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REF: Expediente No. 06 094108- Solicitud de Patente de Invención a favor de **THE PENN STATE RESEARCH FOUNDATION**

JUAN PABLO CADENA SARMIENTO, portador de la tarjeta profesional No. 57.492 del C.S.J., obrando en calidad de apoderado de la Sociedad THE PENN STATE RESEARCH FOUNDATION, de acuerdo con el artículo 46 de la Decisión 486 de la Comisión de la Comunidad Andina, me permito anexar copia de las siguientes publicaciones, para dar cumplimiento al requerimiento notificado por fijación en lista del 29 de octubre de 2010.

1. COLLISSON et al., Treatment of metastatic melanoma with an orally available inhibitor of the Ras-Raf-MAPK cascade. Cancer Research, 15 September 2003, Vol 63, pages 5669-5673.

2. HINGORANI et al., Suppression of BRAF (V599E) in human melanoma abrogates transformation. Cancer Research, 1 September 2003. Vol 63, pages 5198-5202.

3. NICHOLSON et al. The protein kinase B/Akt signaling pathway in human malignancy. Cellular Sinalling, 2002, Vol 14, pages 381-395.

4. ZINDA et al. AKT-1, -2 and -3 are expressed in both normal and tumor tissues of the lung, breast, prostate, and colon. Clinical Cancer Research, August 2001, Vol 7, pages 2475-2479.

5. STAHL JILL M et al: "Loss of PTEN promotes tumor development in malignant melanoma". CANCER RESEARCH, vol. 63, no. 11, 1 June 2003 (2003-06-01), pages 2881-2890, XP002590380.

6. STAHL JILL M et al: "Deregulated Akt3 activity promotes development of malignant melanoma". CANCER RESEARCH, vol. 64, no. 19, 1 October 2004 (2004-10-01), pages 7002-7010, XP002590381 ISSN: 0008-5472

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MXB/LMA
Anexo

[CANCER RESEARCH 63, 5669–5673, September 15, 2003]

Advances in Brief

Treatment of Metastatic Melanoma with an Orally Available Inhibitor of the Ras-Raf-MAPK Cascade[1,2]

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Abstract

The Ras-Raf-MAPK pathway is constitutively activated in the majority of melanomas because of a mutation in the *BRAF* gene. It has been hypothesized that activation of this pathway is crucial for the genesis and maintenance of melanoma and therefore represents an attractive clinical target for metastatic disease. We synthesized a previously characterized MAP kinase kinase inhibitor to test the effect that blocking the Ras-Raf-MAPK pathway would have on the establishment and maintenance of melanoma metastases. Oral administration of CI 1040 inhibited formation of pulmonary metastases and caused rapid regression of established pulmonary metastases in the mouse. Our findings indicate that Ras-Raf-MAPK activation provides crucial signals for the survival of melanoma cells at ectopic sites and that the pharmacological inhibition of this pathway is a promising target for melanoma therapy.

Introduction

Melanoma is a highly aggressive and often fatal malignancy resistant to currently available therapy (1). The recent elevation in melanoma incidence (2) has highlighted the need to understand the molecular underpinnings of this neoplasm. Specific inhibition of oncogenic signaling molecules promises high efficacy with low toxicity if appropriate molecular targets are defined and blocked. This approach has been difficult in solid tumors because of, in part, heterogeneity in potential targets. A high throughput genomic screen recently identified activating mutations of the *BRAF* gene in the majority of melanomas (3). Thus, application of specific kinase inhibitors to melanoma is particularly attractive because of the high prevalence of *BRAF* mutations and the dismal clinical outcome of currently available treatment modalities.

It is now appreciated that the V599E *BRAF* mutation is the most common mutation in human melanoma, occurring in roughly 80% of short-term cultured tumors. Activating mutations of the *BRAF* gene are also present in roughly 80% of nevi (4), which are thought to be premalignant melanocytic lesions. The precise role that *BRAF* and *RAS* mutations play in the initiation and maintenance of malignant melanoma is not understood. Raf kinases play a central role in the Ras-Raf-MEK-MAPK[5] signaling pathway. Raf is activated by GTP-bound ras (5). Once active, raf phosphorylates the MEK kinase (6). Active MEK phosphorylates and activates MAPK, which has multiple targets, and leads to changes in gene transcription and resistance to apoptosis (7).

Mutational activation of *RAS* genes occurs in human melanoma, albeit with low frequency (8). A mouse model has shown that expression of an activated *RAS* allele is sufficient to promote melanomagenesis in an *INK4A*$^{-/-}$ background and that sustained ras signaling is required for melanoma maintenance (9). These studies suggest that *BRAF* mutations may play an important role in initiation of human melanoma, and that sustained Ras-Raf-MAPK signaling may be required for tumor survival *in vivo*. To test this hypothesis, we administered a highly specific MEK inhibitor to melanoma cells *in vitro* and to mice bearing pulmonary metastases of a human melanoma cell line with the V599E *BRAF* mutation. We targeted MEK because it is necessary for many, if not all of the oncogenic functions of raf (5). Moreover, raf kinase inhibitors developed to date may not be fully active against V599E braf because mutations in the *BRAF* gene could lead to resistance as has been described for other drugs developed against kinase domains of oncoproteins (10). In this report, we show that the oncogenic potential of *BRAF* mutations in melanoma can be effectively diminished with adequate inhibition of MEK and that specific targeting of kinases in melanoma may be a feasible and worthwhile clinical approach to management of this disease.

Materials and Methods

Immunohistochemistry. Tissue samples were acquired from archival melanoma samples or tumor-bearing mice with the approval of the UCLA Office for the Protection of Research Subjects board. Sections of formalin-fixed, paraffin-embedded tissue were deparaffinized in xylene and then hydrated through a series of xylene and ethanol washes. Antigen retrieval was performed using 6.5 mM citrate buffer at pH 6.0 in a pressure cooker for 5 min, and 3% H_2O_2 was used to quench endogenous peroxidase activity. After rinsing two times in PBS, the sections were blocked for 1 h at room temperature in 5% normal goat serum (Vector, Burlingame, CA). Localization of phosphorylated MAPK protein expression was examined using a polyclonal rabbit anti-phospho-p44/42 MAPK antibody (New England Biolabs, Beverly, MA) at a dilution of 1:200. The sections were incubated in the primary antibody overnight at 25°C. Biotinylated secondary goat anti-rabbit antiserum was from KPL (Allentown, PA) and was used at 1:100. Staining was done using 3-amino-9-ethylcarbazole and mounted with Permount.

Western Blotting. Immunoblotting of cultured cells was performed as described (11) with antibodies against b-raf (Santa Cruz Biotechnology, Santa Cruz, CA), Phospho-MEK (Cell Signaling, Beverly, MA), Phospho-MAPK (Cell Signaling), Total-MAPK (Cell Signaling), and actin (Sigma, St. Louis, MO).

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[2] Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

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[5] The abbreviations used are: MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; GFP, green fluorescent protein; SCID, sever combined immunodeficient; CCD, charge coupled device; *Fluc*, firefly luciferase.

Cell Lines and Genotyping. A375, SK-MEL-28, CHL, and WM-266-4 cell lines were from American Type Culture Collection. The A375M cell line was a gift from R. O. Hynes (Massachusetts Institute of Technology). All lines were maintained in DMEM (Invitrogen, Carlsbad, CA) + 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Invitrogen). For *BRAF* sequencing, 1×10^5 cells were pelleted, and genomic DNA was prepared using Trizol (Life Technologies, Inc.) exactly as recommended by the manufacturer. PCR-based sequencing of exon 15 was performed using the following intron-flanking primers from IDT (Skokie, IL): BRAF 15 FWD, 5'-CATAATGCTTGCTCTGATAGGA-3'; and BRAF 15 REV, 5'-GGCCAAAAATTTAATCAGTGG-3'.

A375M-F*luc* cells were established by recombinant lentiviral transduction. Briefly, the firefly luciferase cDNA (*Fluc*) along with a downstream EMCV-IRES sequence was subcloned into pCS-CG lentivector using the *Nhe*I and *Eco*47III sites upstream of the *GFP* gene, resulting in pCS-CG-F*luc*-I-GFP. Recombinant lentivirus was produced by standard calcium phosphate cotransfection of 293T cells along with pΔVPR and pVSVG (12). A375M melanoma cells (1×10^6) were seeded in 10-cm plates and incubated with lentiviral supernatants filtered from virus-producing cultures in the presence of Polybrene (8 μg/ml). Stable cell pools were selected by FACS sorting for GFP fluorescence.

Soft Agar Assays. A375M cells were suspended in 0.3% Noble agar with complete DMEM, plated in duplicate in dishes coated previously with 0.5% base agar, and maintained at 37°C. Both base and suspension agar contained CI 1040 or DMSO at the indicated concentration. On day 21, colonies >0.2 mm in diameter were counted.

Cell Cycle Analysis and Apoptosis. Cells were treated with 1 μM CI 1040 or DMSO in the presence of 10% serum for 24 h and then processed for cell cycle analysis by propidium iodide staining as described (11). Apoptosis was assessed by the Annexin V assay kit (Oncogene, La Jolla, CA) exactly as described by the manufacturer.

Animal Studies. All animal studies were approved by the UCLA Animal Research Committee. Beige SCID mice, 8 weeks of age, were injected with 7×10^5 A375M-F*luc* cells via the lateral tail vein on day 0. Mice were treated with 50 mg/kg of CI 1040[6] resuspended in a 8:1:1 PBS:ethanol:Cremophore by gavage twice per day for the first 14 days after injection ($n = 4$, Early Treat), days 15–28 ($n = 3$, Late Treat), or vehicle ($n = 4$, Control). Mice were imaged by i.p. injection of 100 μl of D-luciferin (30 mg/ml; Xenogen) and imaged using a cooled CCD camera (IVIS). Imaging data were quantified as described (13) by averaging the region of interest from the maximal photon emitting exposure.

Results

To evaluate the frequency of Ras-Raf-MAPK pathway activation in melanoma, we immunostained melanomas with a phospho-specific antiserum to MAPK and found the pathway to be activated in the majority of samples tested (14 of 16; 87.5%; Fig. 1*A*), consistent with a recent report (14).

To address the role of *BRAF* mutations in mediating the high level of MAPK phosphorylation in melanoma, we evaluated several melanoma cells lines with known *BRAF* mutations. We found that the WM-266-4, SK-MEL-28, and A375 cells lines all exhibited high levels of phosphorylated MAPK in both the presence and absence of serum, indicating that the Ras-Raf-MAPK pathway was constitutively activated in these cell lines (Fig. 1*B*). These findings are consistent with the reported mutations in the 599 codon of *BRAF* in these cell lines (3). In contrast, the CHL melanoma cell line, with wild-type *Ras* and wild-type *BRAF* alleles, down-regulated MAPK phosphorylation after serum withdrawal (Fig. 1*B*).

To evaluate the therapeutic potential of inhibiting the Ras-Raf-MAPK pathway, we synthesized CI 1040,[6] a specific, p.o. available, small molecule inhibitor of the MEK kinase (11) that does not inhibit raf. We found that CI 1040 inhibited MAPK phosphorylation in all

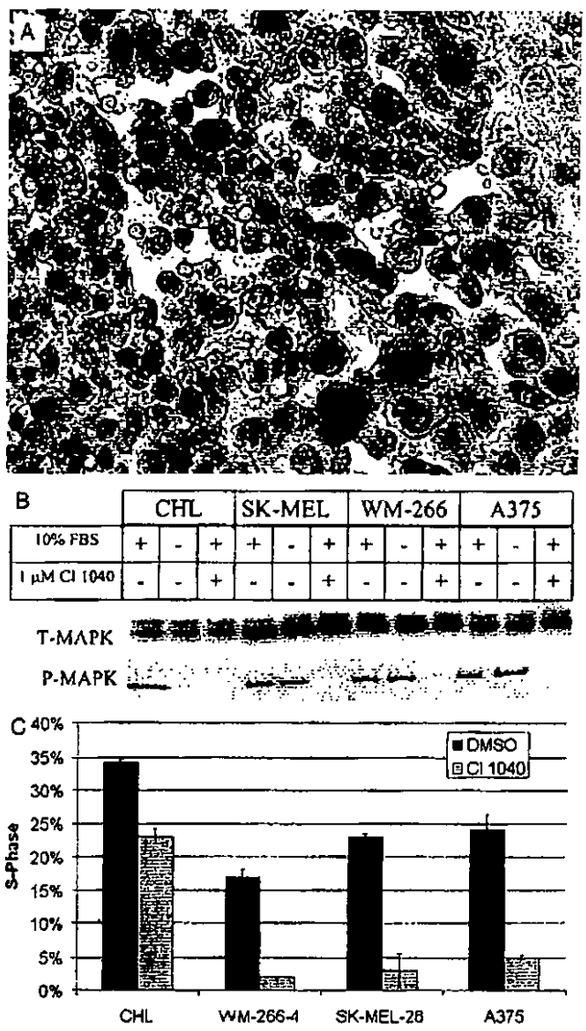
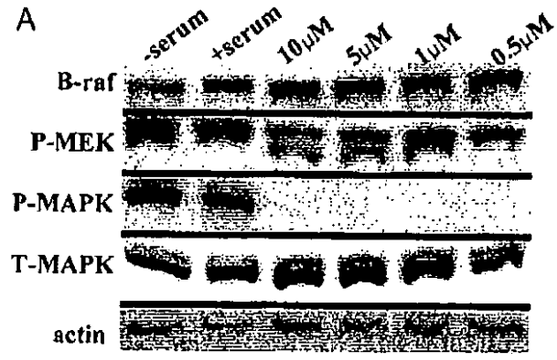


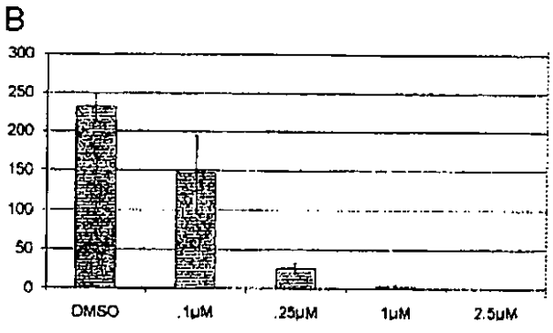
Fig. 1. The Raf-MAPK pathway in melanoma. *A*, immunohistochemistry of a representative primary melanoma stained for phospho-MAPK. *B*, immunoblots of CHL, SK-MEL, WM-266-4, and A375 cell lysates, blotted for total MAPK (*T-MAPK*) or phosphorylated MAPK (*P-MAPK*) in the presence or absence of serum and CI 1040. *C*, quantitation of cell proliferation in the presence (*gray columns*) or absence (*black columns*) of 1 μM CI 1040. *Bars*, ±1 SD.

cell lines tested, indicating that a constitutively active braf required MEK activity to up-regulate MAPK phosphorylation (Fig. 1*B*). To address the possibility that activating *BRAF* mutations contribute to cellular proliferation independent of MEK, we evaluated the ability of CI 1040 to inhibit proliferation of the above cell lines. CI 1040 potently inhibited proliferation of all three lines harboring mutant *BRAF* but had minimal effect on the CHL line with wild-type *Ras* and *BRAF* genes (Fig. 1*C*). This finding indicates that CI 1040 may be specifically potent against cells dependent on constitutive MEK activation for proliferation.

To model the effects of the V599E *BRAF* mutation in living animals, we sought a melanoma cell line that contained this mutation and was well characterized with respect to metastasis *in vivo*. We first confirmed that the A375M melanoma cell line (15) harbored the V599E mutation similar to its parental line (data not shown). We then

[6] Details on the synthesis of CI 1040 are available online at: http://www.mikedcrm.med.ucla.edu/CI1040Synthesis.htm.





C

	DMSO	1μM CI 1040	10μM CI 1040
16h	1.2% (0.1)	5.53% (0.2)	8.1% (0.6)
24h	1.3% (0.1)	7.2% (0.4)	11.8% (0.9)

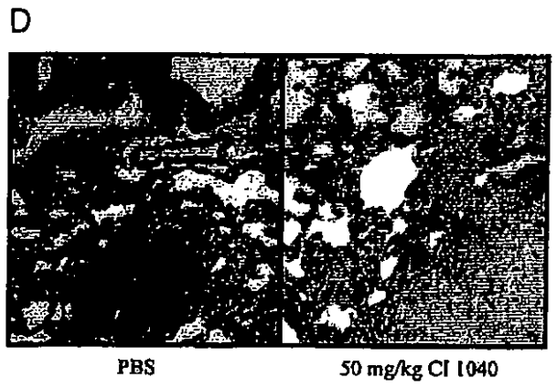


Fig. 2. Results of MEK inhibition on A375M cells. *A*, A375M cells treated with the indicated concentration of CI 1040 and immunoblotted for B-raf, Phospho-MEK (*P-MEK*), Phospo-MAPK (*P-MAPK*), or Total-MAPK (*T-MAPK*). *B*, number of colonies in soft agar assays performed with A375M cells in the presence of various concentrations of CI 1040 after 21 days. *Bars*, ±1 SD. *C*, invaded A375M cells in Matrigel chamber assays with various concentrations of CI 1040 or DMSO control after 48 h (*top images*) or percentage of annexin V positive (*bottom table*) at given time points. *D*, explanted tumor-bearing mouse lungs after treatment with CI 1040 (*left panel*) or PBS (*right panel*) were immunostained for Phospo-MAPK (*P-MAPK*).

treated A375M cells with various concentrations of CI 1040 and observed inhibition of MAPK phosphorylation, whereas total MAPK levels were unaffected (Fig. 2*A*). Of note, B-raf protein levels were not decreased by CI 1040 treatment. Phosphorylation, and thus activation of MEK, was increased by treatment with CI 1040, as reported previously (16).

Anchorage-independent growth is a hallmark of the malignant phenotype. A375M cells plated in soft agar were unable to proliferate when incubated with CI 1040, whereas untreated cells grew robustly (Fig. 2*B*). Ras-Raf-MAPK signaling has been reported to increase invasion of tumor cells (7). The A375M cell line has been selected for high metastatic potential in a mouse xenograft model (15). We incubated A375M cells with CI 1040 in Matrigel invasion chambers and found that CI 1040 treatment inhibited invasion in a dose-dependent manner (Fig. 2*C*). Finally, the Ras-Raf-MAPK pathway has been implicated in cell survival. Incubation of A375M cells with CI 1040 caused a dose- and time-dependent apoptosis (Fig. 2*C*). These *in vitro* data suggest that in the A375M cell line, constitutive activation of the Ras-Raf-MAPK pathway is essential for full expression of the malignant phenotype.

CI 1040 has been reported to be a p.o. effective MEK inhibitor (11). We found that administration of a single oral dose of CI 1040 to A375M tumor-bearing SCID mice at 50 mg/kg inhibited MAPK phosphorylation within 2 h (Fig. 2*D*), thereby indicating that the drug is both bioavailable and effective at this dosage and route of administration.

To further examine the sequelae of MEK inhibition on melanoma in living animals, we established A375M cells stably expressing the firefly luciferase (*Fluc*) and *GFP* genes using a bicistronic lentivirus carrying both reporters (12). The resultant A375M-*Fluc* cells expressed a consistent level of *Fluc* over a 1-month period as described (12) and performed indistinguishably from parental A375M cells in soft agar and invasion assays (data not shown), indicating that lentiviral transduction was both stable and did not appreciably alter the behavior of this cell line. A375M-*Fluc* cells were then injected into SCID mice via the lateral tail vein, and pulmonary metastasis formation was monitored by serial bioluminescent optical imaging with a cooled CCD over 1 month. Initial experiments revealed that light emission accurately reflected tumor burden (data not shown), as shown in similar systems (12).

A375M-*Fluc* cells injected into untreated control mice were rapidly trapped in the pulmonary vasculature. The majority of these cells died over the next 24 h, most likely because they were unable to extravasate and survive at this ectopic site. Metastases became detectable at day 7 and grew exponentially in the lungs thereafter (Fig. 3). In contrast, mice treated with CI 1040 from the day of cell injection (day 0) until day 14 showed no detectable metastases on day 14 (Fig. 3). However, after cessation of CI 1040 administration on day 14, some tumors reemerged by days 21 and 28 (Fig. 3*B*). In parallel experiments, we treated established metastases with CI 1040 for 14 days, beginning at 14 days after tail vein injection. These established tumors rapidly regressed upon CI 1040 administration (Fig. 3).

Discussion

In this report, we show that Ras-Raf-MAPK inhibition by a small molecule holds promise for both the treatment and prophylaxis of metastatic melanoma. Our data support the hypothesis that specific

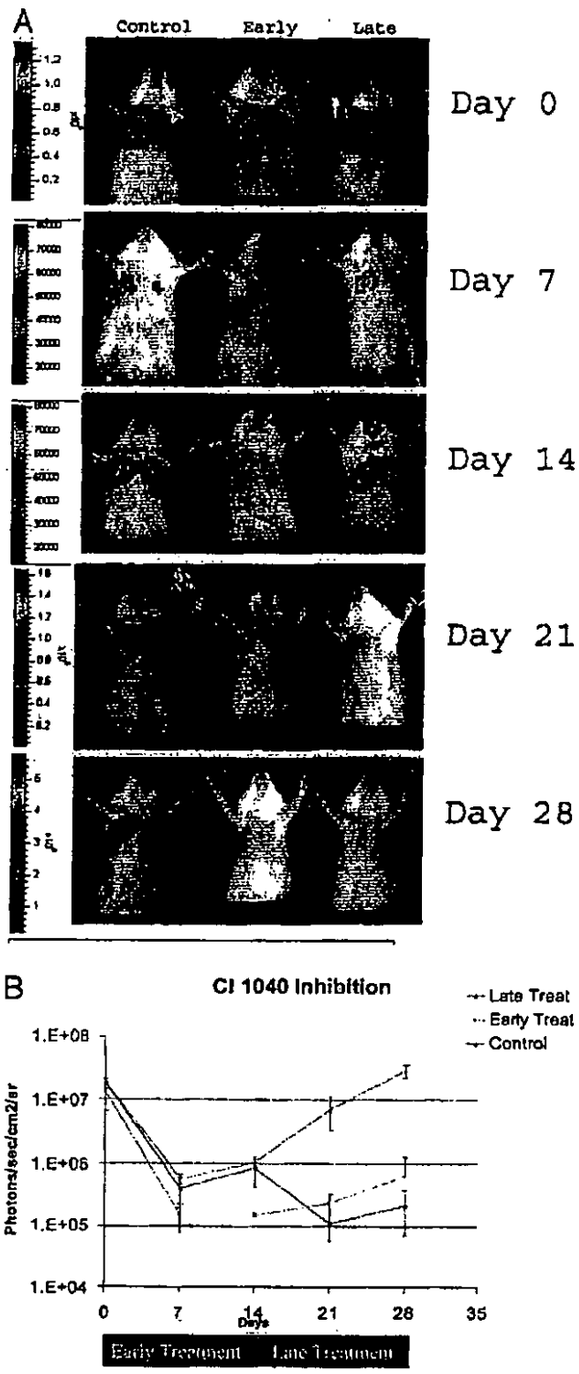


Fig. 3. MEK inhibition is effective *in vivo*. *A*, A375M-*Fluc* cells were injected into SCID mice via the lateral tail vein. Serial images of a representative mouse from each group is pictured. Mice were treated with either PBS (*Control, left column*), CI 1040 at 50 mg/kg, p.o. twice per day either from days 0–14 (*Early Treat, middle column*) or days 15–28 (*Late Treat, right column*). On the indicated days, mice received injections i.p. with 3 mg of D-luciferin and placed in a cooled CCD camera for imaging. *B*, results were averaged from the peak light-emitting exposure from each group and graphed in logarithmic scale.

kinase inhibition downstream of a commonly occurring molecular defect, *i.e.*, Ras-Raf-MAPK activation attributable to mutation of the *BRAF* gene, can provide effective therapy for metastatic melanoma. A well-defined murine transgenic melanoma model has shown that ras activation is sufficient to promote melanomagenesis in an *INK4A*$^{-/-}$ background and that withdrawal of activated ras, by tet-inducible system manipulation, results in rapid tumor regression (9). Complementary work has demonstrated widespread sensitivity of human melanoma cell lines to a MEK inhibitor *in vitro* (17) but did not correlate this susceptibility to tumor genotype, nor was *in vivo* efficacy assessed. Extensive study of both ras and raf has shown that in addition to transducing signals to MEK, these molecules have additional targets (5). Our results suggest that MEK activation is necessary for the survival of melanomas harboring either activating *RAS* or *BRAF* mutations.

Previous work has shown a cytostatic effect of CI 1040 on s.c. tumors harboring *RAS* mutations with resumption of growth upon cessation of the drug (11). Using serial optical imaging, we document regression of metastases treated with CI 1040. However, some A375M cells survive treatment, as evidenced by the regrowth of metastases after drug withdrawal. Serial imaging of mice allowed us to differentiate rapid tumor growth at treatment cessation from slow but continuous growth in the presence of drug, with the imaging data demonstrating the prior scenario.

Our results demonstrate that pharmacotherapy targeted downstream of deregulated *BRAF* or *RAS* signaling may be a viable approach to melanoma management. Additionally, this work suggests that the malignant phenotype resulting from mutational b-raf activation might be susceptible to targeted inhibition of MEK. Approaches aimed at polarizing the cellular response to MEK inhibition toward a cytocidal rather than cytostatic outcome hold promise. For example, combining MEK inhibition with another agent, such as the staurosporine analog UCN-01, dramatically enhances apoptosis (18). Neovascularization is also dependent on MEK signaling, and inhibition of raf in the neovasculature is an emerging antitumor approach (19). CI 1040 also may affect angiogenesis in this manner and could account in part for the efficacy of CI 1040, although we did not directly assess this possibility in the present study. Additionally, targeted inhibition of kinases in cancer has been shown to rapidly give rise to resistance in the clinic (10). The A375M cell line harbors a naturally occurring *BRAF* mutation and could be adapted to select for mechanisms of resistance to MEK inhibition and thus provide a framework for interpreting clinical observations with MEK inhibitors.

The V599E *BRAF* mutation is highly prevalent in both premalignant melanocytic nevi and melanoma, making it the earliest known genetic change in this disease. An emerging theory of cancer progression to metastasis views early events in carcinogenesis as predictive of later metastatic ability (20). The observation that metastatic-like transcriptional profiles exist within primary tumors supports the concept that the genetic changes in a primary tumor are sufficient for metastasis (21). Our findings further support this theory by demonstrating that the earliest known genetic change in melanoma is necessary for a fully metastatic phenotype, and therapy targeted against this change can reverse the metastatic progression of this disease.

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Advances in Brief

Suppression of BRAF^{V599E} in Human Melanoma Abrogates Transformation[1]

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Abstract

Activating mutations in the *BRAF* serine/threonine kinase are found in >70% of human melanomas, of which >90% are *BRAF*^{V599E}. We sought to investigate the role of the *BRAF*^{V599E} allele in malignant melanoma. We here report that suppression of *BRAF*^{V599E} expression by RNA interference in cultured human melanoma cells inhibits the mitogen-activated protein kinase cascade, causes growth arrest, and promotes apoptosis. Furthermore, knockdown of *BRAF*^{V599E} expression completely abrogates the transformed phenotype as assessed by colony formation in soft agar. Similar targeting of *BRAF*^{V599E} or wild-type *BRAF* in human fibrosarcoma cells that lack the *BRAF*^{V599E} mutation does not recapitulate these effects. Moreover, these results are specific for *BRAF*, as targeted interference of *CRAF* in melanoma cells does not significantly alter their biological properties. Thus, when present, *BRAF*^{V599E} appears to be essential for melanoma cell viability and transformation and, therefore, represents an attractive therapeutic target in the majority of melanomas that harbor the mutation.

Introduction

Malignant melanoma will afflict >50,000 people in the United States this year and result in >7,000 deaths (1). The incidence of melanoma is rising among the most rapidly of all malignancies (2). When diagnosed early, melanoma is highly curable by wide surgical excision. However, in patients with deep local invasion, or with spread to lymph nodes or distant sites, the disease is highly resistant to all forms of therapy. The median survival for patients with metastatic melanoma is 6–9 months (3).

Recently, activating mutations in the *BRAF* gene were described in a majority of melanomas and benign nevi, suggesting an important role for this oncogene in melanocyte biology and disease (4–6). More than 60% of malignant melanomas were found to contain a specific mutation, *BRAF*^{V599E}, the product of which possesses constitutive kinase activity. *BRAF* is a member of the Raf family of serine/threonine kinases, along with *CRAF* and *ARAF*, which serve as immediate effectors of the *ras* GTPases (7). Activation of the Raf/MEK[3]/ERK, or MAPK, signaling cascade promotes cellular proliferation and survival. The highly homologous Raf family members have overlapping but distinct biochemical activities and biological functions. We therefore sought to determine whether Raf family members, and specifically *BRAF*^{V599E}, are required in melanoma cells for maintenance of the transformed state. Accordingly, the biochemical signaling properties and cellular phenotypes of melanoma cells were assessed after depletion of B-Raf, B-Raf^{V599E}, and C-Raf proteins by RNAi.

Materials and Methods

RNAi Sequences and Preparation. Small inhibitory duplex RNAs (PROLIGO, Boulder, CO) were prepared and reconstituted in annealing buffer as described (8, 9). The sense strands of the siRNA duplexes were as follows: Lamin A/C: CUggACUUCCAgAAgAACATT; Com-4: AgAAUUggAUCUggAUCAUTT; Mu-A: gCUACAgAgAAAUCUCgAUTT; C1: UgUgCgAAAUggAAUgAgCTT. Duplex shRNA oligos were cloned into the *Hind*III and *Bgl*II sites in pSUPER.retro (Oligoengine, Seattle, WA), and insert fidelity was confirmed by sequencing both strands with the following primers: forward – ttatccagccctcactcc; reverse – gtgttctgggaaatcacc. The sense strands of the shRNA pSUPER.retro inserts were as follows:

Com-1: gatccccTGGATACCGTTACATCTTCttcaagagaGAAGATGTAACGGTATCCAttttggaaa.

Com-2: gatccccTCCCAGAGTGCTGTGCTGTttcaagagaACAGCACAGCACTCTGGGAttttggaaa.

Com-3: gatccccTTGGTTGGGACACTGATATttcaagagaATATCAGTGTCCCAACCAAttttggaaa.

Com-4: gatccccAGAATTGGATCTGGATCATttcaagagaATGATCCAGATCCAATTCTttttggaaa.

Mu-A: gatccccGCTACAGAGAAATCTCGATttcaagagaATCGAGATTTCTCTGTAGCttttggaaa.

Mu-B: gatccccGAGAAATCTCGATGGAGTGttcaagagaCACTCCATCGAGATTTCTCttttggaaa.

C1: gatccccTGTGCGAAATGGAATGAGCttcaagagaGCTCATTCCATTTCGCACAttttggaaa.

BRAF cDNA. Human wild-type *BRAF* and *BRAF*^{V599E} were cloned from mRNA and sequenced to confirm fidelity. 5′ HA epitope tags were cloned into both cDNAs by PCR. Full-length *BRAF* cDNAs were subsequently cloned into pBABE.puro.

Cell Culture and Transfection. WM793 melanoma cells were derived from a vertical growth phase tumor as described previously (10), and HT1080 and HEK cells were obtained from American Type Culture Collection. Cells were cultured under standard conditions (37°C in humidified atmosphere containing 5%CO_2) and grown in DMEM supplemented with 25 mM HEPES (pH 7.4), 10% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). To achieve transient suppression of gene expression, cells were plated in six-well dishes at 50–60% confluency and transfected with 5 μg of duplex RNA plus 6 μl of OLIGOFECTAMINE (Life Technologies, Inc., Carlsbad, CA) per the manufacturer's recommendations and as described (8, 9). The specificity of the targeting sequences was determined by transient cotransfection of HEK cells with pBABE.puro.HA-tagged *BRAF* or pBABE.puro.HA-tagged *BRAF*^{V599E} and shRNA vectors (11). For stable transfection experiments, cells were plated at 50–80% confluency in 100-mm dishes and transfected with 4 μg of plasmid DNA and 12 μl of Fugene 6 (Roche, Indianapolis, IN) per the manufacturer's instructions. Twenty-four h after transfection, cells were selected in media containing 2 μg/ml Puromycin for 60–72 h and then collected for biochemical and cellular assays.

Immunoblotting. Adherent cells were washed with ice-cold PBS and lysed and scraped in boiling SDS lysis buffer (10 mM Tris, 1% SDS, 50 mM NaF, and 1 mM VO4). Lysates were boiled for 5 min, the DNA was sheared, and insoluble debris was removed by microcentrifugation (14,000 rpm for 10 min).

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[3] The abbreviations used are: MEK, mitogen-activated protein kinase kinase; ERK, extracellular signal-regulated kinase; siRNA, small interfering RNA; HEK, human embryonic kidney; HA, hemagglutinin; shRNA, short hairpin RNA; MAPK, mitogen-activated protein kinase; RNAi, RNA interference; TBS, Tris-buffered saline.

Protein concentrations were determined with bicinchoninic acid (Pierce, Rockford, IL). Samples (15 μg of total protein per lane) were resolved by reducing SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore, Bedford, MA). Membranes were blocked and incubated with primary antibodies in TBS [150 mM Tris-HCL (pH 8.0) and 150 mM NaCl] + 3% BSA for antiphospho antibodies or 5% nonfat dry milk/TBS for other antibodies. The membranes were subsequently washed (TBS/0.1% Tween 20), incubated with horseradish peroxidase-conjugated secondary antibodies, and washed again before being processed with enhanced chemiluminescence plus (Amersham Biosciences, Little Chalfont, United Kingdom). Membranes were probed sequentially for the indicated proteins after washing in stripping buffer [50 mM Glycine (pH 2.5) and 0.05% Tween 20] for 15 min at 55°C. Primary antibodies were procured from the following sources: antiphosphorylated and total MEK (Cell Signaling, Beverly, MA), anti-HA (Sigma, St. Louis, MO), and anti-Lamin A/C (Vector Laboratories, Burlingame, CA) and antibodies against actin, B-Raf, and C-Raf were all obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

Proliferation, Apoptosis, and Transformation Assays. After selection, shRNA-transfected cells were plated onto glass coverslips in media. The next day, cells were incubated with 1 mM BrdUrd (Sigma) for 4 h, and positive nuclei detected with anti-BrdUrd FITC per manufacturer's instructions (Roche, Indianapolis, IN). Apoptosis was detected with the In Situ Cell Death Detection kit per the manufacturer's instructions (Roche). Three high-powered fields were counted manually to determine the percentage of cells in S phase and the degree of apoptosis, respectively. Nuclear staining was detected with 4′,6-diamidino-2-phenylindole (Sigma). Soft agar assays were performed by plating 50,000 cells/60-mm dish in 0.34% agar/media suspension over a solidified 0.5% agar layer. Dishes were replenished every 5–7 days.

Results

The recent development of facile methods for both transient and stable suppression of gene expression by RNAi has provided powerful tools for the study of mammalian cell genetics (8, 11, 12). These methods exploit a conserved biological response to short duplex RNA that results in post-transcriptional gene silencing (13). To evaluate the role of *BRAF* expression in human melanoma cells, we generated a series of stably expressing vectors against discrete regions of the human *BRAF* coding sequence (Fig. 1*A*). We then tested the specificity of these reagents in HEK cells that had been transiently cotransfected with HA epitope-tagged wild-type and mutant *BRAF* vectors (Fig. 1*B*). Targeting sequences common to both wild-type and mutant alleles (Com-1, 2, 3, and 4) caused various degrees of knockdown of *BRAF* expression in HEK cells as demonstrated by immunoblots against the expressed protein, with Com-4 being the most effective. A mutant-specific vector (Mu-A) was also created that essentially completely abolished $BRAF^{V599E}$ expression while keeping wild-type *BRAF* expression intact.

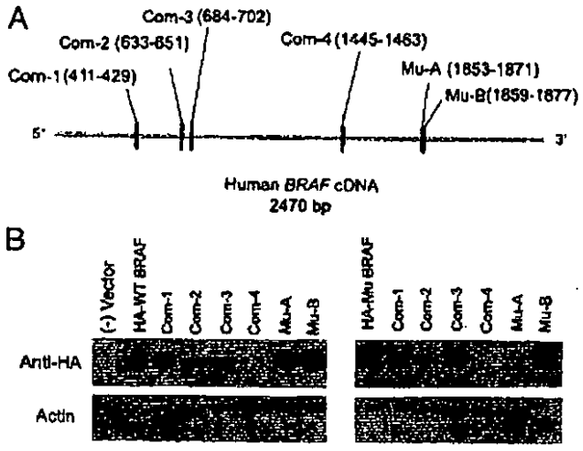

Fig. 1. Design and characterization of vector-based RNAi of human *BRAF*. *A*, schematic representation of human *BRAF* cDNA demonstrating sites selected for targeting. *B*, efficacy of shRNA vectors in suppressing *BRAF* and $BRAF^{V599E}$ expression. Subconfluent adherent HEK cells were either not transfected (−) or cotransfected with pBABE.puro.HA-*BRAF* (*left panels*) or pBABE.puro.HA-$BRAF^{V599E}$ (*right panels*), and the series of shRNA plasmid constructs were directed against *BRAF*. After transfection (48–72 h), whole cell extracts were prepared and analyzed for HA expression; actin was used as a loading control. This experiment was performed three times with similar results.

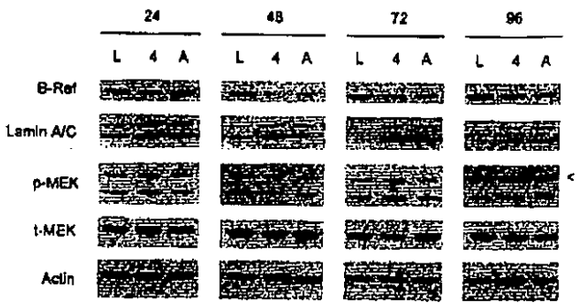

Fig. 2. Transient suppression of wild-type and mutant *BRAF* by siRNA in WM793 cells. Subconfluent cell cultures were transfected with siRNA directed against *Lamin A/C* (*L*), *BRAF* (*4*), and $BRAF^{V599E}$ (*A*). Whole cell lysates were then prepared at 24-, 48-, 72-, and 96-h post-transfection and analyzed for specific protein expression by immunoblotting with the indicated antibodies. The position of the M_r 45,000 phospho-MEK 1/2 proteins is indicated; the identity of the faster migrating band is unknown. This experiment was performed three times with similar results.

After establishing the efficacy and specificity of various RNAi constructs described above, we designed corresponding duplex siRNA species to study the effects of endogenous *BRAF* knockdown in melanoma cells because of the higher transfection efficiencies achievable by this method and the ability to obviate the need for antibiotic selection. WM793 human melanoma cells were derived from a vertical growth phase tumor (10) and have been shown to harbor the $BRAF^{V599E}$ allele (14). Com-4 and Mu-A siRNA significantly inhibited *BRAF* expression and its attendant downstream signaling as measured by phosphorylated MEK levels, although these effects were relatively short lived and reversed by 72–96 h after transfection (Fig. 2). Transfection of siRNA against Lamin A/C as a control revealed efficient and more durable suppression of target expression, reflecting either greater efficacy or stability of this transfected RNA duplex or perhaps lower endogenous levels of lamin mRNA synthesis.

Despite the marked biochemical sequelae to *BRAF* knockdown by siRNA, the effects were transient and rapidly reversible, and there were no overt phenotypic consequences. Thus, to study the potential biological consequences of more durable perturbations of these pathways, we used our panel of shRNA vectors to stably knock down *BRAF* in melanoma cells. Transfection of Com-4 and Mu-A shRNA vectors into WM793 cells effectively and stably suppressed *BRAF* expression and MEK phosphorylation (Fig. 3*A*). Cells transfected with control vector had no discernible effect on these parameters. On the other hand, although it was possible to stably suppress *BRAF* expression by Com-4 in HT1080 human fibrosarcoma cells, no corresponding inhibition of MEK phosphorylation was observed, indicating that these cells activate the MAPK pathway by another mechanism. As expected, transfection with Mu-A had no discernible effect on B-Raf levels because these cells do not contain the $BRAF^{V599E}$ mutation.

Several cellular consequences to stable *BRAF* suppression were readily apparent. First, the morphology of WM793 cells changed dramatically, becoming much larger and flatter and also less refractile (Fig. 3*B*). In addition, far fewer WM793 cells were recovered after

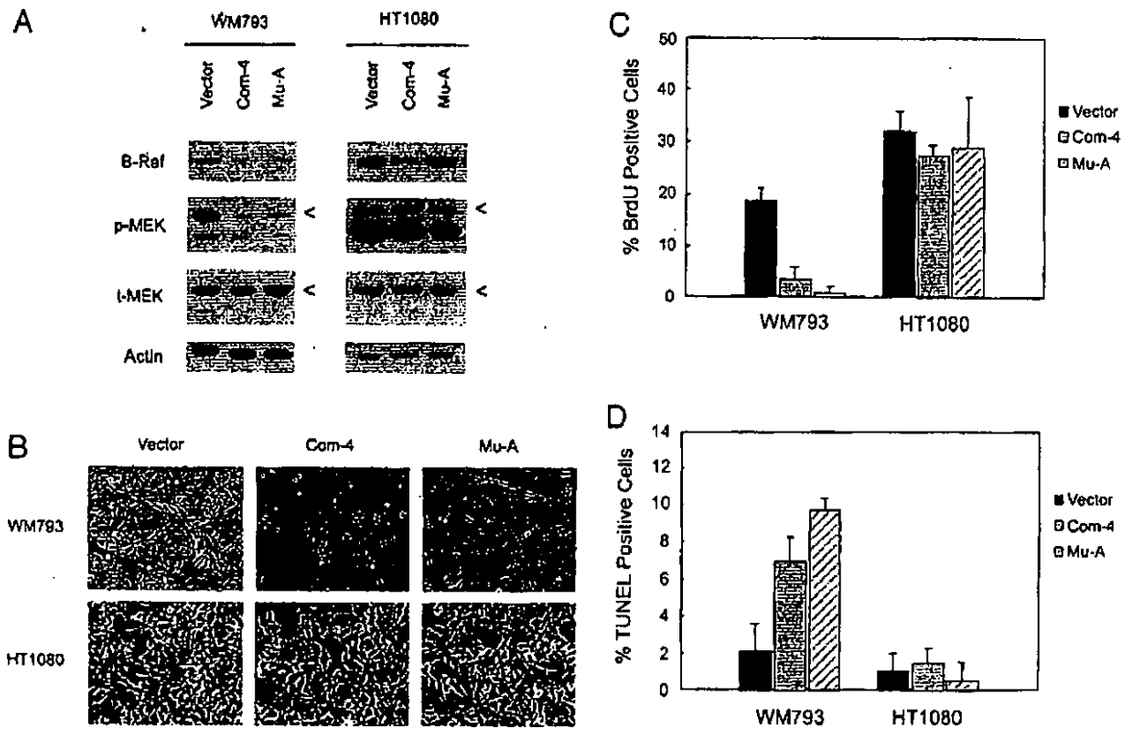

Fig. 3. Effects of stable suppression of endogenous *BRAF* and *BRAF*^{V599E} in WM793 and HT1080 cells. Subconfluent cultures of WM793 or HT1080 cells were transfected with pSUPER.retro (vector), shRNA directed against *BRAF* (Com-4), or shRNA directed against *BRAF*^{V599E} (Mu-A). After selection in puromycin, the biochemical and biological properties of these cells were assessed. *A*, immunoblot analysis of B-Raf, activated MEK (*p-MEK*), total MEK (*t-MEK*), and actin levels. The positions of the *M*, 45,000 phospho- and total-MEK 1/2 proteins are indicated. *B*, phase contrast microscopy of cells in culture (magnification: ×100). *C*, proliferative index as measured by BrdUrd incorporation. *D*, degree of apoptosis as assessed by TUNEL reactivity. BrdUrd and TUNEL data are expressed as the means +/− SD of direct counting of three high-powered fields. These experiments were performed twice with similar results.

transfection with Com-4, as compared with control vector or Mu-A, suggesting that wild-type B-Raf function may also be important for the viability of these cells. No appreciable differences in morphology or cell number were noted in parallel transfections of HT1080 cells (Fig. 3*B*). In addition, Com-4 and Mu-A-transfected WM793 cells exhibited markedly lower proliferative rates; the percentage of cells in S phase was ~4 +/− 2% and 0.8 +/− 1%, respectively, as compared with 19 +/− 2% for cells transfected with empty vector (Fig. 3*C*). The proliferation of HT1080 cells was not affected by transfection with any of these vectors, again despite achieving significant knockdown of B-Raf levels (Fig. 3*C*). WM793 cells also exhibited increased levels of apoptosis after stable suppression of *BRAF*, whereas human fibrosarcoma cells again remained unaffected by similar manipulations (Fig. 3*D*).

These results demonstrated that *BRAF*-dependent signaling was necessary for the optimal proliferation and survival of human melanoma WM793 cells and dispensable for human fibrosarcoma cells. We wondered whether these effects were specific for the *BRAF* family member of the Raf kinases or extended to the heretofore more extensively studied homologue, *CRAF*. We therefore generated *CRAF*-specific duplex siRNA species and stably expressing shRNA vectors and tested their abilities to suppress *CRAF* expression and inhibit downstream phosphorylation of MEK. As shown in Fig. 4*A*, knockdown of C-Raf protein levels by siRNA followed a slower time course than that of B-Raf and remained more durably suppressed. Notably, however, there appeared to be no effect on MEK phosphorylation despite nearly complete suppression of *CRAF*. Thus, at least in WM793 melanoma cells, *CRAF* appears not to be required for MEK activation. A stably expressing shRNA vector directed against the same sequence similarly knocked down *CRAF* expression with no attendant affect on MEK phosphorylation (Fig. 4*B*).

Finally, the ability to manifest anchorage-independent growth, an established feature of cellular transformation, was assessed in melanoma cells after the knockdown of *BRAF*, *BRAF*^{V599E}, or *CRAF*. WM793 cells transfected with empty vector readily formed colonies in soft agar (Fig. 4*C*). Cells in which C-Raf levels had been stably knocked down formed colonies almost as readily. Transfection with either Com-4 or Mu-A, however, essentially completely abrogated the ability of these cells to manifest anchorage-independent growth. Therefore, *BRAF* is uniquely required for cellular transformation in WM793 cells.

Discussion

Oncogenesis is generally viewed as a multistep process characterized by the progressive acquisition of genetic mutations and functional capabilities (15). The hope for curative therapies lies in the proposition that key genetic events exist which represent unique points of vulnerability for cancer cells. Indeed, despite the complexity of genetic and epigenetic alterations in cancer cells and their microenvironment, recent evidence demonstrates that the specific inhibition of one, or perhaps a few, critical pathways in tumor cells may be sufficient to kill them and provide significant clinical benefit. As examples, treatment of patients with stable phase chronic myeloge-

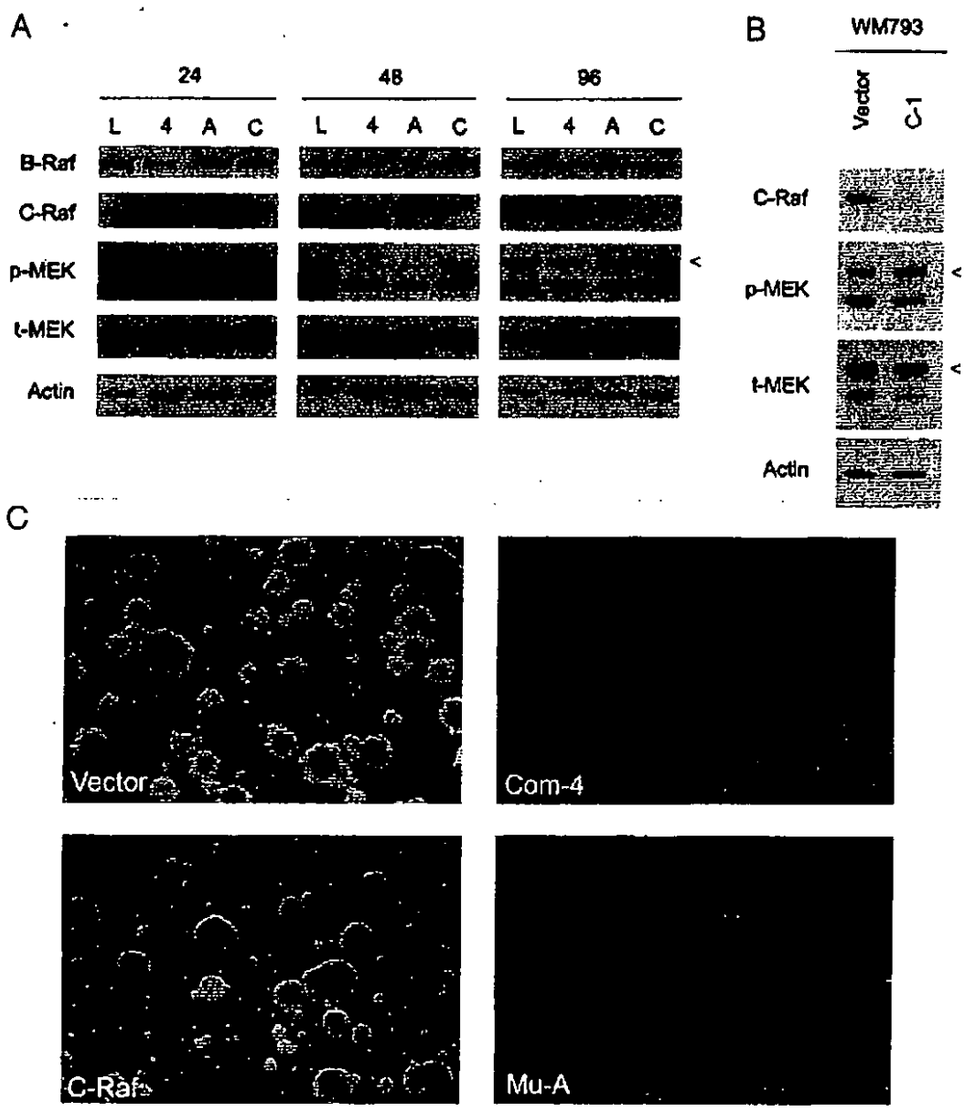

Fig. 4. Effects of transient and stable suppression of *BRAF* or *CRAF* expression in WM793 cells. In *A*, WM793 cells were transfected with siRNA directed against *Lamin A/C* (*L*), *BRAF* (*4*), *BRAF*^{V599E} (*A*), and *CRAF* (*C*) and analyzed for specific protein expression at 24, 48, and 96 h post-transfection. In *B*, WM793 cells were stably transfected with pSUPER.retro (vector) or shRNA directed against *CRAF* (C-1), subjected to antibiotic selection, and assessed for knockdown of C-Raf, activated MEK (*p-MEK*), total MEK (*t-MEK*), and actin levels. The positions of the *M*, 45,000 phospho- and total-MEK 1/2 proteins are indicated. In *C*, WM793 cells were stably transfected with pSUPER.retro, Com-4 shRNA against BRAF, C-1 shRNA against C-Raf, or Mu-A shRNA against *BRAF*^{V599E} and plated onto semisolid media. Colonies were counted and photographed after 30 days of growth (magnification: ×40). These results are representative of two independent experiments.

nous leukemia or advanced gastrointestinal stromal tumors with imatinib mesylate, a small molecule inhibitor of the *ABL* and *KIT* tyrosine kinases, respectively, induces dramatic remissions with minimal toxicity (16, 17). Importantly, the responses of chronic myelogenous leukemia and gastrointestinal stromal tumor patients to imatinib correlates with the drug's ability to inhibit the kinase activities of Bcr-Abl and mutant c-kit (18, 19), respectively. These results suggest that essential pathways may exist in other malignancies and that biochemical confirmation of the effectiveness of molecularly targeted therapies may be predictive of clinical efficacy.

Our findings suggest that the *BRAF*^{V599E} mutation commonly found in malignant melanomas may represent a therapeutic target analogous to *BCR-ABL* and *KIT*. We have demonstrated here that knockdown of *BRAF* expression and inhibition of downstream signaling in WM793 human melanoma cells causes growth arrest and promotes apoptosis under adherent conditions, and prevents colony formation in suspension. These observations have been preliminarily extended to a second melanoma cell line known to contain the *BRAF*^{V599E} mutation (data not shown). These effects were specific to *BRAF*, as suppression of *CRAF* failed to inhibit downstream phosphorylation of MEK and did not appreciably alter the biological properties of these cells. Moreover, these effects were specific to melanoma cells, because human fibrosarcoma cells were impervious to suppression of *BRAF* expression.

Currently, a Raf kinase inhibitor, BAY 43–9006 (20), is undergoing worldwide clinical evaluation in Phase I and II trials in patients with a variety of malignancies, including melanoma. However, BAY 43–9006 inhibits both B-Raf and C-Raf kinase activities,[4] and any beneficial or adverse effects of treatment may therefore result from simultaneous inhibition of both kinases. Our results suggest that targeted inhibition of B-Raf specifically in such tumors may be equally efficacious and perhaps associated with less toxicity.

That *CRAF* expression was dispensable for the transformed phenotype in human melanoma cells was somewhat surprising. As the first of the three Raf family isoforms identified, a large body of evidence exists exploring the transforming properties of *CRAF* in mammalian cell systems. These properties, however, are exquisitely dependent on cellular context (7); for example, although a constitutively active form of *CRAF* can readily transform NIH 3T3 cells, it is unable to do so in RIE-1 cells (21). More recent experiments involving targeted disruption and mutation of Raf isoforms in mice implicate B-Raf as the more potent activator of MEK in many cell and tissue types (22). The minimal effects on transformation of *CRAF* suppression in the melanoma cells studied here suggest that this isoform may not always be the predominant effector of MAPK signaling in human cells either.

In summary, we find that suppression of *BRAF*^{V599E} in WM793 human melanoma cells abrogates their transformed phenotype and conclude, therefore, that agents that specifically inhibit activated *BRAF*, and not *CRAF*, might be particularly efficacious in melanomas, and perhaps other tumor types, that harbor activating mutations in this proto-oncogene.

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Review article

The protein kinase B/Akt signalling pathway in human malignancy

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Abstract

Protein kinase B or Akt (PKB/Akt) is a serine/threonine kinase, which in mammals comprises three highly homologous members known as PKBα (Akt1), PKBβ (Akt2), and PKBγ (Akt3). PKB/Akt is activated in cells exposed to diverse stimuli such as hormones, growth factors, and extracellular matrix components. The activation mechanism remains to be fully characterised but occurs downstream of phosphoinositide 3-kinase (PI-3K). PI-3K generates phosphatidylinositol-3,4,5-trisphosphate (PIP_3), a lipid second messenger essential for the translocation of PKB/Akt to the plasma membrane where it is phosphorylated and activated by phosphoinositide-dependent kinase-1 (PDK-1) and possibly other kinases. PKB/Akt phosphorylates and regulates the function of many cellular proteins involved in processes that include metabolism, apoptosis, and proliferation. Recent evidence indicates that PKB/Akt is frequently constitutively active in many types of human cancer. Constitutive PKB/Akt activation can occur due to amplification of *PKB/Akt* genes or as a result of mutations in components of the signalling pathway that activates PKB/Akt. Although the mechanisms have not yet been fully characterised, constitutive PKB/Akt signalling is believed to promote proliferation and increased cell survival and thereby contributing to cancer progression. This review surveys recent developments in understanding the mechanisms and consequences of PKB/Akt activation in human malignancy. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Akt; Protein kinase B; Apoptosis; Tumourigenesis; Proliferation

1. Introduction

During cancer development, tumour cells acquire a number of phenotypic characteristics that allow them to proliferate both rapidly and limitlessly, invade the surrounding tissue, survive without their normal microenvironment, and finally, metastasise to secondary sites [1]. These features are usually acquired progressively over a protracted period of time as a result of increasing genomic instability that leads to up-regulation of oncogenes and down-regulation of tumour suppressor genes. As a result of intensive worldwide research activity in the last few years, the major signalling pathways that are altered during tumourigenesis, and how these pathways link to dysregulated processes such as proliferation and survival, are being elucidated. The serine/threonine protein kinase, protein kinase B or Akt (PKB/Akt), has emerged as a crucial regulator of widely divergent cellular processes including apoptosis, proliferation, differentiation, and metabolism. Disruption of normal PKB/Akt signalling has now been documented as a frequent occurrence in several human cancers and the enzyme appears to play an important role in their progression. The main purpose of this review is to discuss potential mechanisms by which PKB/Akt signalling could contribute to cancer progression. Several recent reviews contain detailed descriptions of PKB/Akt structure and its molecular mechanism of activation [2–4] and these aspects will be covered only briefly here.

2. Structural features of PKB/Akt proteins

The PKB/Akt story began with the isolation of two genes, named *Akt1* and *Akt2*, which were identified as human homologues of the viral oncogene *v-akt*, previously known to cause a form of leukaemia in mice [5]. Subsequently, three independent studies revealed that *v-akt* and its mammalian homologues encoded a protein kinase with some similarities to protein kinase C (PKC) and protein kinase A (PKA) [6–8]. Its relatedness to PKA and PKC led to it being named PKB by the authors of one of these

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studies. To date, three members of the family have been isolated and these are now referred to as PKBα (or Akt1), PKBβ (Akt2), and PKBγ (Akt3). They are products of distinct genes but are highly related, exhibiting greater than 80% homology at the amino acid level. The three genes are expressed differentially, with PKBα/Akt1 and PKBβ/Akt2 displaying fairly broad and PKBγ/Akt3 more restricted tissue distribution. The major structural features of the PKB/Akt proteins are illustrated in Fig. 1. Each isoform possesses an N-terminal pleckstrin homology (PH) domain of approximately 100 amino acids. Recent detailed structural examination of PKB/Akt PH domains reveals similarity to PH domains found in other signalling molecules that bind 3-phosphoinositides [9,10]. This, together with evidence from earlier in vitro studies, indicates that the PH domain mediates binding of PKB/Akt to 3-phosphoinositides (see Section 3.1). The PH domain is followed by the kinase catalytic domain, which shows a high degree of similarity to those found in PKA and PKC [11,12]. Also present in this region is a threonine residue (T308 in PKBα/Akt1) whose phosphorylation is necessary for activation of PKB/Akt. Following the kinase domain is a hydrophobic C-terminal tail containing a second regulatory phosphorylation site (S473 in PKBα/Akt1). Phosphorylation at T308 and S473 occurs in response to growth factors and other extracellular stimuli and is essential for maximal PKB/Akt activation [13] (see Section 3.1). PKB/Akt may also be phosphorylated on S124 and T450 but neither of these sites appears to regulate PKB/Akt activity and their phosphorylation does not change following cell stimulation [13].

Splice variants of PKBγ/Akt3 lacking the Ser472 phosphorylation site have been identified [14,15]. The human variant, PKBγ1, appears to be regulated differently from that of full-length PKBγ/Akt3 in a manner that suggests that the absence of the second phosphorylation site limits its maximal potential for both membrane translocation and catalytic activation [15].

3. Regulation of PKB/Akt activity

3.1. Phosphoinositide 3-kinase (PI-3K)-dependent activation of PKB/Akt

Although originally suspected of being involved in the regulation of cell growth, its central role in signalling only became apparent when PKB/Akt was shown to be a downstream target for PI-3K [16,17]. The cellular activation of PKB/Akt is dependent upon the generation of inositol-containing membrane lipids phosphorylated by PI-3K at the D3-OH group on the inositol ring. A number of studies have documented PI-3K-independent activation of PKB/Akt in various cell systems (see Ref. [4]) but, as the physiological significance of these findings remains uncertain, they will not be discussed further here.

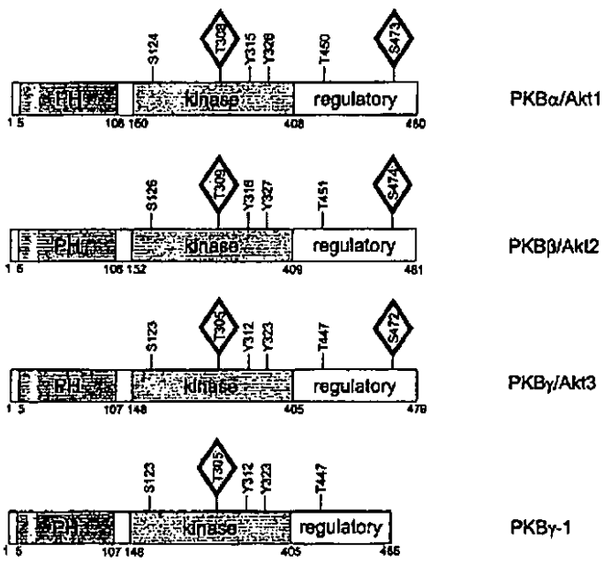

Fig. 1. Domain structure of the three human PKB/Akt isoforms and the PKBγ/Akt3 splice variant. Each isoform consists of an N-terminal PH domain containing a region (shown in hatched bars) for binding inositol phospholipids, a kinase domain, and a C-terminal regulatory domain. Residues contained within diamonds are serine and threonine sites inducibly phosphorylated in response to various cell stimuli. Other residues in PKBα/Akt1 known to be phosphorylated constitutively but not thought to regulate catalytic activity are also highlighted. Tyrosine residues Y315 and Y326 have recently been described as having a potential role in the regulation of PKBα/Akt1 activity. The equivalent phosphorylation sites in PKBβ/Akt2, PKBγ/Akt3, and PKBγ1 have been obtained from sequence databases. Further details are given in Section 2.

PKB/Akt is activated downstream of Class 1_A and Class 1_B PI-3K, which are activated by tyrosine kinase and G-protein-coupled receptors, respectively. Following its recruitment to these receptors in the plasma membrane, PI-3K is activated and phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) on the 3-OH group generating the second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP_3). PIP_3 levels are tightly regulated by the action of phosphatases such as PTEN, which removes phosphate from the 3-OH position, and SHIP, which dephosphorylates at the 5-OH position (see Section 4.4). PIP_3 does not activate PKB/Akt directly but instead appears to recruit PKB/Akt to the plasma membrane and to alter its conformation to allow subsequent phosphorylation by the phosphoinositide-dependent kinase-1 (PDK-1) (Fig. 2).

PDK-1 is a 63-kDa serine/threonine kinase containing a C-terminal PH domain that binds with high affinity to 3-phosphoinositides. PDK-1 phosphorylates PKB/Akt in the activation loop, which regulates access to the catalytic site of PKB/Akt. Phosphorylation of this site (T308 in PKBα/Akt1) in vitro is enhanced by 3-phosphoinositides and it has been suggested that the lipids induce both a favourable conformation of PKB/Akt (and possibly PDK-1) allowing access to the acceptor phosphorylation site and also colocalisation of the two proteins in the lipid microenvironment [18,19].

Although phosphorylation at T308 partially activates PKB/Akt [13], full activation of PKB/Akt requires phosphorylation on a second site (S473 in PKBα/Akt1) located in the regulatory tail. The mechanism mediating S473 phosphorylation remains controversial. Balendran et al. [20] showed that, following phosphorylation of PKB/Akt at T308, PDK-1 converts to a S473 kinase as a result of its interaction with the singly phosphorylated PKB/Akt and a fragment of a protein known as PRK-2. Modification of S473 has also been shown to occur through autophosphorylation [21]. Other findings suggest that S473 is modified by a distinct kinase or PDK-2. Although in most situations phosphorylation of PKB/Akt at S473 in intact cells occurs in tandem with that at T308, a number of studies have shown that phosphorylation at the two sites can occur independently [13,22]. Furthermore, PDK-1 null embryonic stem cells maintain the ability to undergo S473 phosphorylation [23] and a recent study showed that S473 phosphorylation could be stimulated by insulin in the absence of T308 phosphorylation and PKB/Akt activation, indicating the existence of a ligand-activatable PDK-2 [24]. The integrin-linked kinase (ILK) was shown to phosphorylate S473 [25] but a subsequent report suggested that ILK acts only as a facilitator and does not phosphorylate PKB/Akt directly [26]. A more recent study showed that ILK could phosphorylate PKB/Akt on S473 and that the kinase activity of ILK was essential for S473 phosphorylation in cells. A novel ILK-specific inhibitor also blocked S473 phosphorylation of PKB/Akt [27]. These studies strongly suggest that ILK plays some role in the activation process but whether it phosphorylates PKB/Akt directly remains an open question. Clearly, the regulation of PKB/Akt is complex and, at this stage, not fully understood. Although there is evidence for independent regulation of S473, because phosphorylation at this site alone has not been shown to increase PKB/Akt activity, the physiological significance of these findings is unclear.

Finally, a recent report documented evidence that PKB/Akt activation requires the phosphorylation of two tyrosine residues in the activation loop (Y315 and Y326 in PKBα/Akt1) [28]. These modifications were shown to be dependent on src family tyrosine kinases. If confirmed then these findings add further to the complexity of PKB/Akt regulation and could be particularly relevant in cancer cells where src family kinases are frequently overexpressed.

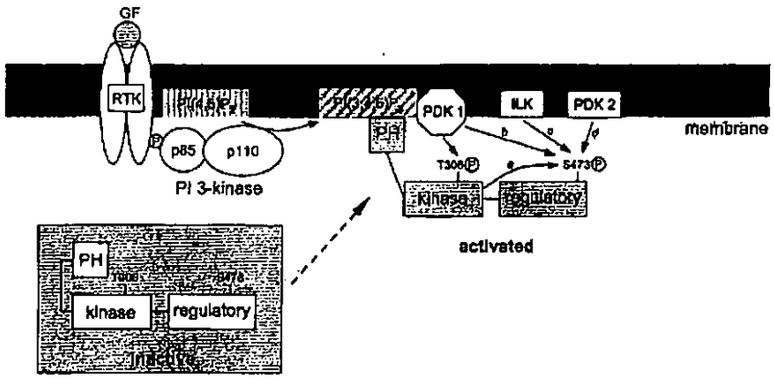

Fig. 2. Mechanism of activation of PKB/Akt. In unstimulated cells PKB/Akt is not phosphorylated on T308 or S473 and resides mainly in the cytosol. Following growth factor (GF) activation of receptor tyrosine kinases (RTKs, or other cell surface receptors, not shown), PI-3K is recruited to the receptor and activated resulting in the production of PIP_3. This recruits PKB/Akt to the membrane where it is phosphorylated on T308 within the catalytic domain by PDK-1 and on S473 within the regulatory domain by an ill-defined mechanism, possibly involving (a) autophosphorylation, (b) PDK-1, (c) ILK, or (d) an unidentified PDK-2. PKB/Akt is then released from the membrane and translocates to other subcellular compartments. Further details are given in Section 3.1.

3.2. Regulation of PKB/Akt dephosphorylation

Theoretically, the activation status of PKB/Akt in cells will depend upon the balance between the "on" signals generated by elevated PIP_3 levels and the influence of "off" signals that lead to PKB/Akt dephosphorylation. Several lines of evidence indicate that PKB/Akt phosphatases participate in both the positive and negative regulation of PKB/Akt. PKB/Akt can be activated by treating cells with inhibitors of protein serine/threonine phosphatases such as okadaic acid [29]. Since activation of PKB/Akt by okadaic acid was reported to be resistant to inhibition of PI-3K [29], a protein phosphatase, possibly PP2A, may act to constrain PKB/Akt in a dephosphorylated inactive state in resting cells. More recent studies suggest that additional interacting proteins, such as the 90-kDa heat-shock protein Hsp90, are necessary to prevent phosphatase access during the activation of PKB/Akt [30].

Attenuation of PI-3K activation leads to a rapid dephosphorylation at S473 and (more slowly) T308, accompanied by loss of PKB/Akt activity, indicating that PIP_3 constrains phosphatase action as well as promotes PDK-1 activation [31]. The inactivation of PKB/Akt by ceramide [32] and osmotic stress [33,34] occurs predominantly via S473 dephosphorylation by an okadaic acid-sensitive phosphatase. Dephosphorylation of T308 appears to occur independently [32] and a recent study suggests, controversially, that PDK-1 participates in the dephosphorylation of T308 by an unknown mechanism [35].

4. PKB/Akt in human cancer

4.1. Gene amplification

As noted above, Akt was first identified as a component of a fusion product of the retroviral oncogene *v-akt* that causes leukaemia in mice [6]. No modified or mutated *Akt* genes have been found in mammals. However, a number of studies have discovered *PKB/Akt* gene amplifications in human cancers. The work that originally identified *Akt* as a potential human oncogene detected amplification of *PKBα/Akt1* in a single gastric carcinoma [5]. *PKBβ/Akt2* gene amplification has been found in ovarian, pancreatic, gastric, and breast tumours [36,37]. *PKBβ/Akt2* amplification was particularly associated with high-grade aggressive ovarian tumours and appears to occur as part of the frequent amplification of the 19q13.1–q13.2 chromosomal region [38]. One study documented PKBγ/Akt3 mRNA overexpression and selective activation of the protein by growth factors in hormone-independent breast and prostate cancer cell lines [39]. Overall, these studies indicate that *PKB/Akt* gene amplification, particularly *PKBβ/Akt2*, may be a frequent occurrence in several human cancers. However, although one study reported increased PKBβ/Akt2 protein expression in pancreatic tumours with corresponding *PKBβ/Akt2* gene amplification [40], there remains little documentation of PKB/Akt protein levels and activation in clinical material.

4.2. PI-3K gene amplification

The *PIK3CA* gene, which encodes the p110α catalytic subunit of PI-3K, is located on chromosome 3q26, a region that is frequently amplified in a number of human cancers. Recent studies have confirmed amplification of *PIK3CA* in ovarian [41] and cervical [42] tumours. Furthermore, corresponding cell lines harbouring this alteration display enhanced PI-3K catalytic activity and growth that is strongly suppressed by PI-3K inhibitors, suggesting that PI-3K is oncogenic at least in these tumour types.

4.3. Activation of upstream regulators of PI-3K

PI-3K is activated as a result of the ligand-dependent activation of tyrosine kinase receptors, G-protein-coupled receptors, or integrins. Receptor-independent activation can also occur, for example, in cells expressing constitutively active Ras proteins [43,44]. As cell surface receptors are commonly overexpressed or constitutively active in many human cancers (see Ref. [45]), downstream signalling pathways are often activated as a result. One of the most extensively studied examples is the erbB2 tyrosine kinase receptor, which is overexpressed as a result of gene amplification in a large number of breast and other cancers (see Ref. [46]). Although erbB2 overexpression is associated with particularly aggressive disease and poor patient prognosis, studies with transgenic mice indicate that erbB2 by itself may be insufficient for transformation [47]. ErbB2 is an orphan receptor with no defined ligand, which instead acts as a dimerisation partner for other members of the erbB family. ErbB2-containing heterodimers are potent activators of multiple signalling pathways involved in proliferation, invasion, and survival [48]. Studies in breast cancer cells, primary breast tumours, and transgenic mice all indicate that erbB2, when overexpressed, is constitutively associated with erbB3 [47]. Because erbB3 possesses seven phosphorylatable tyrosine residues that act as binding sites for the SH2 domains of the p85 regulatory subunit of PI-3K [49], erbB2–erbB3 dimers strongly activate the PI-3K–PKB/Akt pathway. This provides a basis for data showing that tumour cells overexpressing erbB2 display constitutive PKB/Akt activity [50]. Indeed, recent data indicate that the PKB/Akt pathway may play a major role in stimulating proliferation and survival in erbB2-overexpressing cells (see Section 5.2).

4.4. PIP_3 phosphatases

PTEN (also known as MMAC1 or TEP1) is a dual-function lipid and protein phosphatase that was originally identified as a tumour suppressor gene frequently mutated in

the advanced stages of a number of human cancers, particularly glioblastoma, endometrial, and prostate cancers. In addition, germline mutations in PTEN give rise to the rare autosomal dominant inherited human cancer syndrome known as Cowden's disease, which is associated with increased risk of developing breast and other cancers (see Ref. [51]). The results of studies in which PTEN has been overexpressed in various cell lines suggest that PTEN acts as a tumour suppressor by inhibiting cell growth [52] and increasing susceptibility to apoptosis and anoikis [53]. The main physiological lipid substrate for PTEN is PIP_3, the product of PI-3K. PTEN dephosphorylates PIP_3 at the D3 position and thus acts as a negative regulator of PI-3K signalling. Indeed, PTEN-null embryonic fibroblasts display elevated PIP_3 levels and constitutive PKB/Akt activity, indicating that PTEN acts to constrain the pathway in unstimulated cells [54]. Absence of PTEN also strongly correlates with activation of PKB/Akt in tumour cell lines [55,56]. Conversely, reexpression of PTEN in cells lacking PTEN down-regulates PKB/Akt phosphorylation [57] as well as reversing the phosphorylation of PKB/Akt cellular substrates such as BAD [58] (see Section 5.1.2). Although PTEN is believed to negatively regulate PKB/Akt activation primarily through PIP_3 dephosphorylation, recent studies indicate additional mechanisms. PTEN expresses protein phosphatase activity against a number of substrates and recently was shown to dephosphorylate ILK [59], which is implicated in the mechanism through which PKB/Akt is activated (see Section 4.1).

Because PTEN, through both its lipid and protein phosphatase activities, probably regulates a number of signalling pathways, an important question is to what extent does the PKB/Akt activation seen in PTEN-deficient cells contribute to increased growth and resistance to apoptosis? This question has recently been addressed in a study by Weng et al. [60], who showed that the antigrowth effects of PTEN are mediated by both PKB/Akt-dependent and -independent pathways, whereas the proapoptotic effects of PTEN are mostly PKB/Akt-dependent. The mechanisms by which PKB/Akt mediates PTEN-induced growth arrest and apoptosis remain unclear but a recent study demonstrated a major role for forkhead transcription factors (see Section 5.1.1) [61].

Although PTEN, because of its frequent alteration in human cancers, has attracted most interest, it is clear from recent work that cells possess additional mechanisms for down-regulating PIP_3 signalling. SHIPs are SH2-domain-containing inositol phosphatases that dephosphorylate PIP_3 at the 5′ position (see Ref. [62]). Two forms of SHIP have been identified to date; SHIP is expressed predominantly in haematopoietic cells, whereas SHIP-2 is more widely expressed. Recent studies have implicated SHIPs as crucial negative regulators of myeloid cell activation [63,64] and glioblastoma cell proliferation [65]. In both cases, the effects of SHIP have been linked to inactivation of PKB/Akt. Although there are no reports documenting loss of SHIP or SHIP-2 in human tumours, the above studies indicate that such alterations could have a major impact on PKB/Akt signalling and should be investigated.

5. Cellular processes regulated by PKB/Akt

The number of identified physiological PKB/Akt substrates is expanding rapidly. Predicting whether a protein is phosphorylated by PKB/Akt has been aided by studies defining a minimal consensus peptide sequence Arg-Xaa-Arg-Xaa-Xaa-[Ser/Thr]-Hyd (where Xaa is any amino acid and Hyd is a bulky hydrophobic amino acid) for phosphorylation by PKB/Akt [66,67]. A survey of the Swiss-Prot database in February 2001 using Scansite identified this sequence 551 times in 446 protein entries. Clearly, there must be additional higher-order structural requirements for PKB/Akt recognition and phosphorylation of substrates in vivo. Subcellular location is also important. In resting non-stimulated cells, the majority of PKB/Akt resides in the cytoplasm. Activation of PKB/Akt occurs at the plasma membrane and has been shown in several studies to be followed by its translocation to both the cytosol and nucleus [68–72]. It is noteworthy that many of the substrates of PKB/Akt are proteins that function in the nucleus (see below). A list of proteins, suggested by studies in intact cells to be physiological substrates of PKB/Akt, is shown in Table 1. In most cases, the functional consequences of PKB/Akt phosphorylation have been defined. Linking this information with the known cellular functions of some of these proteins has led to conclusions that PKB/Akt regulates apoptosis, proliferation, and other processes. In this section, we discuss recent progress in this area with an emphasis on how disrupted PKB/Akt signalling may affect the phenotypic behaviour of cancer cells.

5.1. Involvement of PKB/Akt in prosurvival and antiapoptotic mechanisms

The intrinsic capacity of all cells to undergo apoptosis is suppressed by survival signals induced by factors within their immediate microenvironment. Studies conducted over the last 4–5 years conclusively show that PKB/Akt is critical for cell survival triggered by growth factors, extracellular matrix, and other stimuli (see Ref. [3]). For example, dominant negative alleles of PKB/Akt reduce the ability of growth factors and other stimuli to maintain cell survival whereas overexpression of wild type or activated Akt can rescue cells from apoptosis induced by various stress signals [44,73–75]. Although there can be little doubt that PKB/Akt promotes cell survival, the mechanisms involved have only recently begun to emerge. Progress in this direction has come mainly through the identification of PKB/Akt substrates, that either participate directly in the apoptotic cascade or regulate the transcription of pro- and antiapoptotic genes. Targets of PKB/Akt

Table 1
PKB/Akt substrates

Substrate	Phosphorylation site	Effect	References
Prosurvival			
ASK1	Ser83	inhibition of stress-activated kinases	[107]
BAD	Ser136	association of BAD with 14-3-3 proteins; suppression of BAD-induced cell death	[94,95,190]
CREB	Ser133	increased transcription of CREB-regulated survival genes	[90]
Forkhead family (FKHR, FKHRL1, AFX)	Thr24, Ser256, Ser319 (FKHR)	promotes nuclear exclusion; association with 14-3-3 proteins; prevention of transcription of proapoptotic genes	[76–79,191,192]
	Thr32, Ser253, Ser315 (FKHRL1)		
	Thr28, Ser193, Ser258 (AFX)		
IκB kinase	Thr23	induction of NF-κB transcriptional activity	[86,87]
Procaspase-9	Ser196	suppression of caspase-9-induced cell death	[108]
Cell cycle progression			
GSK-3-α, -β	Ser21 (α), Ser9 (β)	inhibition of GSK-3 catalytic activity	[118]
mTOR/FRAP	Thr2446, Ser2448	modulation of mRNA translation?	[135,136]
$p21^{WAF1}$	Thr145	cell cycle progression	[141,142]
Others			
AR	Ser210, Ser790	decreased transcription of AR-regulated genes; modulation of AR-mediated apoptosis	[169]
BRCA1	Thr509	nucleocytoplasmic localisation?	[180]
ER-α	Ser167	increased transcription of ER-regulated genes	[168]
eNOS	Ser1177	activation of eNOS; production of nitric oxide	[155–157]
Nur77	Ser350	reduced transcriptional activity	[193]
c-Raf	Ser259	inhibition of c-Raf signalling	[194]
B-Raf	Ser364, Ser428	inhibition of B-Raf activity	[195]
Telomerase reverse transcriptase subunit	Ser227, Ser824	enhanced telomerase activity	[174]

signalling that are believed to promote cell survival are illustrated in Fig. 3.

5.1.1. Transcriptional regulation of pro- and antiapoptotic genes

One means by which PKB/Akt may promote cell survival is by directly phosphorylating transcription factors that control the expression of pro- and antiapoptotic genes. PKB/Akt appears to both negatively regulate factors that promote the expression of death genes and positively regulate factors that induce survival genes. An example of the former is the forkhead family of transcription factors. The three identified mammalian members of the forkhead family, FKHR, FKHRL1, and AFX, all contain consensus PKB/Akt phosphorylation sequences, which can be effectively phosphorylated by PKB/Akt in vitro [76–78]. Other studies have shown that stimulation of cells with various growth factors leads to forkhead protein phosphorylation via the PKB/Akt pathway [79,80]. Phosphorylation of forkhead proteins by PKB/Akt appears to alter their subcellular location. In the absence of PKB/Akt activation, forkhead proteins reside predominantly in the nucleus where they are able to promote transcription of proapoptotic target genes such as *Fas-L*, *IGFBP1*, and *Bim*, through specific DNA elements in their promoters [77,81]. Stimulation of cells with factors that increase PKB/Akt activity leads to the export of FKHRL1 from the nucleus [76] and its accumulation and sequestration by 14-3-3 proteins in the cytoplasm [77]. The role of 14-3-3 proteins in this process is analogous to their participation in the sequestration of BAD following its phosphorylation by PKB/Akt (see Section 5.1.2). 14-3-3 proteins may therefore have a more generalised role in removing phosphorylated PKB/Akt substrate proteins from their site of action. Mutants of FKHRL1 that cannot be phosphorylated by PKB/Akt do not undergo nuclear export [77]. Recent studies on AFX indicate that although its phosphorylation by PKB/Akt occurs primarily in the nucleus, the phosphorylation has no effect on nuclear export but instead blocks reentry to the nucleus [82] and thereby affects the steady-state distribution of AFX between the two compartments. Thus, modification of AFX and perhaps other forkhead family proteins by PKB/Akt would appear to require translocation of active PKB/Akt to the nucleus. As already mentioned, nuclear translocation of PKB/Akt can be demonstrated in cells treated with growth factors but may only occur as a result of sustained PKB/Akt activation. Assessment of forkhead protein location in cancer cells with constitutive PKB/Akt activity may indicate whether this pathway is important for cell survival in cancer. It is worth noting that, although PKB/Akt appears to play a major role in forkhead phosphorylation, another PI-3K-dependent protein kinase, known as serum and glucocorticoid-regulated kinase (SGK), also phosphorylates these proteins [83]. Indeed, as the patterns of FKHRL1 phosphorylation induced

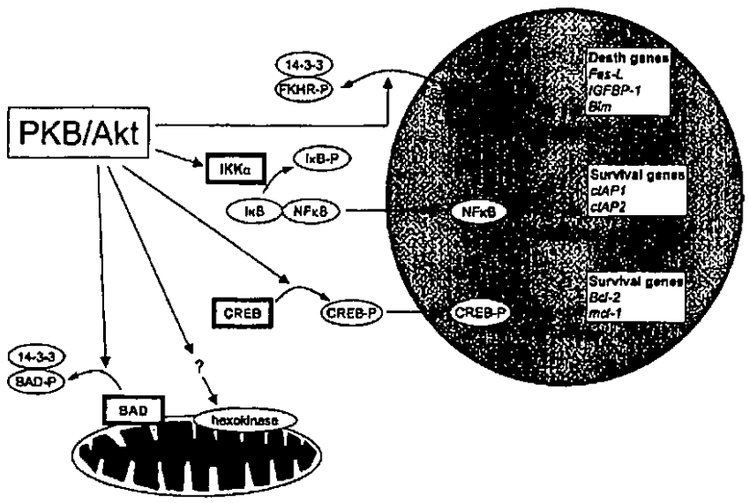

Fig. 3. Regulation of cell survival by PKB/Akt. PKB/Akt promotes cell survival by multiple mechanisms: (1) decreasing the transcription of death genes by phosphorylating forkhead family transcription factors such as FKHR, which promotes their sequestration by 14-3-3 proteins in the cytoplasm, (2) increasing the transcription of survival genes by activating NF-κB and CREB transcription factors, (3) phosphorylating and inactivating the proapoptotic protein BAD, and (4) maintaining mitochondrial integrity by activating hexokinase. The direct PKB/Akt substrates that mediate these events are represented as black boxes. Although substrates are placed in particular subcellular localisations, in most cases, the location in which PKB/Akt phosphorylation takes place is uncertain. Further details are given in Section 5.1.

by the two kinases are distinct, it may be that modification of FKHRL1 function requires the coordinated input from both kinases [83].

A recent study reported a novel function for FKHR as a coregulator of certain steroid receptors including the estrogen receptor (ER) [84]. Intriguingly, overexpression of FKHR inhibited estrogen-driven proliferation in breast cancer cells. Although not tested, one would predict that PKB/Akt signalling could also affect this nuclear function of FKHR by inducing its redistribution to the cytoplasm. This in turn may have major implications for estrogen-driven growth in breast cancer cells.

In addition to negatively regulating forkhead activity, PKB/Akt appears to positively regulate at least two other transcription factors. NF-κB is involved in the regulation of cell proliferation, apoptosis, and survival by a wide range of cytokines and growth factors. Numerous studies also indicate that it is critical in tumourigenesis (see Ref. [85]). Its survival-promoting activity is mediated through its ability to induce prosurvival genes such as *c-IAP-1* and *c-IAP-2*. NF-κB is regulated through its association with an inhibitory cofactor I-κB, which sequesters NF-κB in the cytoplasm. Phosphorylation of I-κB by upstream kinases, known as IKKs, promotes its degradation allowing NF-κB to translocate to the nucleus and induce target genes (Fig. 3). PKB/Akt has been shown to interact with and activate IKKα [86]. Data suggest that PKB/Akt phosphorylates IKKα directly but more importantly PKB/Akt is believed to be essential for IKK-mediated destruction of I-κB and activation of NF-κB [86,87]. These findings indicate that PKB/Akt is a critical regulator of NF-κB-dependent gene transcription and may play a role in promoting survival in cancer cells.

The PKB/Akt pathway has been shown in some cell types to increase expression of the antiapoptotic gene *bcl-2* [88,89]. Induction of *bcl-2* promoter activity by IGF-1 was shown to occur via a PKB/Akt pathway involving the cyclic AMP (cAMP)-response element binding protein (CREB) transcription factor. CREB is a direct target for phosphorylation by PKB/Akt [90] and this phosphorylation occurs on a site that increases binding of CREB to accessory proteins necessary for induction of genes containing cAMP responsive elements (CREs) in their promoter regions. CREB has also been shown to mediate PKB/Akt-induced expression of another antiapoptotic gene *mcl-1* [91].

Finally, a recent report [92] indicates a role for PKB/Akt in regulating the expression of c-FLIP, a caspase-8 homologue that acts as a dominant negative inhibitor of TNF receptor family-induced apoptosis and is commonly found at high levels in tumours. The mechanism by which PKB/Akt induces c-FLIP has not been characterised but work by another group showing that NF-κB promotes c-FLIP expression [93] suggests that regulation of this transcription factor by PKB/Akt may be critical.

5.1.2. Direct phosphorylation of proapoptotic proteins

In addition to influencing the transcription of pro- and antiapoptotic genes, numerous studies indicate that PKB/Akt promotes survival by directly phosphorylating key regulators of the apoptotic cascade. The most widely studied

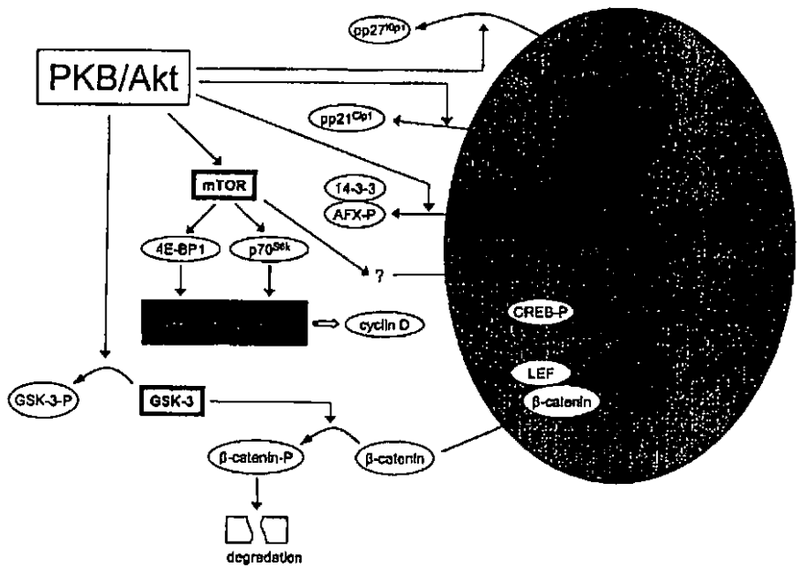

Fig. 4. Regulation of cell cycle progression by PKB/Akt. PKB/Akt promotes cell cycle progression by multiple mechanisms: (1) phosphorylating CDK inhibitory proteins $p21^{CIP1}$ and $p27^{KIP1}$ causing their cytoplasmic accumulation, (2) decreasing $p27^{KIP1}$ transcription by phosphorylating and negatively regulating the forkhead family transcription factor AFX, (3) increasing cyclin D transcription by stabilising β-catenin through the GSK-3 pathway or by activating CREB transcription factor downstream of mTOR/FRAP, and (4) increasing cyclin D mRNA translation. Direct PKB/Akt substrates that mediate these events are represented in black boxes. The subcellular location in which PKB/Akt phosphorylates particular substrates is in most cases uncertain. Further details are given in Section 5.2.

example of this type of regulation involves BAD, a member of the Bcl-2 family, which promotes apoptosis by binding to and antagonising the actions of prosurvival members of the family such as Bcl-2 and Bcl-X_L. PKB/Akt can phosphorylate BAD at residue S136 and this modification promotes the sequestration of BAD by 14-3-3 proteins in the cytosol, thus preventing BAD from interacting with Bcl-2 or Bcl-X_L at the mitochondrial membrane [94,95]. PKB/Akt-induced phosphorylation of BAD may also occur indirectly, through intervening protein kinases such as Raf-1 [96] and $p65^{PAK}$ [97,98]. Although these and other studies indicate that PKB/Akt-dependent phosphorylation of BAD is linked to the promotion of cell survival in some cellular contexts, this mechanism cannot operate universally since BAD is not expressed in all cell types [99]. In addition, more recent studies indicate that phosphorylation of BAD in some cells occurs via PKB/Akt-independent pathways [98,100–105]. Moreover, blockade of PKB/Akt signalling has no effect on BAD phosphorylation or survival in some cell types [100]. Currently, there is little evidence concerning the importance of PKB/Akt-mediated BAD phosphorylation in promoting cell survival or resistance to apoptotic stimuli in cancer cells. The expression of BAD varies widely among tumour cell lines and, although in lines where its expression is high BAD is often constitutively phosphorylated [99], there is no information on whether this is caused by PKB/Akt.

Stress-activated protein kinases (SAPKs) such as JNK are critically involved in the induction of apoptosis following exposure of cells to stimuli such as ionising radiation, heat shock, or osmotic stress (see Ref. [106]). A recent report has provided the first evidence that PKB/Akt may interfere with SAPK signalling and thereby inhibit apoptosis. PKB/Akt was shown to phosphorylate and inactivate ASK1, a kinase that transduces stress signals to the JNK and p38 MAP kinase pathways [107]. This type of cross-talk between survival and apoptotic signalling pathways could be important in malignant transformation, since it is believed that tumour cells must evolve mechanisms to suppress signals that lead to apoptosis [1].

To this point all of the PKB/Akt targets mentioned regulate apoptosis prior to the release of cytochrome *c* from the mitochondria and activation of the caspase cascade that characterises the terminal execution phase of apoptosis. However, data suggesting that PKB/Akt may also influence postmitochondrial events have also been reported. Procaspase-9, the initiator caspase in the caspase cascade, was shown to be a substrate for PKB/Akt [108]. In these experiments, phosphorylation of human procaspase-9 by PKB/Akt blocked its intrinsic protease activity and was shown to be critical for overall regulation of the apoptotic process. In a more recent study by Zhou et al. [109], exogenous PKB/Akt was shown to inhibit the activation of caspases-9 and -3 induced by cytochrome *c* in a cell-free system. However, these authors reported that procaspase-9 was not a direct substrate for PKB/Akt, indicating that PKB/

Akt inhibits caspase activation by modifying an unknown cytosolic factor.

5.2. Cell cycle progression

Early studies showing stimulation of PKB/Akt activity by mitogenic growth factors suggested that PKB/Akt was involved in cell cycle progression. The first direct evidence supporting this idea came from a study showing that overexpression of PKBβ/Akt2 accelerated cell cycle progression and caused transformation in murine fibroblasts [110]. More recent findings support a role for PKB/Akt during G1-S phase progression. Transition of cells through the G1/S checkpoint is regulated by retinoblastoma protein (pRB), which suppresses the transcription of a battery of genes required for G1/S traverse. pRB is phosphorylated and inactivated by cyclin-dependent kinases (CDKs), which, as their name indicates, require cyclins for activation. CDK4, which is dependent on D-type cyclins, is particularly important for pRB phosphorylation in late G1. CDKs are also negatively regulated by a family of inhibitory proteins that include $p21^{CIP1}$ and $p27^{KIP1}$ (see Ref. [111]). A study by Brennan et al. [112] in T cells first indicated that PKB/Akt was involved in the regulation of pRB phosphorylation. More recent evidence indicates that PKB/Akt signalling may do this by controlling the expression or activity of cyclin D, $p27^{KIP1}$ and $p21^{CIP1}$ (Fig. 4).

5.2.1. Regulation of cyclin D

Overexpression of cyclin D1 has been found in many human cancers, particularly breast cancer, where the protein is up-regulated in at least 50% of all cases [113]. Elevated cyclin D1 levels result in shortened cell cycle times and thereby contribute directly to tumour progression. Cyclin D1 expression is controlled via multiple coordinate signals that alter gene transcription, mRNA translation, and protein stability. Accumulating evidence suggests that PKB/Akt can regulate cyclin D1 at each of these levels [114–116].

5.2.1.1. Role of glycogen synthase kinase 3 (GSK-3). GSK-3 was originally characterised as an enzyme important in regulating glycogen synthesis in insulin-responsive tissues [117]. In order to promote glycogen synthesis, insulin causes inactivation of GSK-3 via the PKB/Akt pathway. Phosphorylation of GSK-3-α by PKB/Akt at residue S9 inactivates the enzyme [118] and recent studies have shown that PKB/Akt is essential for GSK-3 inactivation in intact cells [119]. GSK-3 is now known to play diverse roles in many cellular processes including differentiation, proliferation, and transformation (see Refs. [120,121]). Although the mechanisms by which GSK-3 participates in oncogenic transformation are incompletely understood, they are likely to include effects on cyclin D1. Firstly, GSK-3 has been shown to phosphorylate cyclin D1 on a site that promotes its degradation via the proteasome pathway [122], suggesting that PKB/Akt, by inactivating GSK-3, could stabilise cyclin D1 protein. Another target for GSK-3 phosphorylation is β-catenin. In its unphosphorylated state, β-catenin binds to and increases the activity of LEF transcription factors, one of whose gene targets is *cyclin D* [123]. Phosphorylation by GSK-3 increases the degradation of β-catenin via the ubiquitin–proteasome pathway [124] and thereby reduces LEF-mediated transcription. Recent data showing that β-catenin expression correlated with cyclin D levels in breast cancers suggest that this is an important regulatory system in these tumours [125]. In addition, β-catenin mutants that cannot be phosphorylated by GSK-3 are found in some cancers [126,127], supporting the idea that GSK-3 may act as a tumour suppressor by maintaining the turnover of β-catenin. Furthermore, GSK-3 and β-catenin exist in a multimeric complex with at least two other proteins, axin and APC, both of which are essential for GSK-3-mediated phosphorylation and degradation of β-catenin. Inactivating mutations in axin or APC are also strongly associated with tumourigenesis [128,129]. The negative regulation of β-catenin stability by GSK-3 implies that PKB/Akt, by inactivating GSK-3, should promote β-catenin stabilisation, and thereby increase the transcription of LEF gene targets, including *cyclin D1*. Recent studies indicate that such a pathway may operate downstream of the Wnt family of secreted growth factors, some of whose members are strongly implicated in tumourigenesis. PKB/Akt was shown in PC12 cells to participate in Wnt signalling to β-catenin by binding to a GSK-3–axin complex and causing GSK-3 phosphorylation [130]. Whether this is generalised phenomenon is still unclear since other reports argue against a link between PKB/Akt and β-catenin. Thus, activation of PKB/Akt did not alter β-catenin levels in fibroblasts [131] and PTEN was reported to regulate β-catenin stability and LEF activity in prostate cancer cells via a mechanism that did not involve a major contribution from PKB/Akt [132].

Finally, in discussing its potential role in regulating cell cycle progression, it should also be noted that recent studies indicate that GSK-3 inactivation may also be critical for cell survival induced by PKB/Akt [133]. These conclusions were drawn from studies showing that overexpression of GSK-3 caused apoptosis in Rat-1 or PC-12 cells. The substrates of GSK-3 that mediate its effects on apoptosis remain to be identified.

5.2.1.2. mTOR/FRAP. Mitogenic stimuli are known to increase the synthesis of a particular subset of proteins needed for cell growth and proliferation. This is achieved in part through signals that up-regulate the ribosomal translation of the corresponding mRNAs. Among the mRNA species whose translation is increased are c-Myc and cyclin D. The PI-3K-related protein kinase known as mTOR/FRAP plays a major role in regulating protein synthesis by phosphorylating substrates that control mRNA translation (see Ref. [134]). The regulation of mTOR/FRAP is both unclear and controversial. Evidence for an involvement of PKB/Akt in its regulation has come from studies showing that mTOR/

FRAP is a direct substrate of PKB/Akt [135,136]. However, phosphorylation of mTOR/FRAP by PKB/Akt was shown subsequently not to affect either $p70^{S6k}$ or 4E-BP1, two downstream targets of mTOR/FRAP [136], suggesting that additional signals may be required to alter mTOR/FRAP function. Nevertheless, PKB/Akt has been shown to promote the translation of cyclin D mRNA via mTOR/FRAP [116]. Interestingly, a PKB/Akt–mTOR/FRAP pathway may also regulate cyclin D at the transcriptional level by a mechanism that appears to involve CREB and the CRE found in the cyclin D promoter [137]. Evidence that mTOR/FRAP is involved in oncogenic signalling by PKB/Akt has emerged from recent studies showing firstly that constitutive activation of PKB/Akt in prostate cancer cell lines coincided with increased basal phosphorylation of mTOR/FRAP [136] and, secondly, that the oncogenic potentials of both PI-3K and PKB/Akt in an avian fibroblast system, are mediated by mTOR/FRAP [138].

Thus, evidence is accumulating that PKB/Akt, by phosphorylating downstream targets such as GSK-3 and mTOR/FRAP, plays a role in regulating cellular cyclin D levels. However, since other signalling pathways also participate, an important unanswered question concerns the degree to which PKB/Akt signalling contributes to the overall regulation of cyclin D expression, particularly in the large number of breast and other cancers where cyclin D levels are elevated.

5.2.2. $p21^{CIP1}$ and $p27^{KIP1}$

$p21^{CIP1}$ acts as an inhibitor of CDKs, and thereby inhibits progression through the G1 phase of the cell cycle. Multiple upstream signals, notably from the Ras-ERK and Rho pathways, appear instrumental in causing the reduction in $p21^{CIP1}$ levels necessary for cell cycle progression [139]. Although the inhibitory action of $p21^{CIP1}$ on CDKs occurs in the nucleus, it has emerged recently that $p21^{CIP1}$ can localise to the cytosol and participate in the protection of cells from apoptosis induced by cytotoxic drugs. The mechanism appears to involve the activation of ASK-1, a kinase that negatively regulates SAPK signalling [140]. Interestingly, ASK-1 may also be a direct substrate for PKB/Akt (see Section 5.1.2). Recent work has shown that $p21^{CIP1}$ is a direct substrate of PKB/Akt and that this may regulate the subcellular localisation of $p21^{CIP1}$. In one study, using erbB2-expressing breast cancer cells, in which PKB/Akt was constitutively active, phosphorylation of $p21^{CIP1}$ by PKB/Akt correlated with its exit from the nucleus and with increased cell cycle progression [141]. The other study showed that phosphorylation of $p21^{CIP1}$ by PKB/Akt reduced its association with CDKs and also abrogated its binding to PCNA, a protein required for DNA replication [142]. However, these authors failed to find any evidence for redistribution of $p21^{CIP1}$ in association with its phosphorylation by PKB/Akt. Redistribution to the cytosol may therefore be a feature of cells overexpressing erbB2.

$p27^{kip1}$ expression is also down-regulated during G1 and the protein is often expressed at low or undetectable levels in many human cancers. Regulation of $p27^{kip1}$ is complex and occurs at transcriptional, translational, and posttranslational levels. Regulation of $p27^{kip1}$ gene expression is mediated in part by AFX/forkhead transcription factors [143,144], which, as discussed in Section 5.1.1, are major substrates for PKB/Akt. Since PKB/Akt-mediated phosphorylation of AFX sequesters it in the cytoplasm, sustained activation of the PKB/Akt pathway, for example, in cells with nonfunctional PTEN, may cause reduced $p27^{kip1}$ transcription. Indeed, recent reports indicate that one mechanism by which PTEN induces growth arrest is by up-regulating $p27^{kip1}$ [53,145–147], although this may not occur in all cellular contexts [148].

In addition to a possible role in regulating $p27^{kip1}$ gene transcription, a recent conference report [149] provided evidence that $p27^{kip1}$ can be phosphorylated by PKB/Akt directly and that this modification leads to sequestration of $p27^{kip1}$ in the cytoplasm in a similar fashion to that described for $p21^{CIP1}$. Interestingly, the modulation of $p27^{kip1}$ subcellular localisation was also shown to occur in erbB2-overexpressing breast cancer cells. Whether these events are a particular consequence of erbB2 signalling remains to be determined.

5.3. Hypoxia, nutrient supply, and angiogenesis

In solid tumours, the effectiveness of chemotherapy is often limited by factors such as poor vascularisation, gradients of proliferation, and changes in the tumour microenvironment, which vary according to distance from a blood vessel [150]. As such, hypoxic regions can develop in areas with limited oxygen and nutrient supply. The ability to tolerate hypoxia and withstand apoptosis may be required for tumour progression. PKB/Akt, with its critical role in cell survival, may have an important function in adaptation to these adverse environmental changes. In addition, PKB/Akt signalling appears to play an important role in the development of angiogenic capability that is necessary for tumours to expand their mass. For example, evidence is emerging that PKB/Akt, activated in response to proangiogenic stimuli such as angiopoietin-1 [151,152] and vascular endothelial growth factor (VEGF) [153], can prevent endothelial cell apoptosis [154]. PKB/Akt phosphorylates and activates endothelial nitric oxide synthase (eNOS) [155–157] allowing neovascularisation and VEGF-induced endothelial cell migration [158]. Other components of the PI-3K–PKB/Akt pathway involved in angiogenesis include PTEN, which induces the secretion of thrombospondin-1, a negative regulator of angiogenesis [159].

A key regulator of the response to hypoxia is the transcription factor hypoxia inducible factor-1 (HIF-1α). HIF-1α, which can also be activated, in addition to hypoxia, by insulin [160] or growth factors [161], induces the transcription of various genes involved in angiogenesis

including VEGF and glucose transporters (GLUTs). Recent studies in breast and prostate cancer cells have shown that activation of PKB/Akt may lead, via mTOR/FRAP, to the stabilisation of HIF-1α and consequently increased production of VEGF [161–163].

The role of PKB/Akt in glucose metabolism has been well documented. In addition to its role in stimulating glycogen synthesis by phosphorylating GSK-3 (see Section 5.2.1), PKB/Akt promotes translocation of GLUT4 to the plasma membrane and induces GLUT1 gene transcription (see Ref. [164]). A recent study by Izuishi et al. [165] investigated the role of PKB/Akt in tolerance of cells to nutrient depletion. These authors hypothesised that such tolerance may allow tumour progression under hypovascular conditions. They found that high cellular PKB/Akt expression was associated with an ability of liver and pancreatic cancer cell lines to tolerate nutrient deprivation and demonstrated, using antisense PKB/Akt RNA constructs, that this tolerance was partially dependent upon PKBα and PKBβ but not PKBγ. One possible explanation for these findings derives from recent work by Gottlob et al. [166]. In this study, examining the regulation of early events in the apoptotic pathway, the ability of PKB/Akt to block apoptosis was shown to be dependent upon glucose availability. Moreover, PKB/Akt caused increased mitochondrial hexokinase activity, which in turn preserved mitochondrial integrity by maintaining coupling between ATP synthesis and glucose metabolism. If such a model is universally applicable, it may be that in conditions where glucose supply is limiting, PKB/Akt can prevent apoptosis by stimulating hexokinase activity, as well as by increasing the transport of glucose through up-regulation of GLUTs.

5.4. Other substrates with potential involvement in tumourigenesis

5.4.1. Estrogen receptor (ER)

The growth and survival of ER positive breast tumours is promoted by estrogens as evidenced by the ability of antiestrogenic drugs such as tamoxifen to regress such tumours. The ligand-bound ER recruits coactivator proteins and stimulates the transcription of target genes such as c-*myc* and c-*fos* via response elements in their promoters. Many ER positive tumours that initially respond to antiestrogen therapy eventually develop resistance to these drugs and exhibit estrogen-independent growth. It is believed that such growth is stimulated by factors such as EGF and IGF-1 through their ability to cause ligand-independent ER activation. Recent studies suggest that the PKB/Akt pathway mediates these actions [167] and that it may do so, at least in part, by phosphorylating the ER directly causing increased transcription of ER-regulated genes. Additionally, PKB/Akt-mediated phosphorylation of the ER correlated with cellular resistance to tamoxifen [168], suggesting that up-regulation of PKB/Akt may contribute to antiestrogen resistance.

5.4.2. Androgen receptor

In a similar manner to the ER, the androgen receptor (AR) mediates the actions of androgens by binding to genes with androgen response elements. This ligand-dependent activation of the AR is believed to mediate the growth-promoting actions of androgens and may also play a role in androgen-induced apoptosis. Like the ER, the AR is also subject to ligand-independent regulation induced by peptide growth factors such as EGF and IGF-1. A recent study has shown that the AR is phosphorylated on two sites by PKB/Akt and that this leads to inhibition of AR activity and blockade of androgen-induced apoptosis in a prostate cancer cell line [169].

5.4.3. Telomerase reverse transcriptase

Unlike most normal human cells, telomerase is frequently expressed in tumours and this is believed to be one mechanism by which cancer cells maintain their telomeres and circumvent replicative senescence [170]. The human telomerase complex comprises a reverse transcriptase (TERT) catalytic subunit that uses an RNA component (TR) to add TTAGGG repeats to chromosome ends. It has emerged recently that TERT is subject to phosphorylation events that alter telomerase activity [171–173]. TERT contains two PKB/Akt consensus sites and a recent study by Kang et al. [174] provided evidence that both sites were phosphorylated by PKB/Akt leading to increased telomerase activity in a melanoma cell line. While no reports of similar findings in other types of cancer cell have yet appeared, these data place PKB/Akt in a potentially new role; promoting the immortalisation of cancer cells by preventing replicative senescence.

5.4.4. Breast cancer susceptibility gene 1 (BRCA1)

Susceptibility to early-onset breast and ovarian cancers is often caused by germline mutations in the *BRCA1* gene [175]. *BRCA1* has been shown to slow tumour growth [176] and, although its precise cellular function has not been clearly defined, it appears to be required for DNA recombination and repair [177,178]. *BRCA1* encodes a 220-kDa predominantly nuclear protein that is subject to phosphorylation events that may alter function [179]. Recently, heregulin, a growth factor which can stimulate breast cancer cell growth, differentiation, and survival, was shown to increase the phosphorylation of *BRCA1* [180], although the functional consequences of this have not been reported.

6. Role of PKB/Akt in tumourigenesis: evidence from transgenic studies

From the preceding discussion it is clear that PKB/Akt is involved in many signalling pathways that could have a major impact on processes that are abnormally regulated in cancer. Despite this, the question of whether tumours need

to up-regulate the PKB/Akt pathway in order to develop and progress remains largely unanswered. It is quite likely that different cancers evolve different mechanisms to deal with the need to proliferate indefinitely and survive environmental stresses. Nevertheless, the frequency of activation or overexpression of PKB/Akt in human cancers indicates that PKB/Akt serves an important function in a large number of cases. The question of whether PKB/Akt itself is tumourigenic has been addressed by overexpressing the protein in various host systems. Whereas enforced overexpression of wild type PKBβ/Akt2 was shown in one study to transform NIH-3T3 fibroblasts [110], this did not occur in Rat-1 fibroblasts [181] and none of the three rodent wild type PKB/Akt isoforms caused transformation of chicken embryo fibroblasts [182,183]. Nevertheless, membrane-targeted, constitutively active versions of each PKB/Akt isoform are sufficient for oncogenic transformation of these cells [182,183]. The role of PKB/Akt in the transformation of epithelial cells, from which the vast majority of human cancers develop, has only recently begun to emerge from the results of studies using transgenic mice. Targeted overexpression of PKBα/Akt1 in the mammary gland caused hyperplasia but not dysplasia or neoplasia [184]. Transgenic mice expressing a mammary gland-targeted constitutively active PKB/Akt (both regulatory phosphorylation sites mutated to aspartic acid residues), exhibited defects in the normal process of gland involution (as observed in a separate study [185]) but did not develop tumours [186]. However, crossing of these animals with mice bearing a mammary gland-targeted mutant middle T antigen uncoupled from PI-3K, produced mice that developed tumours more rapidly than those animals with mutant middle T alone. Interestingly, strong phosphorylation of FKHR and induction of cyclin D was observed in tumours from the crossed animals but not in the mice bearing activated PKB/Akt alone. These investigators' study also noted that the cross-bred mice did not develop metastatic tumours, suggesting that additional events are required for metastatic progression.

Overall, the phenotypes of PKB/Akt transgenic mice suggest firstly that mere overexpression of PKB/Akt is not tumourigenic, at least in the mammary gland. Constitutive activation of PKB/Akt may be sufficient for tumour formation in some contexts but it is more likely that activated PKB/Akt cooperates with other oncogenic pathways to promote tumour progression. That PKB/Akt is involved more in cancer progression than its initiation is supported by another recent study of prostate cancer. Using protein microarrays to examine molecular markers of disease progression in microdissected tissue, Paweletz et al. [187] found that PKB/Akt was activated to a higher degree as cells progressed from normal to hyperplastic and then invasive stages. PKB/Akt activation was accompanied by loss of markers of apoptosis, supporting the contention that PKB/Akt contributes to enhanced survival during tumour progression.

7. Future prospects

As a result of intensive study in the last 4–5 years, our knowledge of PKB/Akt and its role in promoting cell survival and proliferation has expanded rapidly. Nevertheless, a number of major questions still remain unanswered. For example, the mechanism of activation of PKB/Akt, particularly the regulation of the S473 site, is still unclear. In addition, although PKB/Akt is strongly implicated in the regulation of survival and cell growth, the physiological substrates of PKB/Akt that drive these processes remain to be fully characterised. A number of strong candidates have emerged, such as GSK-3 and forkhead proteins, but their overall importance, compared to other potential substrates, is not yet clear. It seems likely that cellular context will play a role, with PKB/Akt substrates subserving distinct functions in different cell types. Moreover, many of the proteins and downstream cellular processes thought to be modulated by PKB/Akt are subject to simultaneous regulation by other signalling pathways. The response of cells to PKB/Akt activation may therefore be dependent upon the levels of activity of these other signals, a situation that may be particularly important during tumour progression. As we have attempted to highlight in this review, PKB/Akt signalling is frequently up-regulated in human tumours. However, the results of transgenic studies indicate that this is insufficient by itself to cause tumour initiation. Instead, PKB/Akt activation appears to be involved in tumour progression, most likely through interactions with other disrupted signalling processes. Whether PKB/Akt is necessary for progression is a vital question. If it is the inhibition of PKB/Akt, either directly or by interfering with its upstream regulators, is likely to represent an effective and widely applicable anticancer strategy. Indeed, as the vast majority of existing anticancer drugs work by inducing apoptosis, one would predict that inhibition of PKB/Akt could lower the threshold doses of drugs required to kill cells. Recent studies in cell lines [188,189] support this possibility.

Although the PKB/Akt story will probably continue for many years to come, the pace with which knowledge of its cellular functions has accumulated recently indicates that answers to some of the questions mentioned above may be just around the corner.

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AKT-1, -2, and -3 are Expressed in Both Normal and Tumor Tissues of the Lung, Breast, Prostate, and Colon

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ABSTRACT

Purpose: The AKT/PKB kinase controls many of the intracellular processes that are dysregulated in human cancer, including the suppression of apoptosis and anoikis and the induction of cell cycle progression. Three isoforms of AKT have been identified: AKT-1, -2, and -3. Selective up-regulation of AKT-3 RNA expression has been reported in hormone-independent breast and prostate cancer cell lines suggesting that AKT-3 expression may be increased with breast or prostate tumor progression. To determine whether AKT-3 RNA expression is selectively up-regulated in human cancers and whether the patterns of AKT RNA expression may change with tumor development, we examined AKT isoform expression by RT-PCR in human cancer cell lines, primary human cancers, and normal human tissues.

Experimental Design: AKT-1, -2, and -3 RNA expression was examined by RT-PCR. Because up-regulated AKT-3 expression has been implicated in human breast and prostate cancer progression, we also examined AKT-3 expression levels by semiquantitative RT-PCR using matched normal/tumor first-strand cDNA pairs from colon, breast, prostate, and lung cancers.

Results: Our data reveal that the overwhelming majority of both normal and tumor tissues express all three of the AKT isoforms. Moreover, semiquantitative RT-PCR of matched normal/tumor pairs confirmed similar AKT-3 RNA expression levels in both normal and tumor tissue.

Conclusions: Our data show that both normal and tumor tissues express all three of the AKT isoforms and indicate that tumorigenesis does not involve a dramatic shift in the RNA expression patterns of the three AKT isoforms.

INTRODUCTION

The AKT/PKB kinase has been implicated in the genesis and/or progression of numerous human tumors, because AKT regulates many of the key effector molecules involved in apoptosis, anoikis, and cell cycle progression (1). AKT is activated by phosphatidylinositol 3 phosphates, the products of PI-3 kinase activity (2). In addition, AKT activity is commonly dysregulated in a variety of human tumors because of frequent inactivation of the *PTEN* tumor suppressor gene, which negatively regulates phosphatidylinositol 3 phosphate levels (1).

To date, three human isoforms of AKT have been identified, AKT-1, -2, and -3 (2–4). Several studies have found AKT-2 to be amplified or overexpressed in primary tumors and cell lines (5–7). A recent report has shown that AKT-1 and -2 are expressed in a wide array of breast and prostate cancer cell lines but that AKT-3 expression is exclusive to the more advanced, hormone-independent breast and prostate cancer cell lines (8). In addition, AKT-3 RNA expression was selectively up-regulated in the more advanced, ER[3]-negative primary human breast cancers (8). These data therefore suggested that AKT RNA expression patterns may be altered in tumors *versus* normal tissue (8). We have recently shown that AKT-1 and -3 RNA are expressed in both normal prostate tissue and primary prostate cancers, suggesting that patterns of AKT isoform RNA may not be dramatically different in tumor *versus* normal tissue (9).

To clarify whether AKT-3 expression is exclusive to tumors and/or if AKT RNA expression patterns are altered in tumor *versus* normal tissue, we have evaluated AKT isoform expression by RT-PCR in human cancer cell lines, primary human cancers, and normal human tissues. Our data reveal that the majority of the cancer cell lines, primary cancers, and normal tissues express AKT-1, -2, and -3. Moreover, semiquantitative RT-PCR analyses for AKT-3 expression in matched normal/tumor pairs confirm that AKT-3 RNA expression levels are not markedly altered in tumors relative to normal tissue of the lung, colon, prostate, or breast.

MATERIALS AND METHODS

RT-PCR Analysis. Total RNA and poly(A) RNA were isolated using the RNEasy kit and the Oligotex mRNA maxi kit from Qiagen (Chatsworth, CA). Normal tissue total RNA panels I, II, III, IV, and V were obtained from CLONTECH Laboratories (Palo Alto, CA). RT-PCR analysis of the RNA samples was performed with the Titan One Tube RT-PCR kit (Roche

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[3] The abbreviations used are: ER, estrogen-receptor; RT-PCR, reverse transcriptase-PCR.

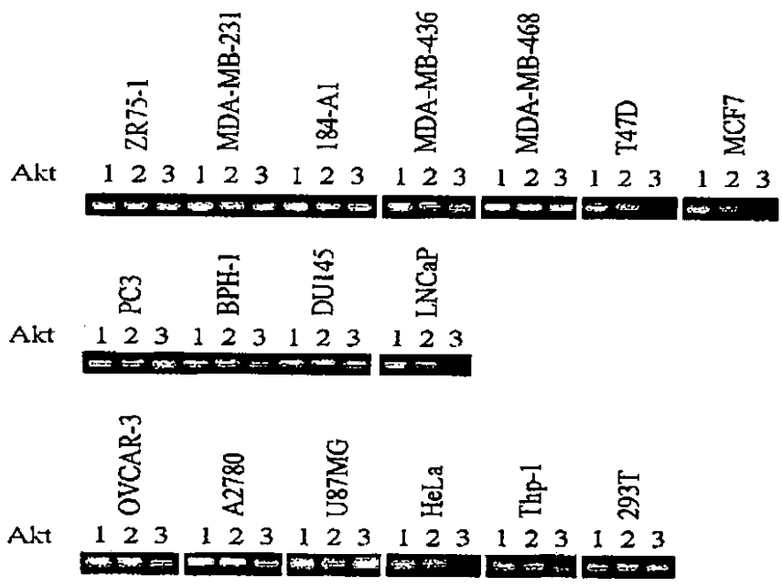

Fig. 1 RT-PCR analyses for AKT-1, -2, and -3 RNA in human cell lines. RT-PCR analysis was performed on 0.25–0.5 μg total or poly(A)+ RNA using primers specific for AKT-1, -2, and -3 (8) for 35 PCR cycles as described (9).

Biochemicals, Indianapolis, IN) as described previously (9) using the specific primer sequences and RT-PCR conditions described for AKT-1, -2, and -3 (8). The SUPERSCRIPT First-Strand Synthesis System for RT-PCR (Life Technologies, Inc.) using 1–5 μg of total or poly(A) RNA with an oligo(dT) primer was used to confirm low abundance or absence of a specific mRNA identified from the One Tube RT-PCR reactions. Controls without RNA and without reverse transcriptase were routinely performed (data not shown). Restriction digestion with *Eag*I or *Pst*I (Life Technologies, Inc.) was used to verify the identity of AKT-1, -2, and -3 RT-PCR products (data not shown). *Eag*I restricts AKT-1 and -3 but not AKT-2, whereas *Pst*I restricts AKT-1 and -2 but not AKT-3.

Semiquantitative RT-PCR. To evaluate semiquantitatively the expression of AKT-3 RNA in tumor *versus* normal tissue, we performed RT-PCR with the High-fidelity PCR kit (Roche Biochemicals) using the aforementioned AKT-3-specific primers on first-strand cDNAs from matched tumor/normal pairs (CLONTECH Laboratories). We specifically evaluated the following tumor/normal pairs: prostate 1 and 3; breast 1 and 2; lung 4 and 5; and colon 2 and 4. To control for differences in template quantity between normal and tumor tissue, we also performed semiquantitative RT-PCR for the ribosomal protein S9 using the specific primers and conditions included with each tumor/normal pair (CLONTECH Laboratories).

RESULTS

Expression of AKT-1, -2, and -3 RNA in Human Cancer Cell Lines. Hormone-dependent breast and prostate cancer cell lines lack expression of AKT-3 (8), whereas many hormone-independent breast cancer cell lines express AKT-3, suggesting that more advanced cancers may selectively up-regulate AKT-3 expression (8). We therefore chose to catalogue expression of each AKT isoform in a large number of cell lines. RT-PCR analysis of 17 different cell lines revealed that only the prostate line LNCaP and the breast cancer cell lines MCF-7 and T47D lacked AKT-3. In the cervical carcinoma line HeLa, AKT3 expression was barely detectable (Fig. 1). All other cell lines, including the immortalized, nontumorigenic breast epithelial cell line 184A1 and the nontumorigenic prostate epithelial cell line BPH-1, expressed AKT-1, -2, and -3. Moreover, the ER-positive breast cancer cell line ZR-75–1 (10) also expressed AKT-3. Therefore, these data suggest that AKT-3 RNA expression is not restricted to cell lines derived from advanced cancers nor to tumorigenic cell lines.

Expression of AKT-1, -2, and -3 RNA in Primary Human Tumor and Normal Tissues. Because the majority of cell lines expressed AKT-1, -2, and -3, we next assessed expression of these isoforms in primary cancer tissues of the most common human solid tumors (colon, lung, breast, and prostate). RT-PCR analyses revealed AKT-1, -2, and -3 expression in each of the 28 tissues from breast, prostate, colon, and non-small cell lung cancers analyzed (Fig. 2). These data from primary human cancer tissues suggest that tumors routinely express all three AKT isoforms, though these tumor samples were not microdissected to separate tumor from normal tissue. As such, it is feasible that these patterns of AKT RNA expression may reflect that from normal tissue, tumor tissue, or both.

We therefore examined RNA expression patterns of the AKT isoforms in normal tissues. Our data revealed that the majority of normal tissues expressed AKT-1, -2, and -3 RNA (Fig. 3*A*). Northern blot analyses verified AKT-3 expression in normal tissues with the highest expression in brain (data not

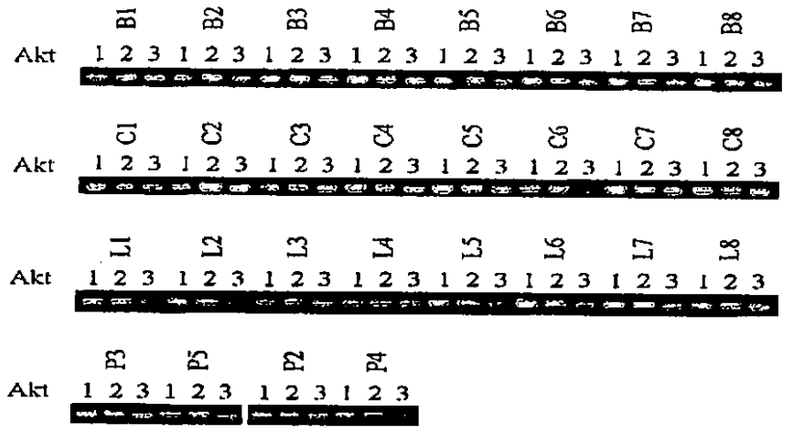

Fig. 2 RT-PCR analysis for AKT-1, -2, and -3 mRNA in primary human cancers. RT-PCR analysis for the AKT isoforms was performed on total RNA from primary human cancer tissues of the breast (*B*), colon(*C*), lung (*L*), and prostate (*P*) using primers specific for AKT-1, -2, and -3 (8) for 35 PCR cycles as described (9).

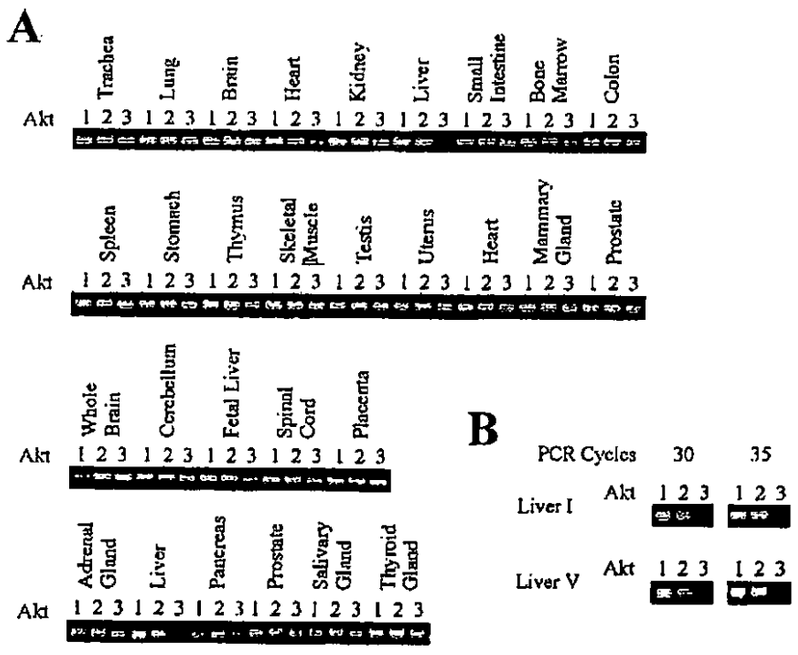

Fig. 3 RT-PCR analysis for AKT-1, -2, and -3 mRNA in normal human tissues. *A*, RT-PCR analysis was performed on CLONTECH total human RNA panels (I–V) using primers specific for AKT-1, -2, and -3 (8) for 35 PCR cycles as described (9). *B*, low level of AKT-3 RNA expression in adult liver was confirmed by performing RT-PCR for 30 or 35 cycles as indicated on first-strand cDNA from two independent adult liver RNA samples (CLONTECH).

shown), as has been published previously (3). Adult liver tissue showed only minimal evidence for AKT-3 RNA expression (Fig. 3*A*), which was subsequently verified in two independent adult liver tissue samples after PCR from first-strand cDNAs for 30 or 35 PCR cycles (Fig. 3*B*). These data are consistent with previously published Northern blot data showing that adult liver had low levels of AKT-3 mRNA (3, 11), whereas fetal liver maintained a relatively high level of AKT-3 mRNA (11). Together, these data indicate that the majority of both normal and tumor tissues express AKT-1, -2, and -3 RNA.

Semiquantitative RT-PCR for AKT-3 Expression in Matched Normal/Tumor Pairs. Our data indicate that normal tissues, with the exception of adult liver, express AKT-1, -2, and -3 RNA. Because up-regulated expression of AKT-3 RNA has been implicated in breast and prostate cancers (8), we wanted to examine AKT-3 RNA expression in tumor *versus* normal tissue in a more quantitative manner. We therefore examined first-strand cDNAs from matched tumor/normal pairs of the breast, prostate, colon, and lung using 30–38 PCR cycles. Our data revealed that both normal and tumor tissues express

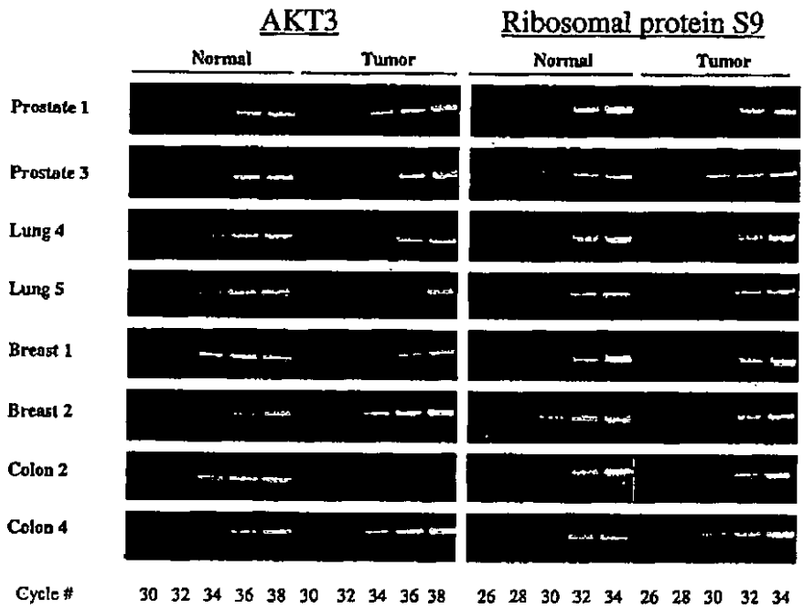

Fig. 4 Semiquantitative RT-PCR for AKT-3 in matched tumor/normal pairs. RT-PCR analysis was performed for AKT-3 on first-strand cDNA from matched tumor/normal pairs (CLONTECH) for the number of PCR cycles indicated. To control for variations in template concentrations, RT-PCR was also performed for a control gene, ribosomal protein S9.

similar levels of AKT-3 RNA (Fig. 4). AKT-3 expression in the samples Prostate 1 and Breast 2 may be elevated in tumor relative to normal but does not appear to be so in the other prostate and breast samples (Fig. 4). Moreover, the colon and lung samples show no apparent differences in AKT-3 levels when controlled for template concentration (Fig. 4). Therefore, these data indicate that there are no consistent differences in AKT-3 expression between normal and tumor tissue. In fact, the differences in AKT-3 signal are largely related to template concentration and are reflected by differences in expression of the control gene, ribosomal protein S9 (Fig. 4).

DISCUSSION

In this report, we have demonstrated that the majority of human tumor cell lines routinely express AKT-1, -2, and -3. As published previously, the hormone-dependent breast cancer cell lines MCF-7 and T47D and the hormone-sensitive prostate cancer cell line LNCaP lack AKT-3 expression (8). Our data now reveal that the hormone-dependent, ER-positive breast cancer cell line ZR-75–1 (10) expresses AKT-1, -2, and -3. Furthermore, the nontumorigenic, immortalized human breast epithelial cell line 184A1 and human prostate epithelial cell line BPH-1 also expressed all three isoforms. These data therefore suggest that AKT-3 expression is not restricted to tumorigenic cell lines or to ER-negative breast cancer cells.

We have also shown that primary human cancers of the breast, colon, prostate, and lung routinely express all three AKT isoforms. Likewise, virtually all normal tissues, with the exception of adult liver tissue (Ref. 3; Fig. 3*B*), express AKT-1, -2, and -3. These data suggest that there may be no differences in the expression of AKT-3 between normal and tumor tissue. However, up-regulated AKT-3 expression has been implicated in the progression of human breast cancers (8). Therefore, we chose to assess AKT-3 expression in normal *versus* tumor tissue more quantitatively. Semiquantitative RT-PCR analysis for AKT-3 RNA expression in matched tumor/normal pairs of the colon, breast, lung, and prostate confirmed that the level of AKT-3 RNA expression in normal tissue is not dramatically different from that of tumor tissue (Fig. 4).

Together, these data indicate that the RNA expression patterns of the AKT isoforms are not specifically related to the neoplastic process. Virtually every normal tissue examined, including prostate and breast tissue, showed AKT-3 RNA expression. Moreover, our data would suggest there may be no particular relationship between hormone receptor status and AKT-3 RNA expression. Normal breast and prostate tissues, which routinely express the estrogen and androgen receptors (12), express AKT-3 RNA as does the ER-positive breast cancer cell line ZR-75–1 (10). In fact, because some tumor cell lines lack AKT-3 expression (*e.g.*, LNCaP, MCF-7, and T47D), though the corresponding normal tissues and nontumorigenic epithelial cell lines express AKT-3, our data suggest that a small fraction of tumors may actually lose AKT-3 expression. Indeed, in our previous study, a small minority of prostate cancer metastases showed no evidence of AKT-3 expression (9).

Because the majority of normal tissues examined expressed RNA for all three AKT isoforms, our data suggest that an AKT isoform-specific inhibitor may not be tumor-specific. However, our data cannot exclude the possibility that there may be differences in the activity of AKT-1, -2, and -3 proteins that are not reflected by the patterns of AKT RNA expression. It is feasible that the different AKT proteins may be differentially activated

or that these activated proteins may have different substrate preferences. Such differences at the protein level may provide a therapeutic opportunity to target tumors preferentially by selectively targeting a particular AKT isoform. However, the data in this report suggest that AKT RNA patterns are not dramatically altered in tumors relative to normal tissue. As such, a broad-spectrum AKT inhibitor may be as effective, or perhaps more effective, as an anticancer therapy than an isoform-specific AKT inhibitor.

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Loss of PTEN Promotes Tumor Development in Malignant Melanoma[1]

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ABSTRACT

Loss of tumor suppressor genes on chromosome 10 plays an important role in the development of 30–60% of melanomas; however, the identity of these genes and the mechanisms by which loss of these genes leads to tumor formation remain uncertain. The phosphatase and tensin homologue deleted from chromosome 10 (*PTEN*) is one of the genes on chromosome 10 whose of which the loss or inactivation may play an important role in melanoma tumorigenesis, but functional studies directly demonstrating *PTEN* involvement in melanomas are necessary to confirm this role. To determine the biological importance of *PTEN* loss in melanomas, we established a novel model in which an intact chromosome 10 was transferred into melanoma cells lacking PTEN protein to express the protein at normal physiological levels and to measure the consequent effects on melanoma tumorigenesis. PTEN expression in these cells retarded tumor development in mice unless, by analogy with loss of heterozygosity, the *PTEN* gene was deleted or inactivated during tumor formation. Mechanistically, *PTEN* loss led to the activation of Akt, which consequently down-regulated the apoptotic pathway of melanoma cells. In contrast, expression of PTEN attenuated Akt activation, thereby increasing sensitivity to apoptotic stimuli in cell culture and *in vivo* in animal models. This model demonstrated that *PTEN* loss is critical for melanoma tumorigenesis and allowed a dissection of the underlying mechanism by which *PTEN* loss facilitated melanoma tumor development. In summary, loss of *PTEN* reduces apoptosis and promotes cell survival, thereby favoring melanoma tumor formation. Thus, these observations provide an etiological basis for *PTEN* loss during the genesis of sporadic melanomas.

INTRODUCTION

Of the three forms of skin cancer, malignant melanoma carries the highest risk of mortality from metastasis (1). The primary sites of metastasis are the lungs, liver, and brain, but this aggressive cancer can invade any organ. Currently, there is no effective long-term treatment for patients suffering from the advanced stages of this disease. This is in part due to a lack of information about the genes causing melanoma and therapies targeted to correct these defects. As in other solid tumors, deletion of genetic material exceeds genomic amplifications, suggesting that loss of cancer suppressor gene function is essential for melanoma tumor development (2). Nonrandom deletion of chromosomes or subchromosomal regions in solid tumors has been used to map the sites of these putative cancer suppressor genes and has facilitated the demonstration that these sites contain functionally inactivated tumor suppressor genes; examples include *INK4A/p16* at 9p21 (3, 4), *RB1* at 13q14 (5), and *PTEN*[3] at 10q23 (6–8).

Loss of cancer suppressor genes on chromosome 10 is an important process in melanoma tumorigenesis and has been reported to contribute to the development of 30–60% of noninherited melanomas (9, 10). The alteration usually entails loss of an entire chromosome 10 homologue, because it is easier for melanoma cells to lose the intact chromosome than to undergo multiple independent mutational or deletional events to eliminate cancer suppressor genes on chromosome 10 (11). Deletion of chromosome 10 has been reported in both early and advanced-stage sporadic melanomas (9, 10), and has been associated with a poor clinical outcome (12). The identity of the melanoma cancer suppressor genes on chromosome 10 remains uncertain; however, evidence from multiple groups suggests that *PTEN*, located at 10q23, might be important in melanoma tumorigenesis and metastasis (11, 13–24).

The *PTEN* gene, also known as *MMAC1* and *TEP1* (6–8), encodes a phosphatase of which the primary function is to degrade the products of *PI3K* by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate at the 3 position (25). Loss of functional PTEN from tumor cells causes accumulation of these critical second messenger lipids, which in turn increase Akt phosphorylation and activity, leading to decreased apoptosis and/or increased mitogen signaling (26–31). Reintroduction of PTEN in cells lacking this protein has been shown to lower Akt activity and induce cell cycle arrest and/or apoptosis; however, it is unknown whether PTEN functions in a similar manner in melanoma cells (32). *PTEN* knockout mice are not viable, whereas *PTEN* heterozygotes survive and develop endometrial, intestinal, thyroid, adrenal gland, and breast hyperplasia, as well as dysplasias and tumors of the skin, gastrointestinal tract, and prostate (33).

Tumor suppressor gene inactivation in noninherited cancers requires two consecutive somatic events targeting each allele through processes that involve a combination of mutation or epigenetic inactivation, and chromosomal loss, deletion, or recombination (34, 35). Unfortunately, the search for genetic lesions of *PTEN* in melanomas has yielded confounding results leaving uncertainty about the role PTEN plays in melanoma tumorigenesis. Mutations or deletions of *PTEN* have been observed in up to 60% of melanoma cell lines; however, only about 10% have been seen in uncultured tumor material (13–22). These observations have led to speculation that one of the *PTEN* inactivating events occurs predominantly through a mechanism other than mutation, such as by epigenetic silencing (36–38) or by altered subcellular localization (39). Alternatively, haploinsufficiency or loss of only one allele could account for the discordance in rates of LOH at 10q23 and biallelic *PTEN* inactivation (40). In support of these possibilities, a recent report has suggested that epigenetic silencing of *PTEN* in melanomas without *PTEN* mutation might occur in as many as 30–40% of metastatic tumors (41); however, the molecular basis of this process in melanomas remains unknown. Therefore, it is possible that epigenetic, mutational, and deletional events could account for PTEN dysfunction in as many as 40–50% of sporadic melanomas (41).

Functional mapping of melanoma suppressor genes on chromosome 10 has also identified *PTEN* as a potentially important factor in the disease (11, 23). The involvement of *PTEN* in melanomas has been demonstrated using an approach termed IVLOH that involved transfer of a normal copy of chromosome 10 into melanoma cells lacking PTEN protein, and then allowing the growth-suppressing

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[3] The abbreviations used are: PTEN, phosphatase and tensin homologue deleted from chromosome 10; PI3K, phosphatidylinositol 3′-kinase, LOH, loss of heterozygosity; IVLOH, *in vitro* loss of heterozygosity; FBS, fetal bovine serum; HA, hemagglutinin A; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; PBS-T, PBS and 1% Tween 20; STS, sequence-tagged site.

genes on the chromosome to force the cells to eliminate the responsible gene(s) *in vitro* through chromosomal fragmentation. Mapping the region of loss suggested that *PTEN* was a candidate melanoma growth suppressor, which was confirmed by subsequent ectopic gene expression studies in cells growing in tissue culture (23). Collectively, the molecular and functional mapping reports could be interpreted as indicating that PTEN plays an important role in melanoma tumorigenesis; however, studies demonstrating the direct involvement of PTEN in melanoma tumorigenesis and the underlying mechanisms by which PTEN regulates melanoma development have not been reported.

Here, we describe the use of a novel, chromosome-based strategy to demonstrate *PTEN* involvement in melanoma tumorigenesis, as well to dissect the underlying mechanism by which loss of functional PTEN aids melanoma tumor development. We used melanoma cells expressing PTEN at normal physiological levels from an introduced chromosome 10 to show that tumor growth in animals is retarded unless, by analogy with LOH (42–44), cells inactivate the functional protein during tumor formation. Targeting PTEN in this manner demonstrated that PTEN loss led to increased Akt activation and stimulated antiapoptotic signaling in melanoma cells. Specifically, loss of PTEN reduced apoptosis rates, thereby aiding cell survival and promoting melanoma tumor development. Thus, these studies provide a mechanistic basis for PTEN loss during the genesis of sporadic melanomas.

MATERIALS AND METHODS

Cell Line, Culture Conditions, and Doubling Times. The melanoma cell line UACC 903, the creation and characterization of the microcell-mediated chromosome transfer hybrid cell lines 29, 36, and 37, as well as the growth conditions for these cell lines, have been reported previously (23). Hybrid cell lines stably maintaining the transferred chromosome 10 in tissue culture were maintained in cell culture using 15% FBS to reduce the negative effect that PTEN exerts on *in vitro* cell growth. Initially, the chromosomally heterogeneous population (from Ref. 23) was flow-sorted to isolate single cells into individual wells of a 96-well plate. These cells were then grown into mass populations in DMEM supplemented with 15% serum. The chromosome 10 donor cell line HA(10)A has been described previously (23). Human melanocytes were obtained from Clonetics (Walkersville, MD). The melanoma cell lines YUDAN-3 and SK-MEL-24 used in these studies were obtained from the American Type Culture Collection (Manassas, VA). The doubling time of UACC 903 cells and the 36A hybrid cell line was estimated by plating 1×10^4 cells in 200 μl of DMEM supplemented with 15% FBS in multiple rows of wells in five 96-well plates. Growth was measured every 24 h over a period of 5 days by performing a colorimetric assay on one plate each day using the Sulforhodamine B Binding Assay (45) and the doubling time calculated. Absorbance was measured at 570 nm using a Perkin-Elmer HTS 700+ Bioassay Plate Reader (Foster City, CA).

Tumorigenicity Assays and Tumor Processing. All of the animal experimentation was performed according to protocols approved by the Institutional Animal Care and Use Committee at the Penn State College of Medicine. Tumor formation was measured in athymic nude mice purchased from Simonson Laboratories (Gilroy, CA) or Harlan Sprague Dawley (Indianapolis, IN). Tumor kinetics were measured by s. c. injection of 5 million cells in 0.2 ml of DMEM containing 10% FBS above both the left and right rib cages of 4–6-week old female nude mice. The dimensions of the developing tumors were measured at weekly intervals using calipers and the sizes estimated in cubic millimeters. For studies to dissect the mechanism by which PTEN inhibited tumor development, the growth of cells lacking (UACC 903 or 36A-R2) or expressing (36A or 36B) chromosomal PTEN was temporally and spatially matched. This was achieved by s. c. injection of 1 million or 20 million of each cell type, respectively, in 0.2 ml of DMEM medium supplemented with 10% FBS at a total of four sites above both the left and right rib cages and rump. Four tumors were harvested every 2 days, sliced into two sections, and processed to achieve maximal morphological or immunohistological results. For morphological analysis, tumors were fixed for 24 h in buffered formaldehyde-fresh low odor 10% formalin from Fisher Scientific (Fair Lawn, NJ), embedded in paraffin, then sectioned and stained with H&E. Frozen tissue specimens for immunohistochemical analysis were prepared by placing the tumor into Peel-A-Way Disposable Embedding Molds from Polysciences (Warrington, PA) containing Tissue-Tek OCT compound from Sakura Finetek (Torrance, CA), followed by slow freezing in liquid nitrogen and sectioning.

Apoptosis Measurements. Apoptosis rates were measured for cells growing in tissue culture and in formalin-fixed, paraffin-embedded tumor sections using the TUNEL TMR Red Apoptosis kit from Roche (Indianapolis, IN). For cell culture studies, 5×10^3 cells were seeded onto glass coverslips in 12-well plates and grown in DMEM supplemented with either 1% or 10% FBS for 48 h. Alternatively, cells growing in DMEM supplemented with 10% FBS were exposed to 1 μM staurosporine for 4 h before paraformaldehyde fixation. For *in vivo* animal studies, formalin-fixed, paraffin-embedded tumor sections were deparaffinized, rehydrated, and digested with 20 μg/ml proteinase K from Fisher Scientific (Fairlawn, NJ) and then incubated with the TUNEL reaction mixture containing terminal deoxynucleotidyl transferase in a humidified dark chamber for 1 h at 37°C. Sections were counterstained with 1 μg/ml Hoechst-33258 for 10 min and mounted. A minimum of 8–10 fields were counted from three to four different tumor sections, and the number of positive cells was expressed as the percentage of apoptotic cells = (number of apoptotic cells/number of cells in each field) × 100%.

Cell Proliferation Rate. The number of proliferating cells in tumor sections was measured by using a purified mouse antihuman Ki67 from PharMingen (San Diego, CA). Formalin-fixed, paraffin-embedded tumor sections were deparaffinized and rehydrated in xylene and a graded series of ethanol, respectively. Tumor sections were incubated in 1% H_2O_2 for 10 min to quench endogenous peroxidase activity. Antigen retrieval was accomplished by incubation in a 0.01 M sodium citrate buffer (pH 6.0) for 10 min at 98°C, followed by cooling to room temperature for 20 min. Sections were blocked for 30 min with 1% BSA, and then incubated with mouse antihuman Ki67 (dilution 1:50) for 1 h at room temperature. After washing with PBS, sections were incubated with biotinylated antimouse IgG for 30 min, washed in PBS, incubated with peroxidase-labeled streptavidin for 30 min, and finally rinsed with PBS-T. The chromogenic reaction was carried out with 3,3-diaminobenzidine from DAKO Corporation (Carpinteria, CA) for 2–5 min and counterstained with hematoxylin. The number of Ki67-positive cells/total number of cells was counted from 8–10 fields. The mean percentage of proliferating cells is reported together with the SE for each.

Vessel Density. The number of vessels in tumor sections was estimated using a purified rat antimouse CD31 (PECAM-1) monoclonal antibody from PharMingen. Frozen, cryostat-cut sections were air dried for 30 min, fixed in acetone (−20°C) for 5 min, and rehydrated in PBS for 5 min. Endogenous peroxidase activity was quenched by incubation for 10 min 1% H_2O_2 in PBS. Each incubation step was carried out at room temperature and was followed by three 3-min washes in PBS-T. Sections were blocked in PBS containing 1% BSA for 30 min and then incubated with rat antimouse CD31 (PECAM-1) antibody at a dilution of 1:500 for 90 min to stain endothelial cells. Sections were then washed in PBS followed by incubation with biotinylated goat-antirat antibody for 30 min. Tumor slices were next incubated with peroxidase-labeled streptavidin from PharMingen for 30 min, then briefly rinsed in PBS-T. Sections were exposed to 3,3-diaminobenzidine and hydrogen peroxide chromogen substrate from Dako Corporation for up to 2 min, rinsed in distilled water, counterstained with Mayer's hematoxylin for 1 min, then dehydrated and coverslipped with a permanent mounting medium. Vessel density was scored by counting the number of CD31-positive vessels in a 3600-μm^2 area of a tumor. Five different areas were counted from each of four different tumors, and the mean number of vessels is reported together with the SE for each.

Sequencing of PTEN DNA and cDNA. The procedure for extraction of high molecular weight DNA from cultured cells has been described previously (46). Total RNA was isolated from cell lines using the TRIzol reagent (Life Technologies, Inc., Carlsbad, CA) and converted to cDNA (23). The DNA or cDNA was used as a template to amplify *PTEN* exon 4 as described previously (23, 47). The amplified *PTEN* exon 4 product was then sequenced on an Applied Biosystems 377XL automated sequencer (Foster City, CA).

Western Blot Analysis. Cells that were 70–90% confluent were washed once with ice-cold PBS followed by the addition of lysis buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 10 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM sodium orthovanadate, 0.1 mM sodium molybdate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 5 μg/ml leupeptin. Whole cell lysates were centrifuged (≥10,000 × *g*) for 10 min at 4°C. Proteins were quantitated using the BCA Assay from Pierce (Rockford, IL). Thirty μg of supernatant per lane were loaded onto a NuPage gel from Life Technologies, Inc. and electrophoresed according to the manufacturer's instructions, followed by transfer of the proteins to polyvinylidene difluoride membrane from Pall Corporation (Ann Arbor, MI). The blots were probed with the appropriate primary antibody according to each supplier's recommendations. Antibodies were obtained from the following sources: anti-PTEN clone 6H2.1 from Cascade Bioscience (Winchester, MA); anti-Akt, anti-phospho-Akt (ser 473), and anti-Caspase-3 from Cell Signaling Technologies, Inc. (Beverly, MA); and α-enolase from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All of the secondary antibodies were conjugated with horseradish peroxidase and obtained from Santa Cruz Biotechnology, Inc. The immunoblots were developed using the enhanced chemiluminescence detection system from Amersham Pharmacia Biotech (Piscataway, NJ). In the PI3K inhibition and apoptotic studies, cells grown in DMEM supplemented with 10% FBS were treated with 50 μM LY-294002 (Alexis Biochemicals, San Diego, CA) for 24 h or 1 μM staurosporine for 4 h, respectively, before harvesting the protein lysates.

Expression of PTEN Using Adeno-associated Viral Constructs. The AAV Helper-Free System from Stratagene (La Jolla, CA) was used to create the recombinant PTEN adeno-associated viruses. Constructs containing PTEN or the catalytically inactive mutant G129R both tagged with HA (generously provided by Dr. Webster Cavenee, Ludwig Institute, San Diego, CA) were transferred into the pAAV-MCS and pAAV-IRES-hrGFP vectors, and viruses were generated according to the Stratagene protocol. On the day before viral infection, 1.5×10^5 cells were plated into six-well plates. After 24 h, the cells were treated for 5–6 h with permissive medium consisting of DMEM supplemented with 10% FBS, 40 mM hydroxyurea, and 1 mM sodium butyrate. Cells were then washed with prewarmed DMEM supplemented with 2% FBS. Increasing volumes of viral lysates (0 μl, 7.8 μl, 15.6 μl, 31.3 μl, 62.5 μl, and 125 μl) suspended in DMEM supplemented with 2% FBS were then added to each well to give a final volume of 1 ml and incubated at 37°C for 1–2 h. An additional 1 ml of DMEM supplemented with 18% FBS was added after the infection to bring the final serum concentration to 10%. Protein from the cells was harvested for Western blot analysis 3 days after infection. For some experiments, the amount of phosphorylated or total protein was quantitated using a Molecular Dynamics Model 100A laser densitometer (Sunnyvale, CA) and the ratio of phosphorylated Akt to total Akt protein used as an indicator of Akt activation.

Statistical Analyses. The statistical analyses of the data were performed using the unpaired Student's *t* test. A P of <0.05 was considered statistically significant.

RESULTS

PTEN Loss Leads to Melanoma Tumor Formation. To establish whether *PTEN* functions as a melanoma tumor suppressor, we developed a novel chromosome-based strategy to measure the effects of PTEN expression on melanoma cell tumorigenicity. We reasoned that melanoma cells expressing PTEN at normal physiological levels from an introduced chromosome 10 could be established and maintained stably in culture under high serum conditions by stimulating cell growth and reducing apoptosis. We predicted that the growth factors present in serum would mask PTEN-mediated apoptosis by stimulating Akt activation thereby suppressing apoptosis (26–31, 48). In contrast, s.c. growth conditions in animals would be such that the presence of PTEN would retard cell growth and tumor development unless, by analogy with LOH (42–44), cells would use genetic strategies to silence the gene thereby allowing tumor development to occur. Targeting *PTEN* in this manner would first confirm involvement of the gene in melanoma tumor development, and second, result in the establishment of genetically related nontumorigenic and tumorigenic cell lines that could be used to determine the underlying mechanism by which PTEN regulates melanoma tumorigenicity.

To create melanoma cell lines that expressed PTEN at normal physiological levels, we used microcell-mediated chromosome transfer to introduce a normal chromosome 10 into the melanoma cell line, UACC 903. Because of a condition called uniparental disomy, this cell line contains two genetically identical copies of chromosome 10 that do not produce PTEN protein because of the same truncating point mutation (T228G) that converts a tyrosine at codon 76 in exon 4 to a stop codon, thereby severely truncating the protein (23). In addition, we reported previously that introduction of chromosome 10 into UACC 903 cells growing in culture in 5% FBS leads to breakage of the transferred chromosome by IVLOH in order for the cells to eliminate the growth-suppressing activity of PTEN (23). To overcome IVLOH for these studies, we designed a strategy to prevent fragmentation of the transferred chromosome by using serum concentrations of 15% to mask the negative effects that PTEN exerts on *in vitro* cell growth. Briefly, we flow sorted the initial chromosomally heterogeneous population (described in Ref. 23) to isolate single cells into individual wells. These cells were then grown into mass populations in medium supplemented with 15% FBS. STS markers that could identify polymorphic alleles in the transferred chromosome 10 were used to verify whether the intact introduced chromosome was retained in the cells, and Western blotting was used to show that these hybrid cells expressed PTEN protein (Table 1). We found that high serum concentrations could effectively mask the growth inhibitory effects of PTEN *in vitro*, allowing establishment of homogeneous stable cell lines containing the intact introduced copy of chromosome 10 that expressed PTEN protein. Even so, the growth rate measured as the doubling time of the cell population remained ~26% longer for the 36A hybrid cell line, doubling every 1.3 days (or 31.5 h), compared with the parental UACC 903 cell line, which doubled about every day (or 25 h). The status of the introduced chromosome 10 and PTEN protein for the parental cell line, initial unstable hybrid cell populations, and five stable hybrid cell lines derived from the initial cell populations is shown in Table 1.

To measure the biological effect of PTEN expression on melanoma tumor development, UACC 903 cells and hybrid cell lines containing the intact introduced chromosome 10 were injected beneath the skin of nude mice. Hybrid cells expressing PTEN were found to be nontumorigenic in comparison with parental UACC 903 cells lacking the protein (Table 2). In all of the cases, tumor development was dramatically reduced for the hybrid cell lines containing the introduced chromosome 10. The majority of injection sites failed to develop tumors. Because tumor development was inhibited completely

Table 1 *Status of transferred chromosome 10 and PTEN protein expression in parental UACC 903 cells and chromosome 10 hybrid cell lines*

Cell line or hybrid name	Retention of the intact transferred chromosome 10	PTEN protein expression
Parental cell line		
UACC 903		none
Unstable hybrids cell lines[a]		
29	–[b]	–
36	–	–
37	–	–
Stable hybrids cell lines		
29A	+	+
36A	+	+
36B	+	+
37A	+	+
37B	+	+

[a] Heterogenous population with each cell retaining a portion of the transferred chromosome 10.

[b] –, unstable retention or partial expression; +, present.

Table 2 *Tumor development by parental UACC 903 melanoma cells and chromosome 10 hybrid cell lines*

Cell line (number of sites injected)	Tumor size (mean ± SE in mm³) Day 7	Day 14	Day 21	Day 28
Parental cell line				
UACC 903 (>20)	143 ± 19	725 ± 83	2587 ± 176	—[a]
Hybrids cell lines expressing PTEN				
29A (8)	5 ± 2	23 ± 5	21 ± 4	97 ± 10
36A (6)	0[b]	0	0	0
36B (20)	36 ± 5	25 ± 5	19 ± 4	16 ± 3
37A (8)	0	0	0	0
37B (8)	0	0	0	0
Revertant cell lines lacking PTEN				
36A-R1 (8)	33 ± 10	176 ± 27	746 ± 56	2014 ± 194
36A-R2 (8)	83 ± 8	331 ± 26	969 ± 74	2665 ± 86

[a] Not available since mice were euthanized at day 21.
[b] 0, no measurable tumor could be detected.

in most cases, the reduced growth rate of 26% observed *in vitro* could not explain the lack of tumorigenicity. Approximately 30% of the injection sites did form tumors after a prolonged latency period (Table 2; Fig. 1). In these instances, tumors larger than 100 mm³ were observed between days 35 and 49, which then proceeded to develop into rapidly growing large tumor masses. The cells constituting two of these tumors, named 36A revertant 1 (36A-R1) and 36A revertant 2 (36A-R2), were re-established in culture and the tumorigenicity of these cells was re-examined (Table 2; Fig. 1). The tumorigenic potential of these cells was found to have reverted to more closely resemble that of the parental UACC 903 cell line, but even so tumor development was delayed by ~1 week. Hence, a melanoma tumor suppressor gene on the introduced chromosome 10 was functionally inactivated during melanoma tumorigenesis.

To determine whether the introduced wild-type *PTEN* gene was specifically targeted for loss during tumor formation, DNA, RNA, and protein were isolated and compared from genetically matched 36A nontumorigenic, and 36A-R1 and 36A-R2 tumorigenic revertant cell lines (Fig. 2). DNA genotyping using markers spanning chromosome 10 at ~10 cM intervals was used to establish whether the intact introduced chromosome was retained in the cells (11). Hybrid cell line 36A, and the revertant cell lines 36A-R1 and 36A-R2 were found to retain all of the polymorphic STS markers present on the transferred chromosome, indicating retention of the introduced chromosome 10. Fig. 2*A* shows this analysis for the markers spanning the distal third of the introduced chromosome 10 in these cell lines. The *PTEN* gene was then examined to determine whether any alteration had occurred to prevent protein expression. The presence of the transferred wild-type *PTEN* gene was determined by PCR amplification of exon 4 from genomic DNA isolated from each cell line followed by sequencing of the product (Fig. 2*B*). Wild-type *PTEN* sequence from the transferred chromosome was present in hybrid 36A and in revertant cell line 36A-R1, but absent from 36A-R2 (Fig. 2*B*). To extend these observations to the transcriptional level, RNA isolated from each cell line was used for reverse transcription-PCR to amplify and then sequence *PTEN* exon 4 to screen for wild-type RNA expression. Fig. 2*C* shows that both wild-type and mutant PTEN RNA expression occurred in the hybrid cell line 36A, but only mutant sequence was observed in both revertant tumorigenic cell lines. This observation was confirmed through Western blotting, shown in Fig. 2*D*, in which PTEN protein was detected in nontumorigenic 36A cells but was absent from the tumorigenic revertant cell lines. These results suggest that the *PTEN* gene in the revertant cell lines was targeted for loss of protein expression by an epigenetic, mutational, or deletional event. Collectively, these data demonstrate *PTEN* is targeted for loss during melanoma tumor formation and that inactivation of the gene occurs at the DNA level.

PTEN Loss Facilitates Cell Survival Thereby Promoting Melanoma Tumor Development. The foregoing experiments showed a consistent relationship between PTEN loss and tumor development; therefore, subsequent studies focused on dissecting the mechanism by which *PTEN* functions as a melanoma tumor suppressor gene. Because PTEN functions as an antagonist of PI3K-mediated signaling by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate at the 3 position, which in turn lowers Akt activity and thereby increases apoptosis rates, we reasoned that cell survival might be a critical function regulated by PTEN in

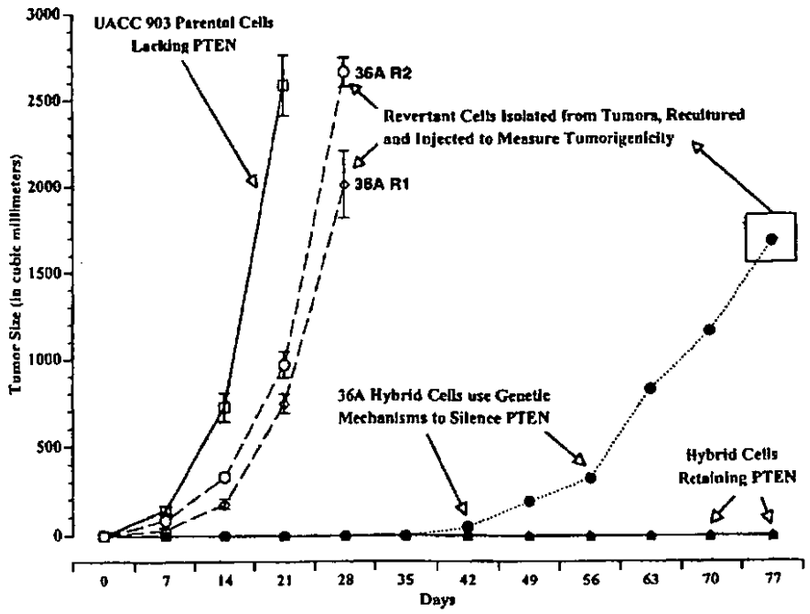


Fig. 1. Tumor development by melanoma cell line UACC 903 and chromosome 10 hybrid cell lines. Size of tumors formed by parental UACC 903 melanoma cells and chromosome 10 hybrid cell lines after injection into nude mice: UACC 903 cells (□); hybrid cells retaining PTEN (◆, ▲); hybrid cells that had lost PTEN (●); revertant cell lines that had lost PTEN and were reinjected into nude mice to measure tumorigenicity (○, ◇). Values are means of a minimum of 16 injection sites in 4 mice per cell line; *bars*, ±SE. No error bars are shown for the hybrid cells that had lost PTEN (●) because this value only represents two tumors.

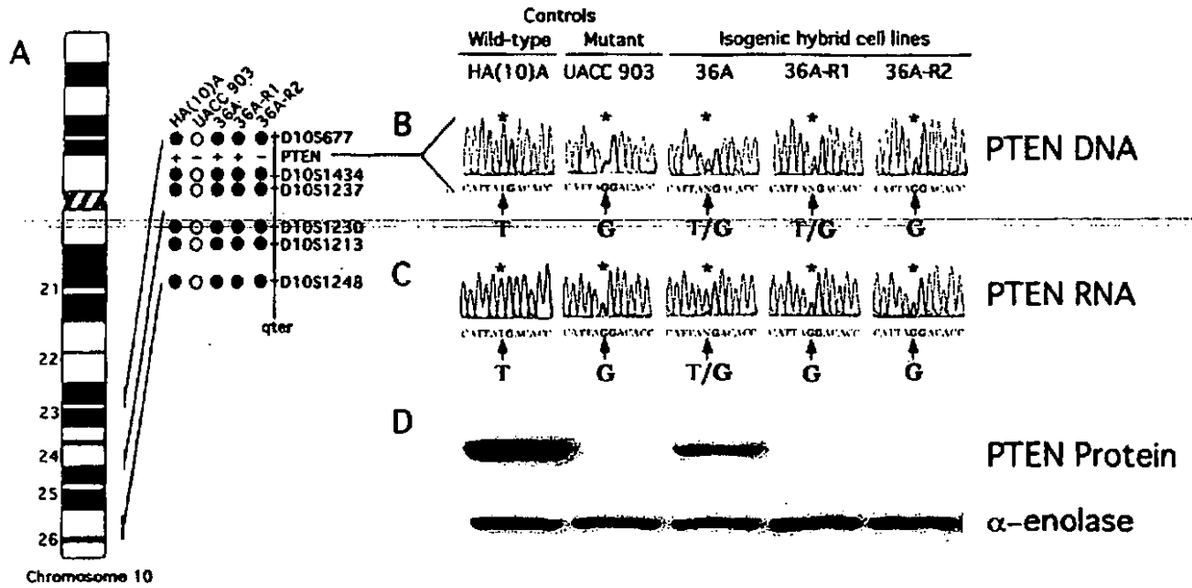


Fig. 2. Status of *PTEN* in UACC 903 cells and chromosome 10 hybrid cell lines. *A*, partial genotypic analysis spanning the distal third of the introduced chromosome 10 is shown for UACC 903 cells, chromosome 10 hybrids, and the donor cell line HA(10)A. STS PCR-based molecular markers spaced at ~10 megabase intervals were determined to be present (●) or absent (○) for donor chromosome 10 alleles in each respective cell line. Hybrid 36A is shown together with revertant cell lines 36A-R1 and 36A-R2. HA(10)A and UACC 903 serve as positive and negative controls, respectively, for the donor chromosome alleles. The order of the markers and the approximate genetic distance between them are indicated, and particular markers are anchored to the chromosome 10 ideogram on the *left* as described previously (11). The presence (+) or absence (−) of wild-type *PTEN* DNA sequence from the introduced chromosome 10 was determined by PCR amplification followed by sequencing of exon 4 from DNA derived from the cell lines. *B*, *PTEN* DNA sequence profiles (from nucleotides 223 to 234) were derived from each cell line or microcell hybrid line. The wild-type profile for HA(10)A is shown for comparison. * show the location of the T to G point mutation occurring at nucleotide 228 in UACC 903 cells. Profiles are also shown for hybrid 36A, and tumorigenic revertant cell lines 36A-R1 and 36A-R2. *C*, PTEN RNA expression in UACC 903 cells and chromosome 10 hybrid cell lines. The partial sequence of the reverse transcription-PCR product for PTEN exon 4 derived from each cell line is shown. *D*, PTEN protein expression in UACC 903 cells and chromosome 10 hybrid cell lines. PTEN protein levels are shown along with the loading control α-enolase. The PTEN antibody recognizes both human and mouse PTEN protein. The HA(10)A cell line expressed elevated levels of PTEN because of the presence of both human and mouse PTEN protein in the cells.

melanomas. To test this possibility and demonstrate a direct relationship between Akt activity and PTEN expression in this model, UACC 903 melanoma cells growing in culture were examined to determine whether cells lacking PTEN had elevated Akt activation that declined significantly after expression of PTEN protein. Initially, the levels of phosphorylated *versus* total Akt protein were measured by Western blot analysis in melanocytes, UACC 903 cells, and matched nontumorigenic and tumorigenic 36A cell lines containing the transferred chromosome 10 (Fig. 3*A*). A phospho-specific antibody (anti-Akt, Ser-473) that recognizes only phosphorylated (active) Akt indicated a dramatic reduction in the activation of Akt in hybrid cells expressing chromosomal PTEN (36A, 29A and 37A) without significant changes in the amount of total Akt protein when compared with parental UACC 903 or 36A revertant cells. Furthermore, it appeared that expression of phosphorylated Akt was lowered to amounts similar to those observed in normal human melanocytes (Fig. 3*A*). Also, comparison of 36A nontumorigenic and tumorigenic revertant 36A-R1 and 36A-R2 cell lines showed that PTEN loss was accompanied by increased levels of phosphorylated Akt similar to those observed in UACC 903 cells lacking the protein. To demonstrate that PTEN reduced Akt activity in a manner similar to that of LY-29004 (49), a known inhibitor of PI3K, UACC 903 cells were treated with the compound, and changes in the levels of phosphorylated *versus* total Akt were measured from densitometric scans of Western blots. LY-294002 treatment reduced the phosphorylated levels of Akt by approximately 60–75% compared with untreated UACC 903 cells. Thus, the observation that Akt phosphorylation were similarly decreased in 36A hybrid cells and after treatment of UACC 903 cells with LY-294002 provided evidence for a direct relationship between Akt and PTEN in this model.

To confirm that PTEN was the primary factor on chromosome 10 responsible for the change in Akt activation, PTEN was ectopically expressed from adeno-associated viruses in 36A-R2 cells, and the effects on Akt phosphorylation (activity) were measured. Increasing volumes of adeno-associated viruses expressing either wild-type or catalytically inactive mutant PTEN protein (G129R) were used to infect 36A-R2 cells to determine whether ectopic PTEN expression could reduce the amount of phosphorylated (active) Akt to levels seen in the original 36A cell population. Three days after infection, expression of PTEN, as well as phosphorylated and total Akt was measured by Western blot analysis. Densitometric scans of the blots were used to quantitate the levels of phosphorylated *versus* total Akt at each viral concentration used to infect the cells. Plots showing Akt activation *versus* increasing PTEN expression are shown in Fig. 3*B* in which ectopic expression of PTEN or PTEN-HA in 36A-R2 cells diminished the phosphorylation (activity) of Akt. In contrast, catalytically inactive PTEN (G129R) or empty virus had minimal effects on Akt activation. These results identify PTEN as the gene on the introduced chromosome 10 that reduced the levels of active Akt in UACC 903 hybrid cells.

To provide additional evidence that PTEN is an important regulator of Akt in other melanoma cell lines in which PTEN expression is altered, the effect of ectopic PTEN expression was also measured in the YUDAN-3 and SK-MEL-24 melanoma cell lines. Changes in Akt phosphorylation (activation) of these cell lines were compared with UACC 903 cells, and the results are shown in Fig. 3*C*. Increasing amounts of wild-type PTEN, but not catalytically inactive G129R PTEN expression, led to decreased phosphorylation of Akt without significant changes in the levels of total Akt protein. Thus, ectopic expression of PTEN consistently reduced active Akt in melanoma cell

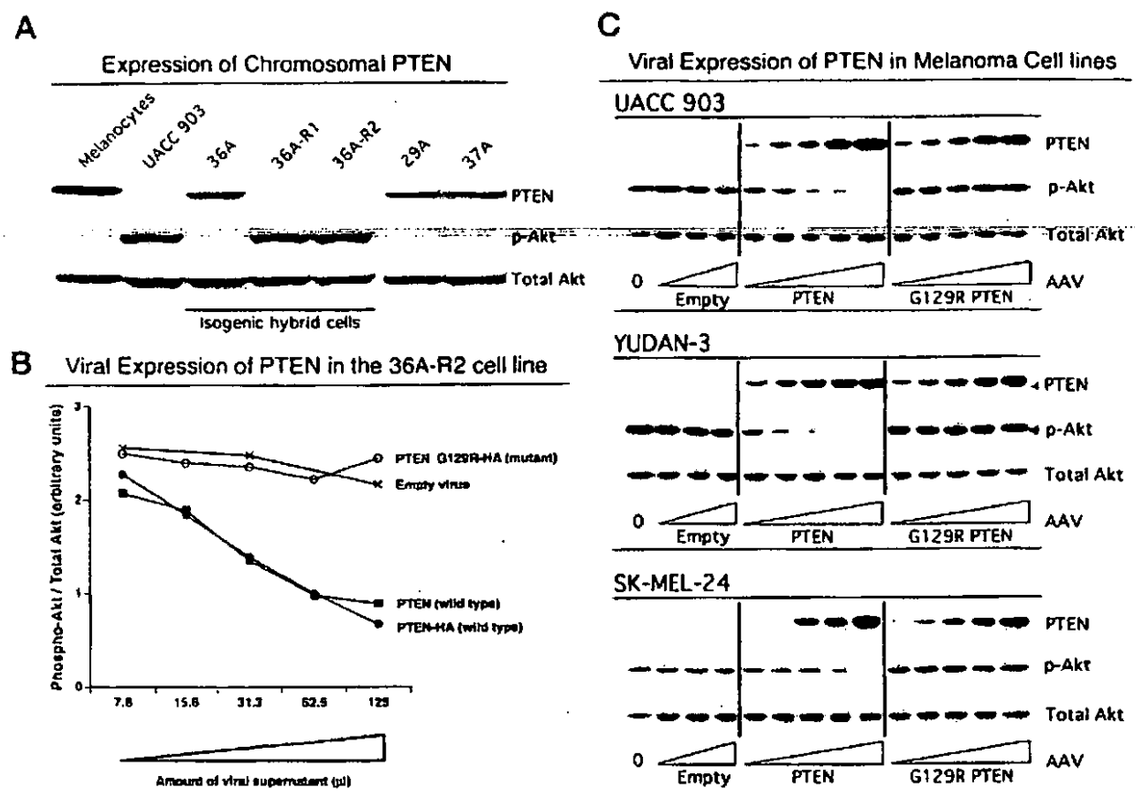


Fig. 3. Regulation of Akt activation by PTEN in melanoma cell lines. *A*, Western blot analysis showing the consequence of chromosomal PTEN expression on Akt phosphorylation (activity). Melanocytes serve as a control for normal tissue. The 36A, 36A-R1, and 36A-R2 are genetically related cell lines are only differing in PTEN expression. *B*, adeno-associated viral expression of PTEN in the revertant 36A-R2 cell line. The ratio of phosphorylated to total Akt was determined after viral infection using increasing volumes of virus expressing PTEN, PTEN tagged with HA, catalytically inactive mutant PTEN G129R, or empty virus. *C*, expression of viral PTEN in the melanoma cell lines UACC 903, YUDAN-3, and SK-MEL-24. The expression of PTEN, phosphorylated-Akt, and total Akt are shown after infection with increasing volumes of viral lysate (7.8, 15.6, 31.3, 62.5, and 125 µl), whereas no infection or infection with the empty virus (7.8, 31.3, and 125 µl of lysate) served as controls. These data are representative of a minimum of three to four separate experiments.

lines lacking functional PTEN protein, indicating that altered PTEN expression or activity plays an important role in regulating Akt activity in melanomas.

To determine whether the altered Akt activity mediated by PTEN led to changes in apoptotic signaling, the level of cleaved caspase-3 was measured after ectopic viral expression of PTEN in melanoma cells. Elevated levels of cleaved caspase-3 indicating higher levels of apoptosis (50) were observed after expression of wild-type, but not mutant G129R PTEN in UACC 903 cells, suggesting that PTEN expression increases apoptosis in melanomas (Fig. 4*A*). To eliminate the possibility that viral overexpression of PTEN led to elevated levels of apoptosis, the apoptotic rates were compared between UACC 903 cells lacking PTEN protein or hybrid cells expressing chromosomal PTEN protein using the TUNEL assay. The percentage of apoptotic cells under different serum conditions and after treatment with the proapoptotic agent staurosporine are shown in Fig. 4. No difference in apoptotic rates was observed between cells lacking or expressing chromosomal PTEN protein when grown under normal conditions in DMEM supplemented with 10% serum (Fig. 4*B*). This is illustrated by comparison of apoptosis rates between the genetically matched 36A and 36A-R2 cell lines that showed no significant difference in apoptosis (Student's t test; $P \geq 0.119$). In contrast, Fig. 4*C* shows that lowering the serum concentration to 1% for 48 h to induce apoptosis led to ~4-fold higher rates of apoptosis in PTEN-expressing cells compared with cells lacking PTEN (Student's t test; $P < 0.001$). Fig. 4*D* shows that similar results were observed after 4 h growth in medium supplemented with 10% serum and 1 µM staurosporine, which led to 2–7-fold higher rates of apoptosis in PTEN-expressing cells *versus* those lacking the protein (Student's t test; $P < 0.001$). This observation was confirmed by Western blot analysis of cell lines treated with 1 µM staurosporine for 4 h (Fig. 4*E*). Hybrid cell lines 36A, 29A, and 37A expressing chromosomal PTEN had elevated levels of cleaved caspase-3 compared with parental UACC 903 cells or the 36A revertant cell lines after treatment with staurosporine. Collectively, these results demonstrate that expression of PTEN increases susceptibility of melanoma cells growing in culture to apoptotic stimuli.

Because the foregoing experiments demonstrated that PTEN sensitized melanoma cells to apoptotic stimuli *in vitro*, subsequent studies focused on establishing whether increased apoptosis was the mechanism underlying *PTEN*-mediated tumor inhibition *in vivo*. Twenty million cells of the nontumorigenic (36A and 36B) or 1 million of the tumorigenic cell lines (UACC 903 and 36A-R2) were injected s.c. into 4–6-week old female nude mice to temporally and spatially match tumor development. Tumor cell masses developing in parallel from each cell type were then harvested every 2 days, starting at day 2 and ending at day 12, and the rates of apoptosis, growth, and vascular development were compared. A comparison of the number of apoptotic cells in tumor masses 4 days after injection into nude mice is shown in Fig. 5*A*. At each time point, more apoptotic cells were

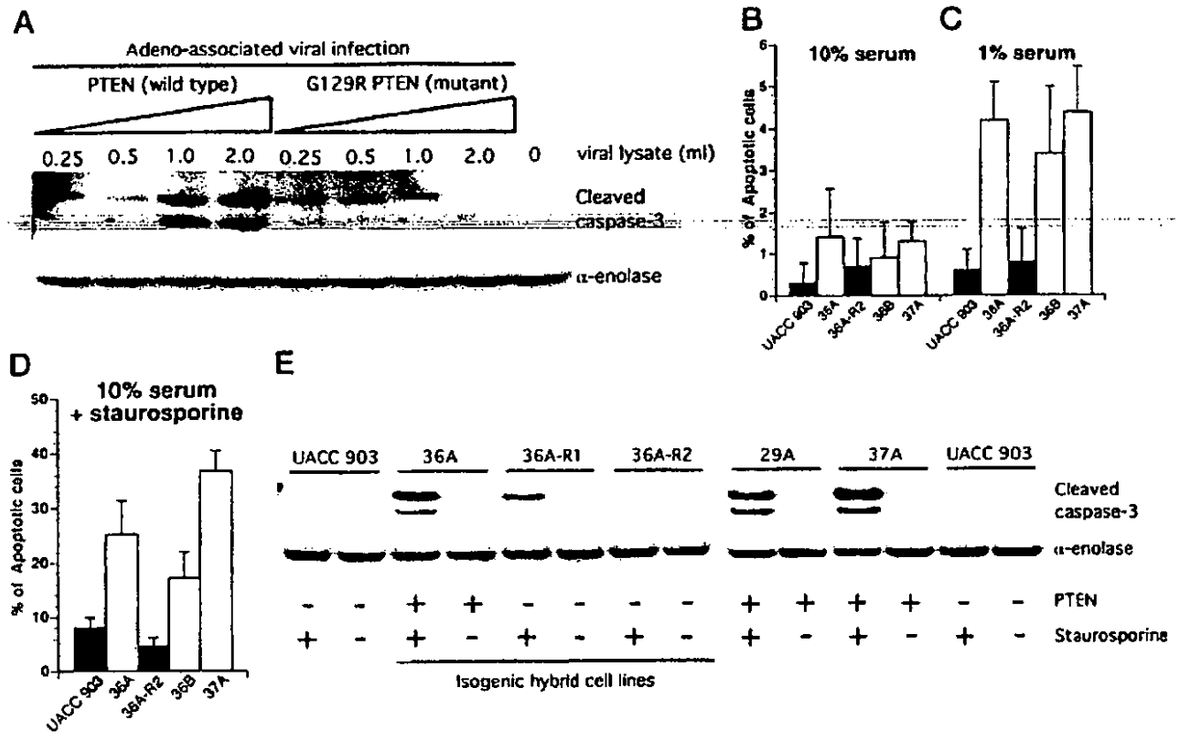
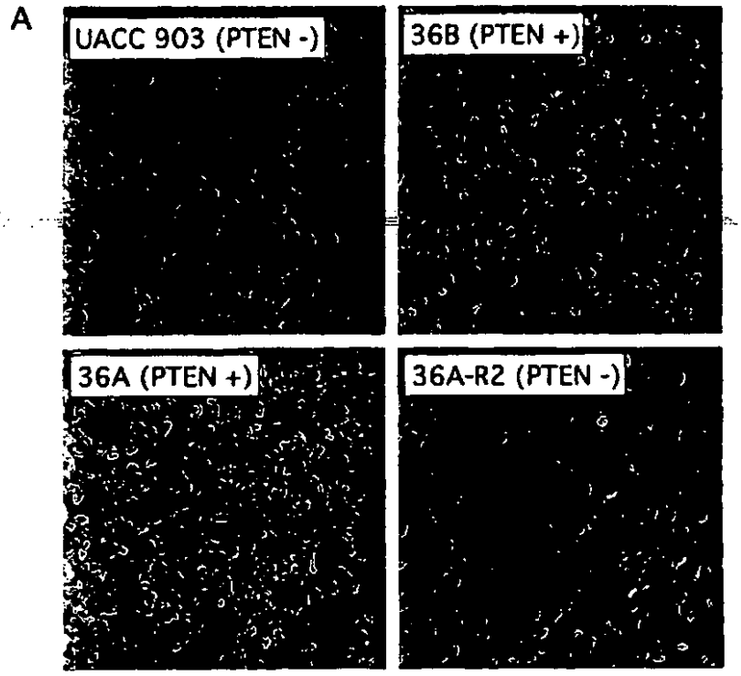


Fig. 4. Measurement of apoptosis in melanoma cell lines expressing ectopic or chromosomal PTEN. *A*, adeno-associated viral expression of wild-type and inactive mutant G129R PTEN in UACC 903 cells. Increasing amounts of viral lysates (0.25, 0.5, 1, and 2 ml) were used for infection. Western blots showing cleaved caspase-3 (17 and 19 kDa products) and α-enolase (loading control). The percentage of apoptotic cells in cell lines UACC 903, 36A, 36A-R2, 36B, and 37A was measured using the TUNEL assay after growth in medium supplemented with: *B*, 10% serum; *C*, 1% serum; or *D*, 10% serum together with 4-h exposure to 1 μM staurosporine. Percentage of apoptotic cells = number of apoptotic cells/total number of cells counted × 100%. Values are means; *bars*, ±SD. *E*, Western blot analyses of staurosporine-treated cells (1 μM for 4 h) using the primary antibody to caspase-3. PTEN protein expression is indicated for each cell line and α-enolase is used as the loading control. Data are representative of a minimum of two separate experiments.

observed in the hybrid 36A and 36B tumor masses than in tumors formed from the UACC 903 or 36A-R2 cell lines. The percentage of apoptotic cells at 2-day intervals for each cell line is quantified in Fig. 5*B*. In general, PTEN-expressing cells (36A and 36B) had 3–6-fold higher rates of apoptosis compared with cells lacking PTEN protein (UACC 903 and 36A-R2). However, day 2 is the most significant time point for these comparisons, because cells have just been introduced to the *in vivo* environment and are reflecting the stimuli experienced in the new environment. Comparison of the apoptotic rates between the genetically matched cell lines 36A and 36A-R2 at each time point were found to be statistically significantly different from one another (Student's *t* test; $P < 0.001$). Although apoptotic rates for all of the cell lines declined to constant levels by day 8, the rates of apoptosis for PTEN-expressing cells remained 2–3-fold higher than cell lines lacking the protein. In contrast to the differences in apoptosis, the rate of growth (Fig. 5*C*) and vascular development (Fig. 5*D*) were not found to be significantly different at day 2. These observations led to the conclusion that elevated levels of apoptosis were inhibiting tumor development by melanoma cells expressing PTEN. In additional support of this conclusion, Fig. 4*C* shows that whereas the rate of cell growth for cell lines lacking PTEN increased steadily from day 4 onwards, the number of dividing cells remained constant in tumor masses established from cells expressing PTEN protein (Student's *t* test; $P > 0.05$). In addition, Fig. 4*D* showed that the number of vessels present in the tumor masses remained relatively constant for all of the cell lines to day 6 after which a steady increase was observed in tumors developing from cells lacking PTEN compared with cells expressing PTEN protein. Thus, these results confirm that the regulation of Akt-mediated apoptosis by PTEN is critical in melanoma tumor development and that PTEN loss reduces apoptosis rates, thereby aiding melanoma tumorigenesis.

DISCUSSION

This study demonstrates the biological role that *PTEN* plays in melanoma tumorigenesis by exploiting chromosome transfer technology together with cancer cell evolution during tumor development to demonstrate the relationship between *PTEN* loss and melanoma tumor development. Because tumor development only occurred with a corresponding loss of PTEN expression, this model demonstrates that altered PTEN pathway signaling in melanomas is not just an *in vitro* phenomenon but important in melanoma tumor development. The melanoma cell line UACC 903 provided an ideal tool for establishing this model, because it had lost a copy of chromosome 10 and duplicated the remaining one that had a mutated PTEN gene (23). Therefore, both copies of the *PTEN* gene had an identical truncating point mutation leaving cells devoid of PTEN protein. The transfer of a normal copy of chromosome 10 into UACC 903 cells by microcell-mediated chromosome transfer resulted in PTEN protein levels at near to normal physiological levels. In addition, the *PTEN* gene was under normal regulatory control with the RNA undergoing appropriate processing although only a single functional copy of the gene was present. Therefore, this model also overcame the growth-inhibitory limitations often encountered after high ectopic expression of tumor suppressor



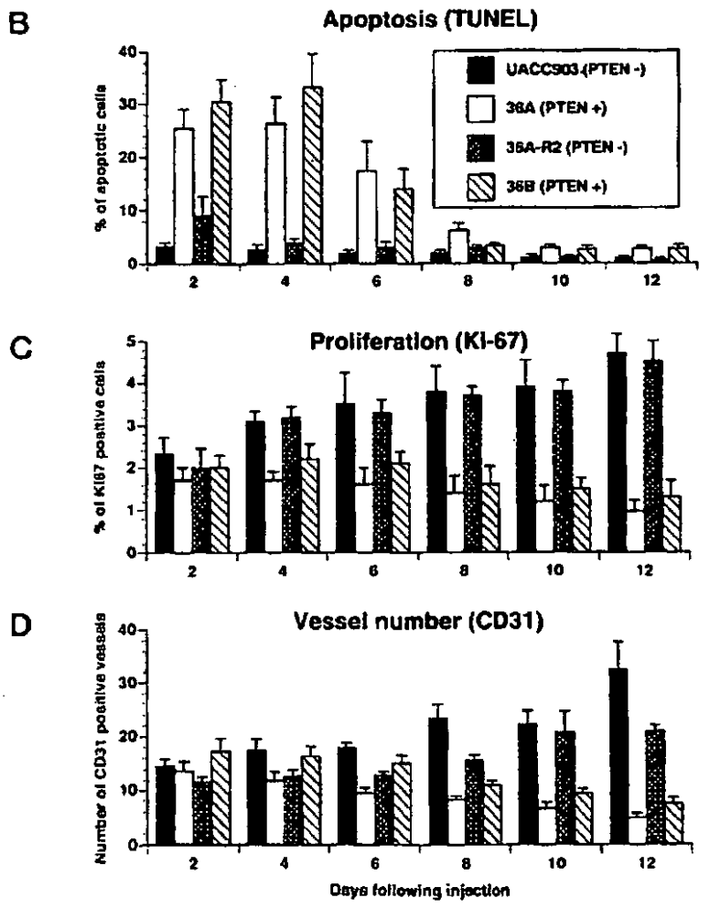


Fig. 5. Measurements of apoptosis, proliferation, and angiogenesis in melanoma tumor masses differing in PTEN expression. *A*, comparison of TUNEL-positive cells in tumor masses derived from UACC 903 parental cells, 36A and 36B cell lines expressing chromosomal PTEN, and the tumorigenic revertant 36A-R2 cell line lacking PTEN protein 4 days after injection into nude mice; magnification, ×200. *B*, apoptotic rates for each cell line were measured at 2-day intervals using TUNEL assays on formalin-fixed paraffin sections of the tumors. *C*, percentage of proliferating cells measured at 2-day intervals by scoring the number of Ki67-positive cells in formalin-fixed paraffin sections of the tumors. *D*, blood vessel density was measured at 2-day intervals by counting the number of CD31-positive vessels in frozen tumor sections. The data shown represent the mean of counts from two to four separate tumors; *bars*, ±SE.

genes in cancer cells using an active promoter element (23, 51). Thus, this is a biologically relevant model where PTEN loss occurs during tumor development *in vivo* and not as a consequence of evolution in tissue culture.

Although PTEN expression was at normal physiological levels in the hybrid cells, the levels were sufficient to exert a negative effect on melanoma cell growth in culture. This was evidenced by the genetic instability or IVLOH that we have reported previously after transfer of chromosome 10 into UACC 903 cells (23). The genetic heterogeneity of the cells undergoing IVLOH made them unsuitable for studying the role *PTEN* plays in melanoma tumor development (11). To overcome this obstacle, the negative effect that PTEN exerts on cell growth in culture was masked using growth factors present in serum to stimulate PI3K (48). As predicted, this led to activation of Akt (26–30) and resulted in antiapoptotic signaling (31). Masking IVLOH using growth factors present in serum resulted in the establishment of cell lines stably retaining the intact introduced chromosome. In contrast, introduction of these cells into an *in vivo* environment unmasked the effects of serum; therefore, cell growth and tumor development were retarded unless the protein expression was lost. Isolation of the cells that lost PTEN during tumor formation resulted in the establishment of genetically matched cell lines that could be used as tools for dissecting the signaling mechanisms leading to the evolution of these cells. Thus, these cell lines were used to confirm the involvement of *PTEN* in melanoma tumorigenesis resulting in a model to demonstrate PTEN regulation of melanoma tumor development.

During melanoma tumor development, it is common for an entire copy of chromosome 10 to be lost from the melanoma cells (2, 10). This occurs because multiple tumor suppressor genes residing on this chromosome must be lost in unison during tumor progression, and this is more efficiently accomplished by chromosome segregation than by multiple mutational and/or deletional events (11). Loss of the intact chromosome has hindered dissection of the role that the individual genes on chromosome 10 play in melanoma tumor development. However, this model has overcome that obstacle by allowing the biological consequence of a single gene to be dissected. Clearly, tumor development *in vivo* occurs after loss of PTEN protein from melanoma cells, thereby demonstrating the importance of this gene in melanoma tumorigenesis. Whereas the tumorigenic potential of the melanoma cells reverted to more closely resemble that of the parental UACC 903 cell line after *PTEN* loss, tumor development remained delayed by ~1 week because of retention of a second cancer suppressor gene located at the tip of the short arm at 10p15.3 (11). Therefore, the biological effect of each gene could be measured using this model. Ectopic expression of PTEN using adeno-associated viruses verified that the model was biologically accurate and that PTEN regulated the same signaling pathways as PTEN expressed from the transferred chromosome 10 present in hybrid cell lines. Hence, this model has allowed the effect of the *PTEN* tumor suppressor gene to be measured individually and in combination with the others on chromosome 10, thereby confirming *PTEN* involvement in melanoma tumorigenesis.

The regulation of cell survival and cell death is an essential process in tumor development (50, 52). The balance of signals that promote or impair cell survival defines tissue homeostasis, and mutated cells that have escaped the constraints of normal growth regulation can lead to tumor development (50, 52). In this report, we have shown that PTEN is one factor regulating melanoma tumor development by controlling Akt-mediated cell survival. In melanocytes, PTEN expression blocks Akt activation, thereby regulating normal cellular apoptotic signaling. In contrast, PTEN dysfunction in melanomas leads to up-regulation of Akt signaling, thereby reducing apoptosis rates. Thus, melanoma cells lacking functional PTEN protein are better suited for surviving in the *in vivo* tumor environment. Therefore, the model we developed demonstrates the role of *PTEN* in melanomas by showing that restoring functional PTEN protein resulted in reduced Akt signaling and consequently prevented tumor growth in animal models.

This model has enabled a better understanding of the role *PTEN* dysfunction plays in melanoma tumor development by supporting and extending existing models for PTEN involvement in melanoma tumor progression. Although studies of cultured melanoma cells have found deletions or mutations of *PTEN* in up to 60% of cell lines, only 10% of patient tumors contain inactivating mutations. However, additional studies have suggested that other mechanisms leading to inactivation of PTEN such as epigenetic alteration or altered subcellular localization might play a more prominent role in melanomas. Taken together, loss of functional PTEN may occur in as many as 40–50% of sporadic melanomas (13–22, 41). For those melanomas expressing catalytically active PTEN at normal physiological levels, it is possible that other members of the PTEN signaling cascade could have altered activity or might be functionally inactivated. This raises the possibility that other potentially important, as yet unidentified, alterations in proteins upstream or downstream of PTEN play an equally important role in cutaneous melanomas lacking *PTEN* alteration. Future studies will determine whether other components of the pathway are also altered. Furthermore, the results presented here suggest that *PTEN* replacement by viral administration may prove to be a useful and important therapeutic approach for inducing apoptosis in melanomas (50, 52). In support of this possibility, a recent report has suggested that adenoviral-mediated *PTEN* gene therapy may be a promising treatment for melanoma tumors, even in those that express normal PTEN protein (53). In conclusion, this study establishes the biological significance of PTEN in the development of sporadic human malignant melanomas by demonstrating the importance of this gene in melanoma tumor formation and the underlying mechanism by which its loss aids this process.

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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 regimes 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/PKBα, Akt2/PKBβ and Akt3/PKBγ, 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 conucleofected 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 μg), 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 NaFl, 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 (≥10,000 × *g*) of lysates, and protein concentration was quantitated using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Protein for immunoprecipitation (100 μg) was incubated with 2 μg of Akt1 or Akt2 or 5 μL 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 μL 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 μL of equilibrated GammaBind G Sepharose beads were incubated with 2 μg of Akt1 or Akt2 antibody, or 5 μL of Akt3 antibody in a volume of 350 μL of lysis buffer at 4°C with constant mixing for ≥2 hours. Microcystin (1 μmol/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 μL of lysis buffer and then incubated with 100 μg of protein in a volume of 350 μL overnight at 4°C with constant mixing. This complex was washed with 500 μL of lysis buffer (3×) and then once with 500 μL 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 μmol/L) from Santa Cruz Biotechnology (Santa Cruz, CA), 37.5 μmol/L ATP, 17 mmol/L $MgCl_2$, 0.25 μCi/μL [γ-^{32}P]ATP, and 90 μmol/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 μL 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 (~12,000 × *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. 1*A*; 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. 1*B*). 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. 1*C*). 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 cells lines (WM115 and SK-MEL-24) to determine which

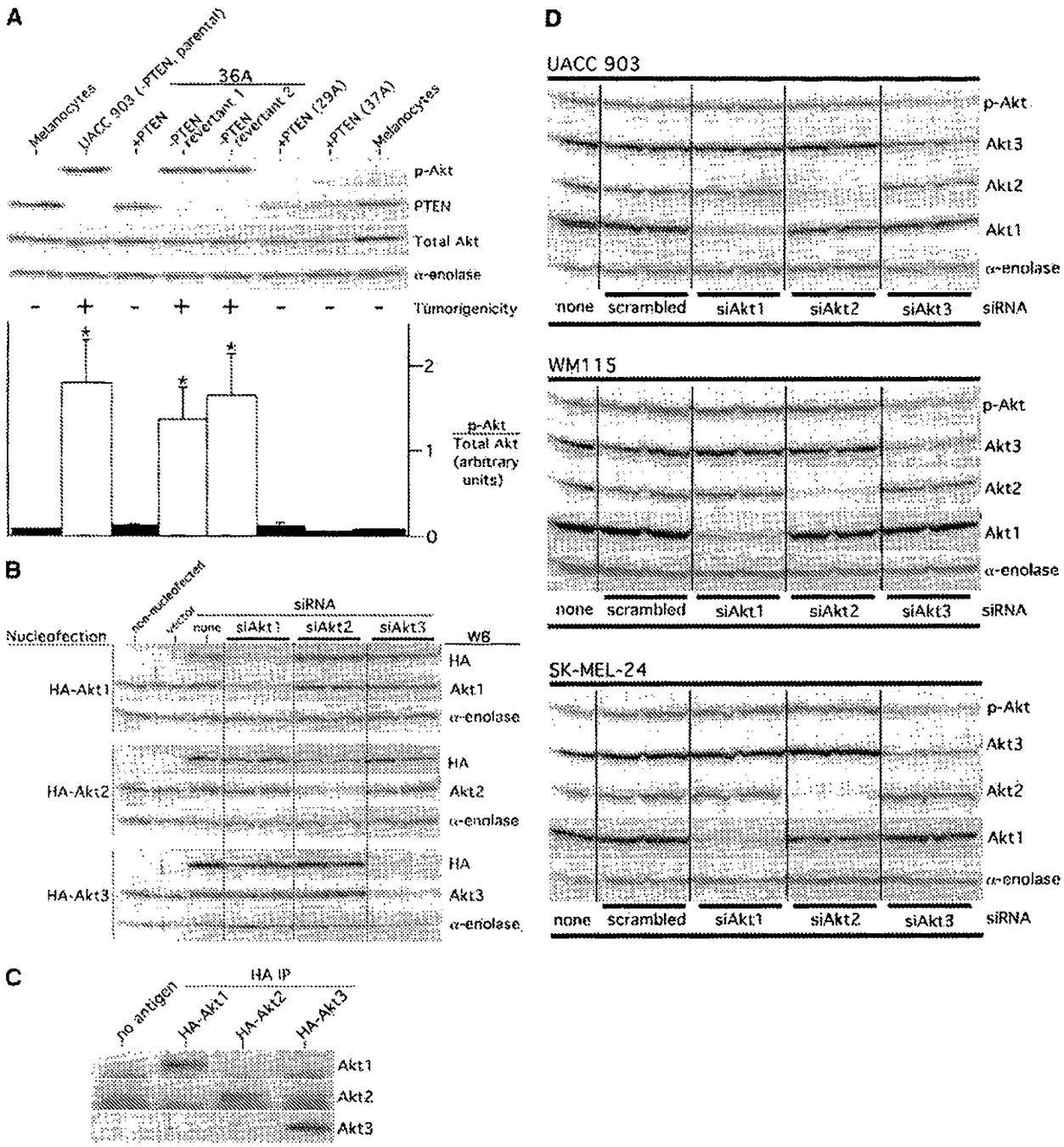
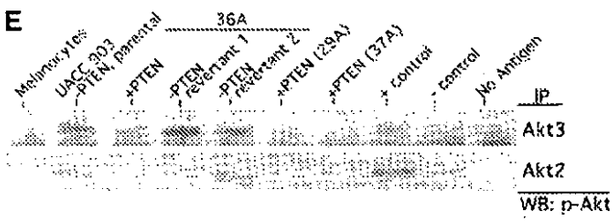


Fig. 1. Identification of Akt3 involvement in malignant melanoma. *A*, Akt activity in the melanoma cell line UACC 903 is regulated by PTEN. Western blot analysis showing expression of phosphorylated-Akt (Ser-473), total Akt, PTEN, and α-enolase (loading control). The 36A, 29A, and 37A cell lines are 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. 1*D*). 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. 1*E*). 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. 1*F*. A



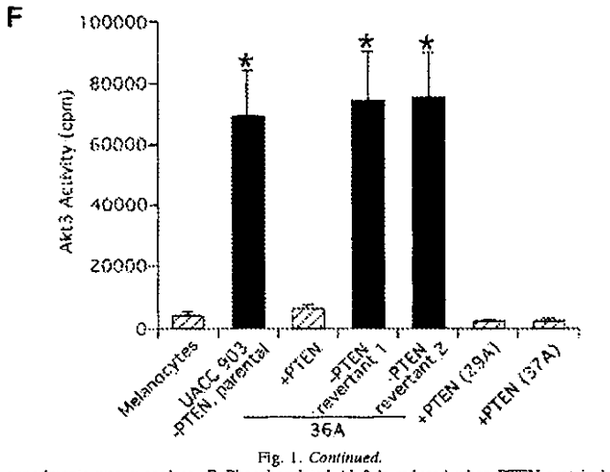


Fig. 1. *Continued.*

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*, ±*SEM*; statistics, one-way ANOVA followed by Dunnet's multiple comparisons *versus* the melanocyte control; *, $P < 0.05$.

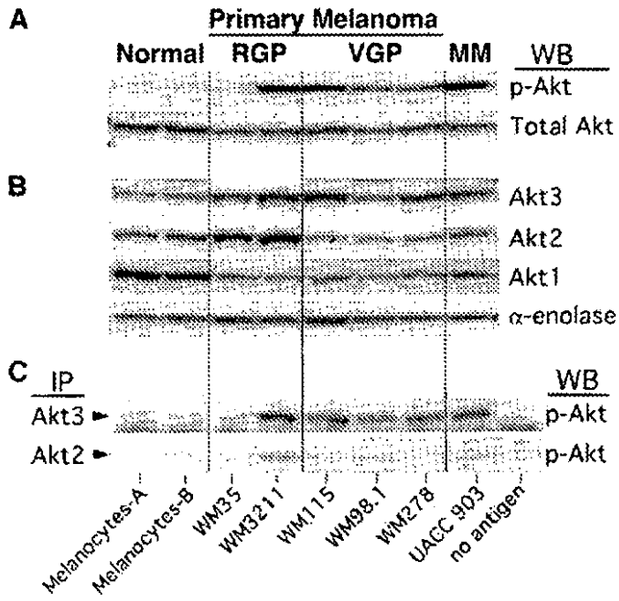


Fig. 2. Increased Akt3 expression and activity occur during melanoma tumor progression. *A*, An increase in the level of phosphorylated (active) Akt (*p-Akt*) occurs during the radial growth phase (*RGP*) in the melanoma tumor progression model. Western blot (*WB*) comparing the amount of phosphorylated 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. 1*F*), 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. 2*A* 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. 2*B*). 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. 2*C*). 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. 2*C*). 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. 2*B*). 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) tumorigenesis 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. 3*A*. 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. 3*B*). 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.

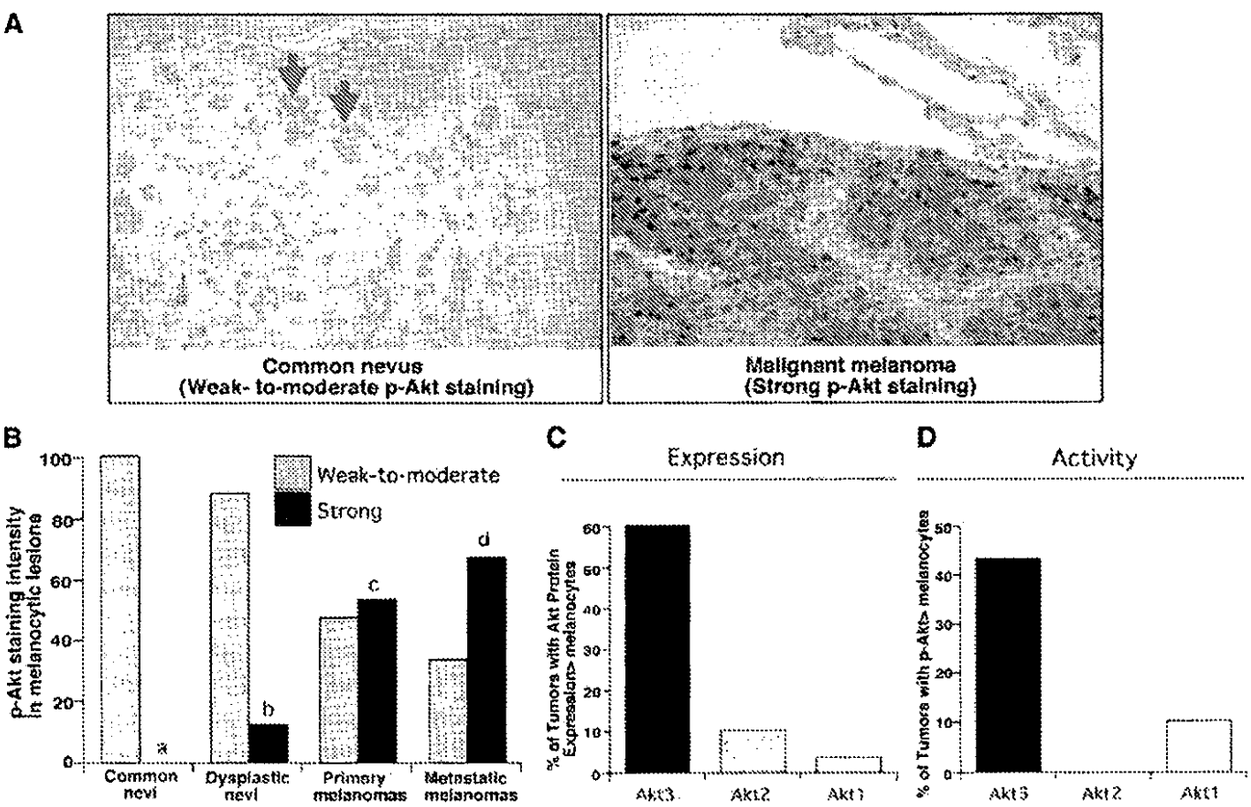


Fig. 3. Akt3 activity is increased in tumors from melanoma patients. *A* and *B*, relative intensity of phosphorylated Akt (*p-Akt*) staining in common nevi, dysplastic nevi, primary melanomas, and metastases from melanoma patients. *A*, Staining intensity was scored as weak-to-moderate or strong. Weak-to-moderate 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. 3*C*). 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. 3*D*). 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. 2*C*) and express PTEN protein. As predicted, Fig. 4*A* and *B* show that siRNA-mediated down-regulation of PTEN led to an increase in total phosphorylated Akt (Fig. 4*A* and *B*, *Lanes 4* and *11*), whereas a scrambled siRNA control exerted a negligible, nonsignificant effect (Fig. 4*A* 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. 4*A* and *B*, *Lanes 5* and *12*), Akt2 (Fig. 4*A* and *B*, *Lanes 6* and *13*), or Akt3 (Fig. 4*A* and *B*, *Lanes 7* and *14*). Only siRNA directed against Akt3 (Fig. 4*A* and *B*, *Lanes 7* and *14*) lowered the level of phosphorylated Akt to that observed in non-nucleofected cells (Fig. 4*A* and *B*, *Lanes 1* and *8*) or cells nucleofected with scrambled siRNA only (Fig. 4*A* 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 μg 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. 4*C*). Cells were then starved of growth factors for 24 hours and replenished with complete media, and then lysates were harvested 30 minutes later.

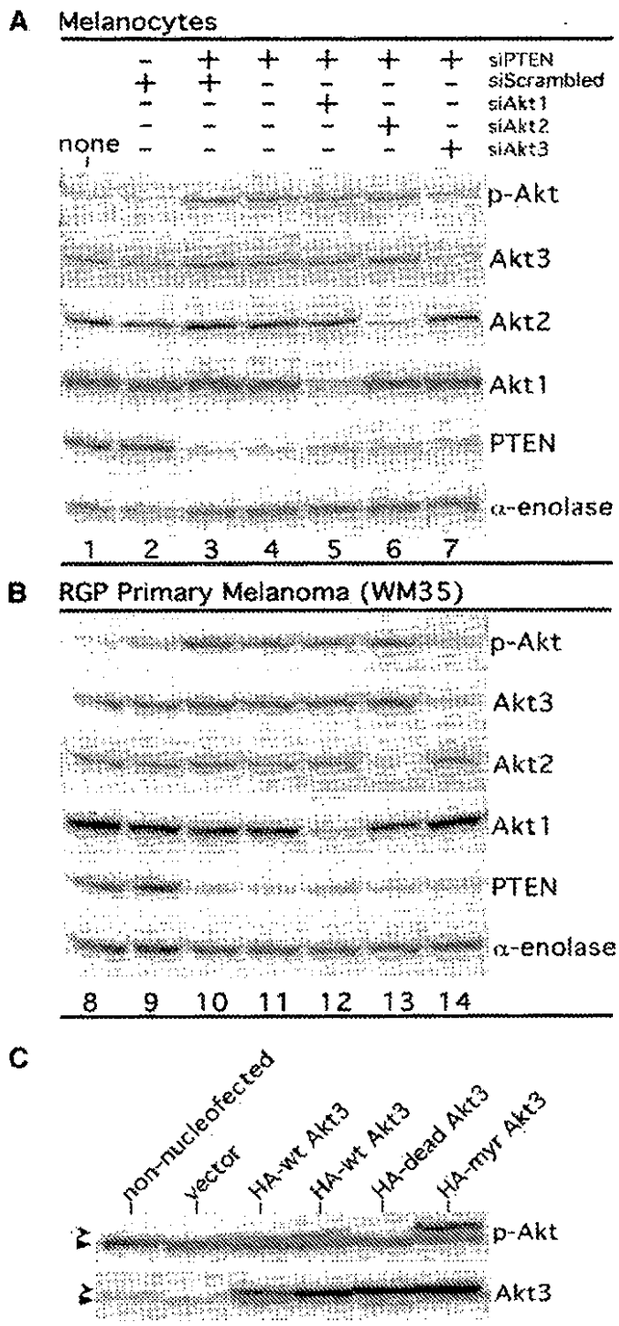


Fig. 4. Mechanism underlying deregulated Akt3 activity in malignant melanomas. Decreased PTEN expression (activity) specifically increases Akt3 activity in melanocytes (*A*) and WM35 [radial growth phase (*RGP*)] cells (*B*). siRNA-mediated reduction of PTEN is shown alone (control) or in combination 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. 5*A* 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. 5*B* 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. 5*E* 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. 5*F*). Thus, either specifically reducing Akt3 activity using siRNA against Akt3 (Fig. 4*B*) or increasing PTEN expression (Fig. 4*A*) 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 NH_2-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, PKCζ-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

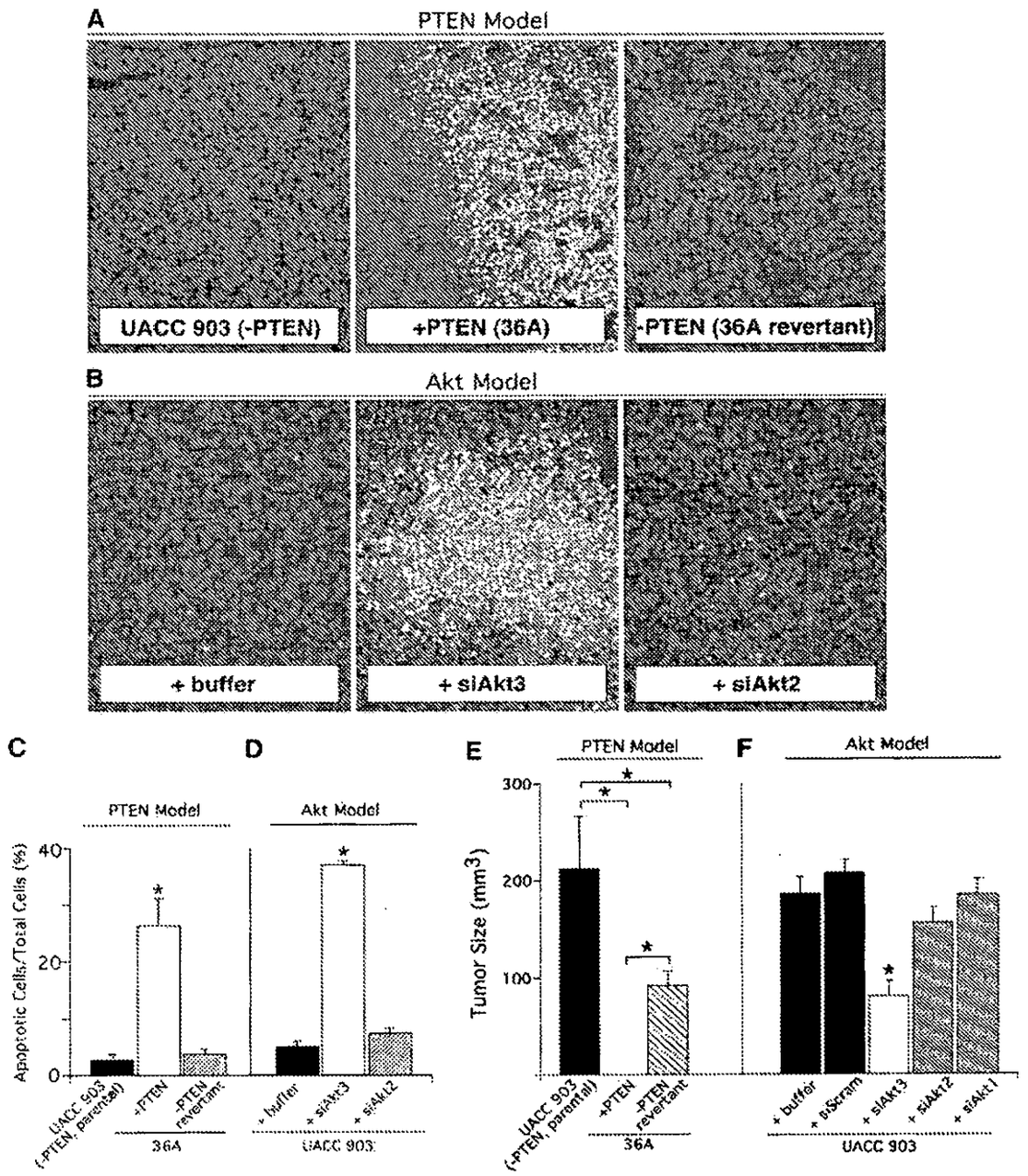


Fig. 5. Increased Akt3 activity promotes melanoma tumor development by reducing apoptosis rates. *A* through *D*, PTEN or siRNA-mediated reduction of Akt3 increases apoptosis in tumors growing in nude mice. Photographs (*A* and *B*) and quantification (*C* and *D*) of TUNEL-positive cells in tumor masses derived from UACC 903 cells expressing PTEN (36A) or nucleofected with siRNA to Akt3 and Akt2; *bars*, ±*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, ×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*, ±*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*, ±*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-

phatc 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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