[CANCER RESEARCH 63, 2881–2890, June 1, 2003]

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

Received 12/5/02; accepted 3/27/03.

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[1] Supported by The Foreman Foundation for Melanoma Research and The Pennsylvania State University Cancer Institute Research Grant Program.

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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) $\times$ 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 ($\geq$10,000 $\times$ *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 mm^3)			
	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 mm^3 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

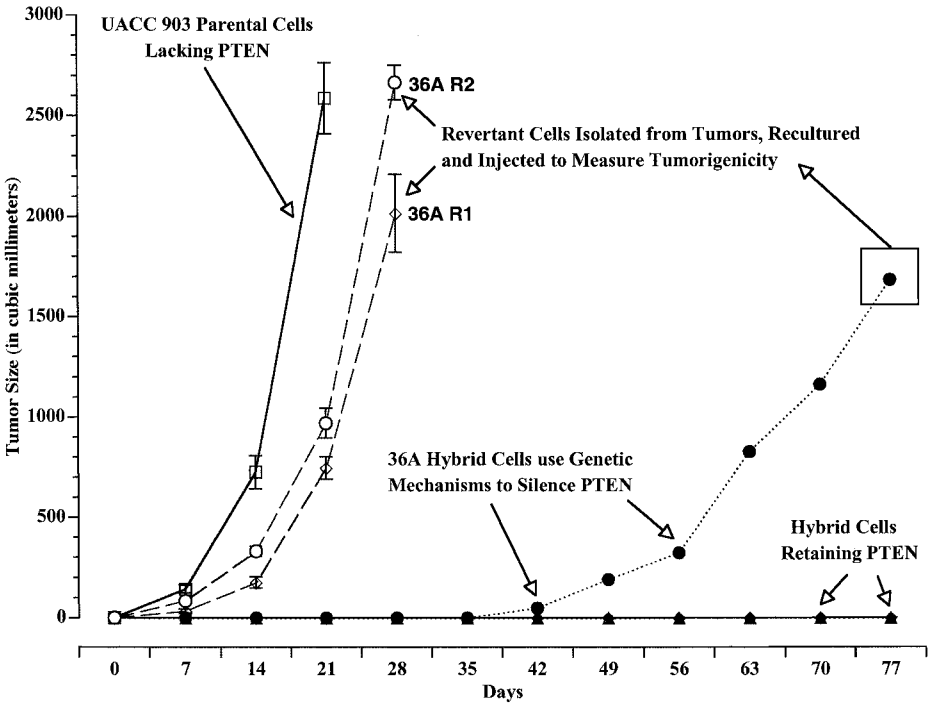

Fig. 1. Tumor development by melanoma cell line UACC 903 and chromosome 10 hybrid cell lines. Size of tumors formed by parental UACC 903 melanoma cells and chromosome 10 hybrid cell lines after injection into nude mice: UACC 903 cells (□); hybrid cells retaining PTEN (♦, ▲); hybrid cells that had lost 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.

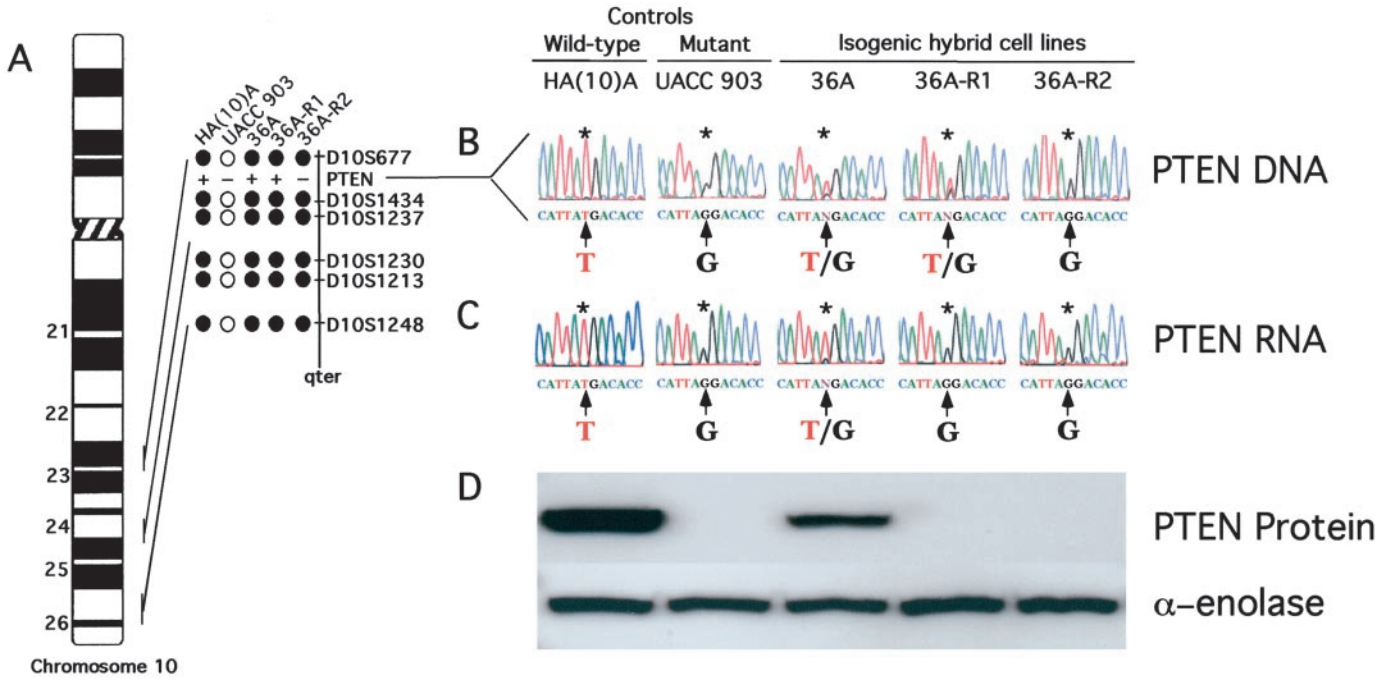

Fig. 2. Status of *PTEN* in UACC 903 cells and chromosome 10 hybrid cell lines. *A,* partial genotypic analysis spanning the distal third of the introduced chromosome 10 is shown for UACC 903 cells, chromosome 10 hybrids, and the donor cell line HA(10)A. STS PCR-based molecular markers spaced at ~10 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

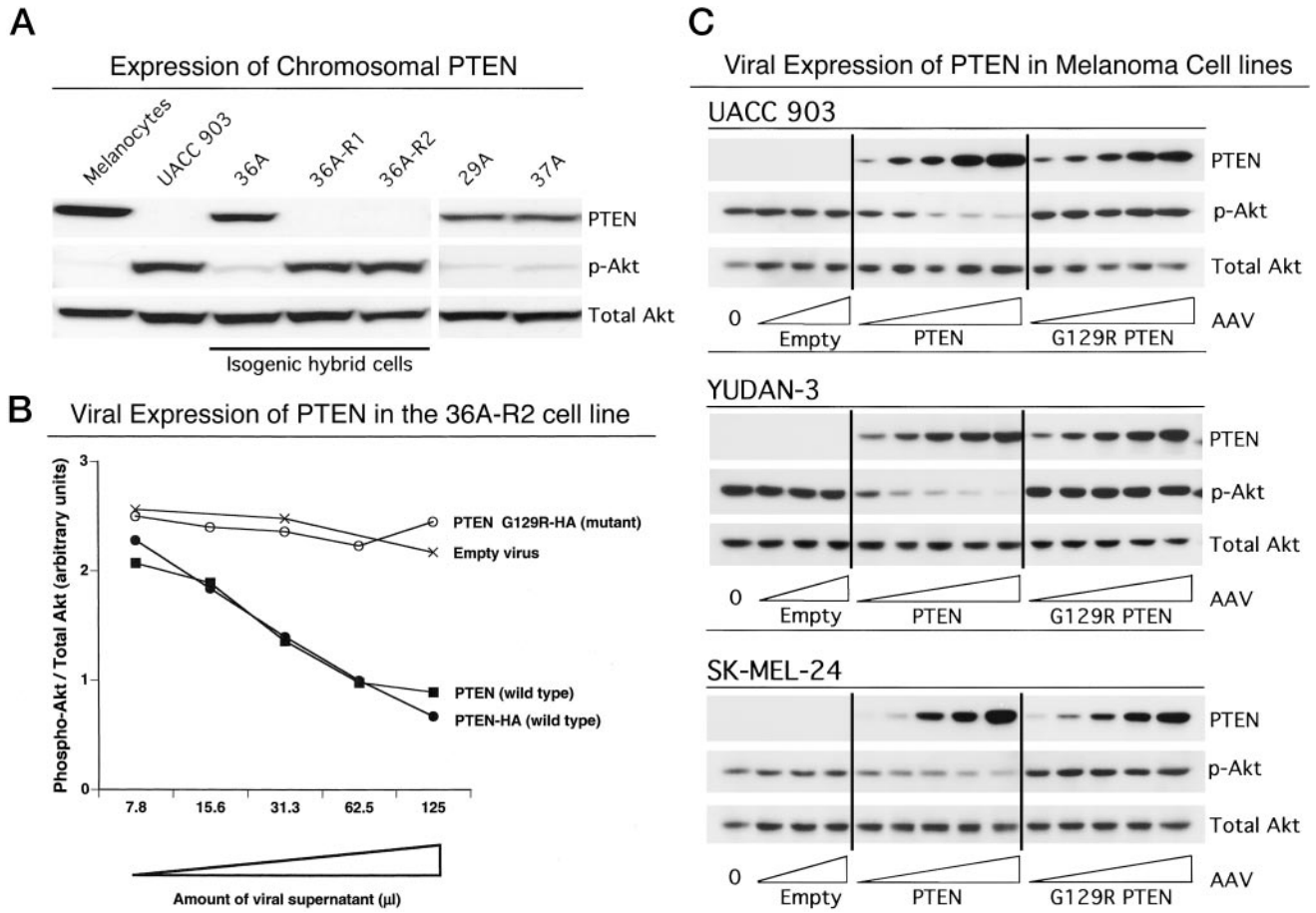

Fig. 3. Regulation of Akt activation by PTEN in melanoma cell lines. *A*, Western blot analysis showing the consequence of chromosomal PTEN expression on Akt phosphorylation (activity). Melanocytes serve as a control for normal tissue. The 36A, 36A-R1, and 36A-R2 are genetically related cell lines are 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

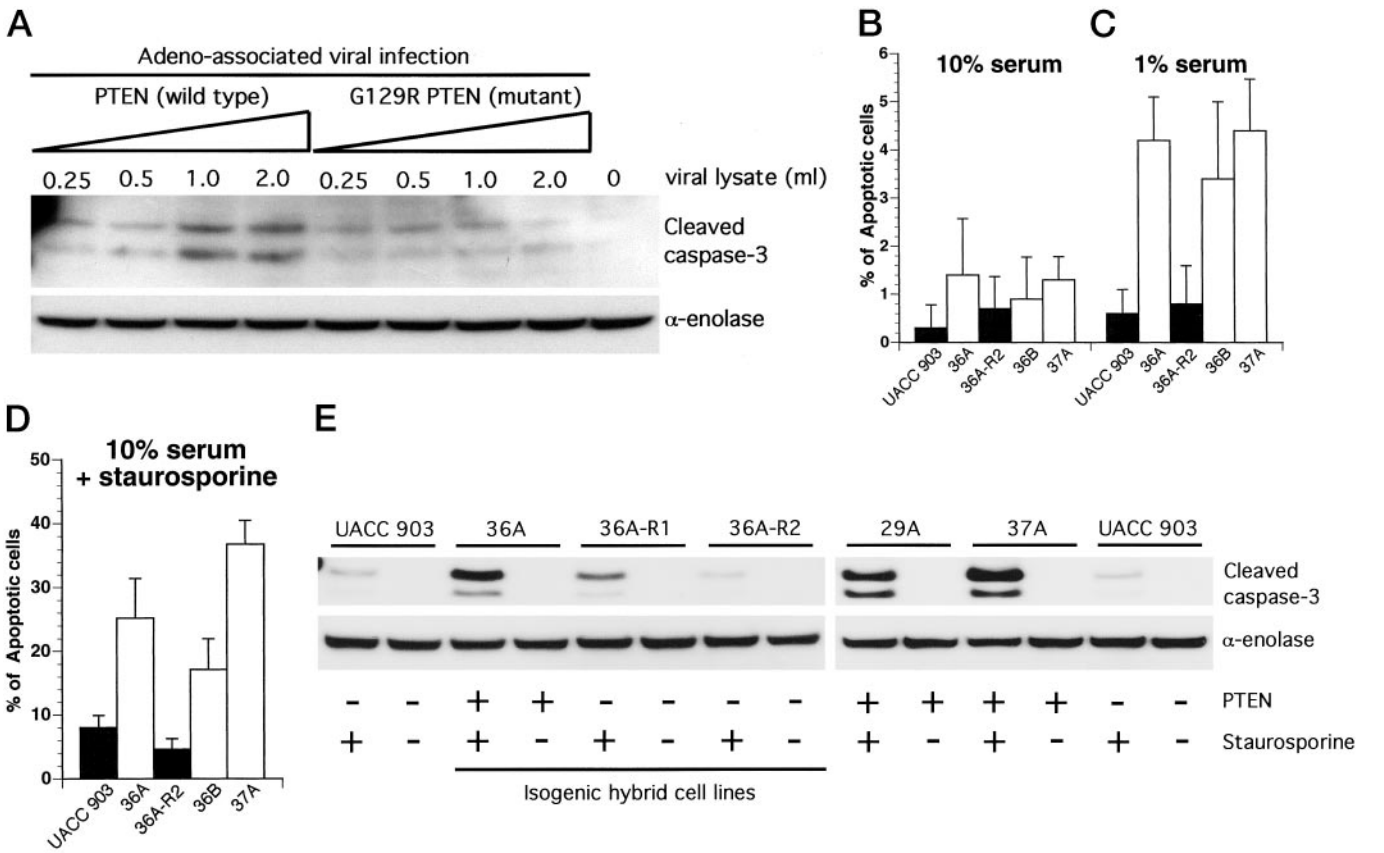

Fig. 4. Measurement of apoptosis in melanoma cell lines expressing ectopic or chromosomal PTEN. *A*, adeno-associated viral expression of wild-type and inactive mutant G129R PTEN in UACC 903 cells. Increasing amounts of viral lysates (0.25, 0.5, 1, and 2 ml) were used for infection. Western blots showing 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

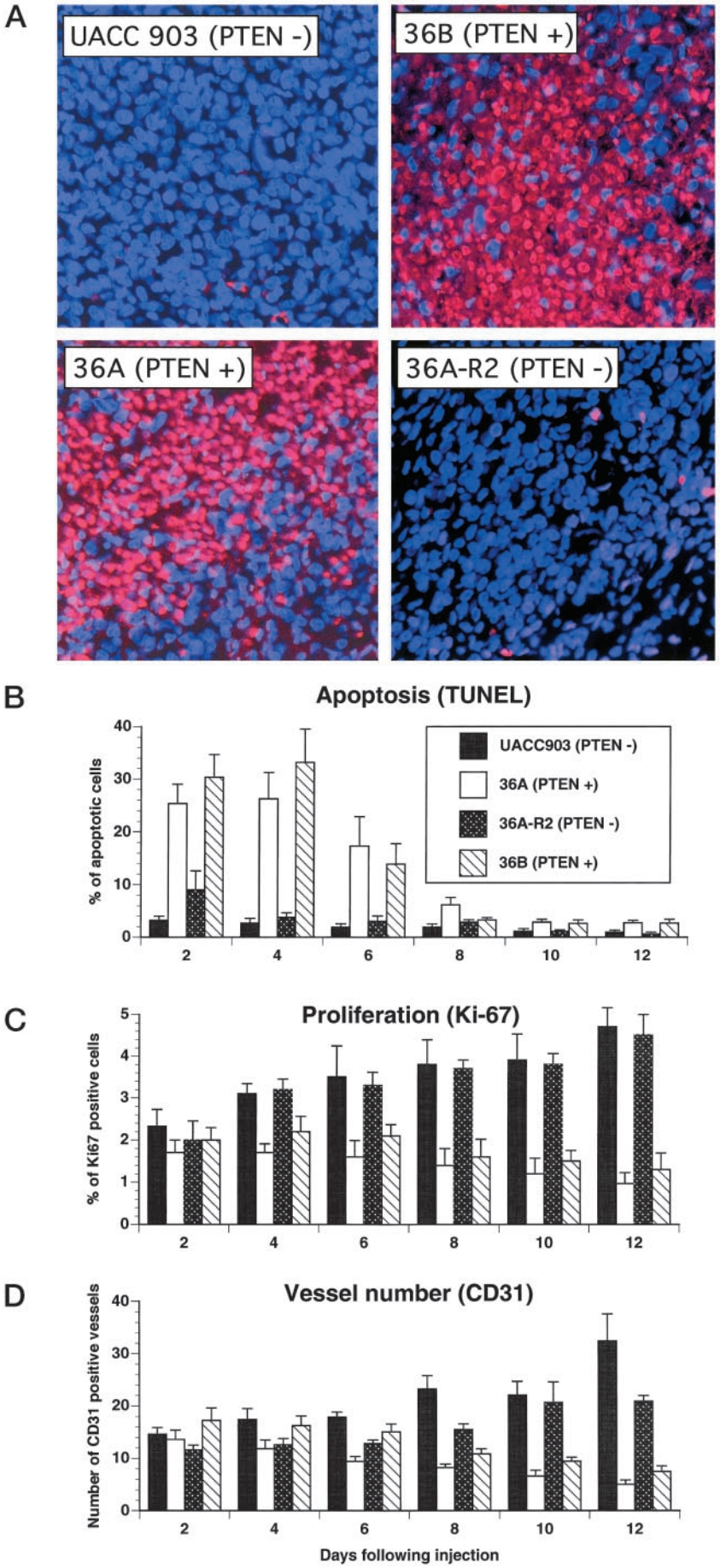

Fig. 5. Measurements of apoptosis, proliferation, and angiogenesis in melanoma tumor masses differing in PTEN expression. *A*, comparison of TUNEL-positive cells in tumor masses derived from UACC 903 parental cells, 36A and 36B cell lines expressing chromosomal PTEN, and the tumorigenic revertant 36A-R2 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.

ACKNOWLEDGMENTS

We thank Dr. Lakshman Sandirasegarane for helpful discussions and for critical reading of this manuscript, Melissa Zimmerman for technical assistance, and Dr. Elliot Vessel for proofreading of this manuscript.

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Cancer Res 2003;63:2881-2890.