation required for effective chemo- or radiotherapy, providing a mechanism to selectively target melanoma cells (4). Therefore, therapeutically targeting Akt3 activity alone or in combination with chemotherapeutic agents could be a potentially important therapy for melanoma patients (4).

In summary, we have identified Akt3 as a specific prosurvival kinase, the increased activity of which in melanoma tumors correlates with tumor progression and provides cells with a selective advantage in the tumor environment.

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