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PI3K Blockade by Ad-PTEN Inhibits Invasion and Induces Apoptosis in Radial Growth Phase and Metastatic Melanoma Cells

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Abstract

Background: Melanoma is an aggressive tumor with a propensity to rapidly metastasize. The PTEN gene encodes a phosphatase with an unusual dual specificity for proteins and lipids. Mutations of PTEN have been found in various human cancers, including glioblastoma, prostate, breast, lung, and melanoma. Here we investigate *in vitro* the effects of blocking PI3K signaling using adenoviral-delivered PTEN (Ad-PTEN) in cell lines derived from both early- and late-stage melanoma.
Materials and Methods: Ad-PTEN transduced melanoma cell lines or normal cells were assayed for cell death, apoptosis, gene expression, invasion and migration, and regulation of angiogenesis.
Results: The PTEN locus from RGP and metastatic melanoma cell lines was sequenced; no coding region mutations were found. Adenoviral transfer of PTEN into melanoma cells containing wild-type PTEN alleles led to tumor-specific apoptosis and growth inhibition, with coordinate inhibition of AKT phosphorylation. Ad-PTEN suppressed cell migration by metastatic melanoma cells with concomitant increase in the level of cell surface E-cadherin. Immunohistochemical and confocal analyses localized PTEN to the cytoplasm and demonstrated enrichment at the cell membrane. Ad-PTEN inhibited angiogenesis as demonstrated by the tube formation assay using human vascular endothelial cells.
Conclusions: These studies indicate that Ad-PTEN can inhibit tumor cells via multiple mechanisms and has pro-apoptotic, anti-metastatic, and anti-angiogenic properties. Thus, PI3K blockade via Ad-PTEN may be a promising approach for the treatment of early- and late-stage melanoma, even in tumors that do not harbor PTEN mutations.

Introduction

Melanoma is an aggressive human cancer whose incidence is increasing more rapidly than any other cancer in the United States (1,2). Based on clinical and histopathologic studies, it has been proposed that melanoma develops and progresses in a sequential fashion. In this model, the first step is the common acquired and congenital nevi with structurally normal melanocytes, followed by dysplastic nevus with structural and architectural atypia. Genetic changes are often associated with progression to radial growth phase (RGP) primary melanoma as well as to the next stage of vertical growth phase or invasive primary melanoma, considered to possess competence for metastatic spread. Unlike most other human cancers, melanoma affects a much younger population and is characterized by an extremely high potential to develop metastases (2,3). Currently, there is no conventional therapy for metastatic melanoma that provides a response rate greater than 20%. New therapeutic regimens for malignant melanoma are therefore urgently needed.

Genetic lesions of chromosome 10 have been observed in 30–50% of early and advanced-stage melanomas (4–7). PTEN was first identified as a candidate tumor suppressor gene at chromosome 10q23, a region frequently deleted or mutated in human glioblastoma, prostate, kidney, and breast cancer (8,9). PTEN was also independently discovered as a novel protein tyrosine phosphatase regulated by tumor growth factor beta (10). Since then, a significant number of PTEN mutations have been reported in other human malignancies including

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endometrial, lung, bladder, testis, head and neck cancers, melanoma, and lymphoma (11–14). In malignant melanoma, mutations of PTEN have been found in approximately 40% of tumors (5,15,16). Germ-line mutations of PTEN have also been found in patients with juvenile polyposis coli, Cowden disease, and the rare Bannaya–Zonana syndrome (18–21), underscoring the critical role of PTEN in the prevention of tumorigenesis. Furthermore, the phenotype of PTEN-knockout mice confirmed the role of PTEN as a tumor suppressor (22,23).

The product of the PTEN gene is a 55-kDa protein with significant homology to protein phosphatases and tensin (8–10). Most of the tumor-associated PTEN mutations are clustered at the phosphatase domain, suggesting that the phosphatase activity of PTEN plays a critical role in controlling tumorigenesis (21,25–26). PTEN is able to dephosphorylate focal adhesion kinase (FAK), a key molecule governing cell–cell adhesion, and Shc, also associated with regulation of cell spreading and migration. The lipid phosphatase activity of PTEN acts on the lipid phosphatidylinositol phosphates, the primary products of the phosphatidylinositol 3-kinase (PI3K) (13,27). The latter activity of PTEN is critical for its tumor suppressor function, because a mutation in Cowden disease specifically abolishes the lipid phosphatase activity of PTEN (28). Furthermore, the phosphorylation of the AKT/PKB oncogene, a protein serine/threonine kinase and a downstream target of PI3K, is also inhibited by PTEN (29–33).

Despite recent advances in our understanding of PTEN mutations in disease progression, the therapeutic value of PI3K inhibition via PTEN augmentation as an anti-melanoma strategy has not been investigated. We report here that the adenoviral transfer of PTEN into melanoma cells that contain wild-type PTEN alleles results in PI3K inhibition, growth inhibition, and apoptosis. This is evident in cells derived from both early-stage as well as metastatic melanoma. Overexpression of PTEN in these cells suppressed the activation of AKT/PKB and increased expression of E-cadherin on the cell surface, and inhibited cell invasion and migration in metastatic melanoma cells. Immunohistochemical analysis of adenoviral-delivered PTEN (Ad-PTEN) transduced cells localized PTEN to the cell membrane and the cytoplasm. Confocal imaging indicated that supraphysiologic expression levels of PTEN resulted in enrichment in adhesion plaques. Furthermore, Ad-PTEN potently inhibited endothelial differentiation, an *in vitro* correlate of angiogenesis. Thus, PI3K blockade via Ad-PTEN can inhibit growth and spread of tumor cells via multiple mechanisms, and may provide a useful tool in the armamentarium to fight this deadly disease.

Materials and Methods

Cell Lines and Cell Culture

Melanoma cell lines used in this study have been previously described (34). Cells were grown in DMEM medium (Gibco, Rockville, MD, USA) with the addition of 10% fetal bovine serum (FBS) and routinely harvested with 0.125% Trypsin −1.3 mM EDTA (Gibco). Normal human epidermal melanocytes (NHEM) and human vascular endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA, USA) and were used in the supplier-recommended media. Experiments were performed during the logarithmic phase of cell growth.

Adenovirus Production

The replication-deficient human type 5 adenovirus carrying the PTEN gene was constructed by subcloning the full-length PTEN cDNA from a plasmid construct, pCDNA3-PTEN (obtained from Dr. David Snary, ICRT) into a shuttle plasmid containing the CMV promoter and SV40 poly (A) elements. The resulting PTEN-containing plasmid, pIN233, was cotransfected with pJM17 into 293 cells to obtain Ad-PTEN virus. Ad-PTEN vector was plaque purified, amplified, and viral DNA purified using the QIAmp DNA purification kit (Qiagen, Valencia, CA, USA). The expression cassette was PCR amplified and the sequence was confirmed. The vector doses are reported in this study in viral particles per milliliter (vp/ml). The stock titers of the vectors used was 1.6×10^{12} vp/ml for Ad-PTEN and 5.5×10^{12} vp/ml for Ad-Luciferase (Ad-luc). These vectors were also tested by plaque assay and had a vp/pfu ratio of approximately 50 and 30, respectively.

Antibodies and Western Blotting Analysis

The following antibodies were used in this study: anti-PTEN was obtained from Oncogene Science (Cambridge, MA, USA). A mouse monoclonal against β-actin was obtained from Sigma (St. Louis, MO, USA). Monoclonal antibodies against AKT and Ser472 phosphorylated AKT were obtained from Cell Signaling (Beverly, MA, USA). Anti–Apaf-1 and anti–caspase-3 were purchased from BD Pharmingen (Los Angeles, CA, USA). To perform Western Blotting analysis, cells were trypsinized and harvested by centrifugation. After washing with ice-cold PBS, cells were lysed with EBC buffer (120 mM NaCl, 10 mM Tris at pH 8.0, 0.1 mM EDTA, 1 mM DTT) containing 0.5% NP-40 (Sigma), and protease inhibitors (Roche, Indianapolis, IN, USA). Cell lysates were normalized before loading onto 4~20% SDS-PAGE gradient gels (Invitrogen, Carlsbad, CA, USA). Proteins were subsequently transferred to nitrocellulose membrane (Invitrogen) and probed with primary and secondary antibodies. The proteins were visualized by the Supersignal

West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA).

Transduction and Cell Proliferation Studies

Tumor or normal cell lines used in this study were infected with Ad-PTEN or Ad-Luc at increasing multiplicity of infection (MOI). Cells were plated at 1–2 $\times$ 10^5 cells/well in a 6-well plate for protein expression or apoptosis assays. Cells were exposed to adenoviral vectors 24 hr post-plating and the viruses were subsequently removed after 1 day of infection. Cultures were assessed for protein expression on day 1 or 2 postinfection and day 3 or 4 for apoptosis and Trypan blue dye exclusion assays.

Annexin V Apoptosis Assay and Trypan Blue Exclusion Assay

Transduced tumor cells were analyzed for apoptosis using the ApoAlert Annexin V-FITC kit (Clontech, Palo Alto, CA, USA) according to manufacturer's instructions. The FITC staining was measured by FACS analysis. Following infection, cells were harvested and stained with 1% Trypan blue (Sigma) to visualize dead cells under light microscopy. The number of blue cells (dead cells) was counted to obtain the percentage of cell killing.

Surface Labeling

Three days after adenoviral infection, cells were trypsinized and harvested by centrifugation. Cell pellets were washed with ice-cold PBS and were resuspended in PBS containing 1:500 E-cadherin antibody (BD Transduction Laboratories, Los Angeles, CA, USA) and incubated on ice. After extensive washing with PBS, cells were again resuspended in PBS containing 1:1000 FITC anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA). After washing again with PBS, cell pellets were resuspended in PBS containing 2% formaldehyde for FACS analysis using a FACScan (EPICS XL-MCL; Beckman Coulter, Fullerton, CA, USA).

Cell Invasion Assay

For the invasion assay, cells were seeded at 5 $\times$ 10^5 cells/well in 6-well tissue culture plates, and infected with Ad-Luc or Ad-PTEN at MOI of 5000 vp/cell. Following transduction, cells were replenished with complete medium. Twenty-four hours after infection, cells were trypsinized, washed in PBS, and resuspended in RPMI 1640 medium supplemented with FBS. Invasion assay was carried out in a 24-well transwell unit (Millipore, Cambridge, MA, USA). Briefly, polycarbonate filters with 8-μm pores were used. The lower chambers of the transwells were filled with serum-free medium and the upper chambers were seeded with 1 $\times$ 10^4 cells from each treatment in triplicate wells. After a 24- and 48-hr incubation, the number of cells that had passed through the filter into the lower wells was counted.

Immunohistochemical Staining for PTEN

Immunostaining of human melanoma cell lines was performed using the mouse monoclonal antibody against human PTEN, as was used in immunoblotting, using the avidin–biotinylated–peroxidase complex method. In brief, the endogenous peroxidase was blocked by immersing slides in 0.3–3% hydrogen peroxidase (Sigma) in methanol for 20–30 min, and then washing in PBS. The avidin–biotin–peroxidase complex (ABC) kit (Vectastain, Vector Laboratories, Burlingame, CA, USA) was then used for staining. Nonspecific binding was blocked by incubating with normal horse serum for 30 min. Slides were blotted to remove excess serum and incubated for 1 hr at room temperature with primary antibody. Monoclonal anti-PTEN antibody was diluted at 1:100 in PBS containing 0.05% Triton X-100 and 0.1–1% BSA. The slides were washed in PBS and then incubated for 30 min with the ABC reagent. After washing in PBS, the immunostaining was developed with the use of 3-amino-9-ethylcarbazole as a chromogen for 10–15 min. Slides were counterstained with hematoxylin (Sigma) and washed with tap water. All slides were mounted using Aqua-Mount (Lerner Laboratories, Pittsburgh, PA, USA). An isotype-matched negative control monoclonal antibody was used to test for background staining. To confirm the antigenicity of samples, we used vimentin antibody (BioGenex, San Ramon, CA, USA) at 1:500 dilution as a positive control.

Genomic Sequencing

Exon-specific primers of PTEN were prepared and the PTEN coding region was sequenced from genomic DNA prepared from melanoma cell lines.

Confocal Microscopy

Cells were grown and transduced on coverslips. After transduction, viruses were removed, and the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature (RT). Cells were subsequently permeabilized with 0.1% ice-cold Triton X-100. Anti-PTEN antibody was diluted in 0.2% fish skin gelatin and incubated with the cells for 20 min at RT. After washing, diluted secondary antibody was added for 1 hr and cells washed. The cells were fixed with Mowiol:DABCO (Calbiochem, San Diego, CA, USA) and visualized with a Zeiss Axiovert 100M equipped with a LSM 510 scanning system.

Endothelial Cell Tube Formation Assay

Tube formation assay was performed according to a protocol from BioSource (BD Systems). Briefly, 250 μl of Matrigel solution (10 mg/ml) was added to each well of 24-well plate. Incubate the plate at 37°C for 5–15 min to allow gel formation. Add 250 μl of medium (MEM containing 0.1% BSA) or test sample in each well along side of well. Add 50,000 endothelial cells (either Ad-PTEN transduced or

Table 1. Melanoma cell lines used in the study

Cell Lines	Melanoma Stage	PTEN Alleles
WM35	RGP	Wild-type
A375	Metastatic	Wild-type
A375-S2	Metastatic	Wild-type
MeWo	Metastatic	Wild-type
WM1342	Metastatic	Wild-type
NHEM	Normal melanocytes	Wild-type*

*Not sequenced.

Mock-transduced) in 50 μl volume (1×10^6 cells/ml) to each well. Incubate at 37°C and observe under microscope at regular intervals (4–24 hr).

Results

Examination of the PTEN Alleles in Melanoma Cell Lines

Five different melanoma lines were analyzed, based on their invasion characteristics: WM35 derived from early stage RGP melanoma; A375, A375-S2, WM1342, and MeWo were derived from malignant metastatic melanoma. All PTEN exons in these cell lines were individually amplified by PCR and sequenced. All five melanoma lines contained wild-type PTEN sequence; no mutations were detected in the coding region (Table 1). Additional alterations in the noncoding regions of the PTEN locus cannot be ruled out. Total cell lysates from all of these cell lines were fractionated by SDS-PAGE and evaluated for endogenous PTEN protein expression. Although none of these cell lines had PTEN coding region mutations, little or no endogenous PTEN protein could be detected by IHC or Western blotting (Figs. 1 and 4), suggesting that additional epigenetic mechanisms might compromise the expression of PTEN in these cancer cells, consistent with the tumor suppressor role of PTEN.

Ad-PTEN Expresses High Levels of Transgene Protein in Melanoma Cells

The full-length PTEN cDNA was subcloned into the replication-impaired adenovirus vector (Ad-PTEN) to express PTEN protein upon infection. Melanoma cells were infected with 5000 MOI of Ad-PTEN or Ad-Luc for 24 hr followed by immunohistochemical analysis using a specific antibody against PTEN. As shown in Figure 1, intense PTEN expression was primarily localized to the cytoplasm and the membrane in WM35,

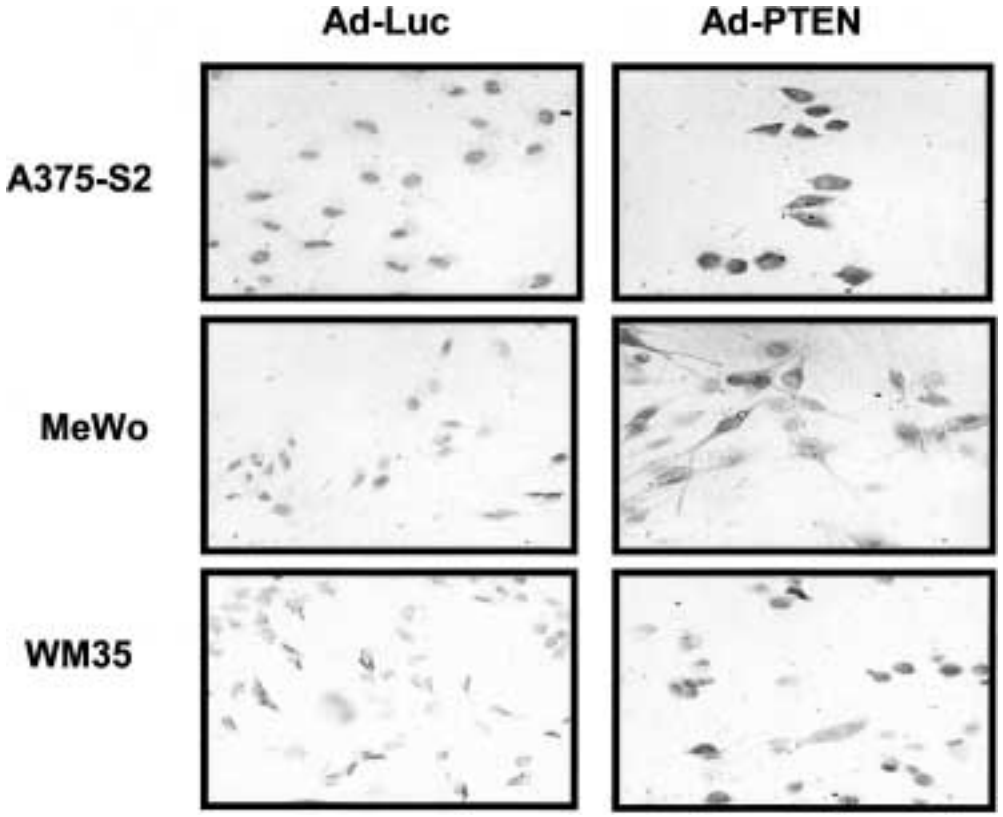

Fig. 1. High-level expression of PTEN after transduction of melanoma cells with Ad-PTEN. WM35 (RGP), A375-S2, and MeWo (malignant melanoma) cells were transduced with Ad-Luc or Ad-PTEN for 24 hr and fixed for immunohistochemistry with anti-PTEN monoclonal.

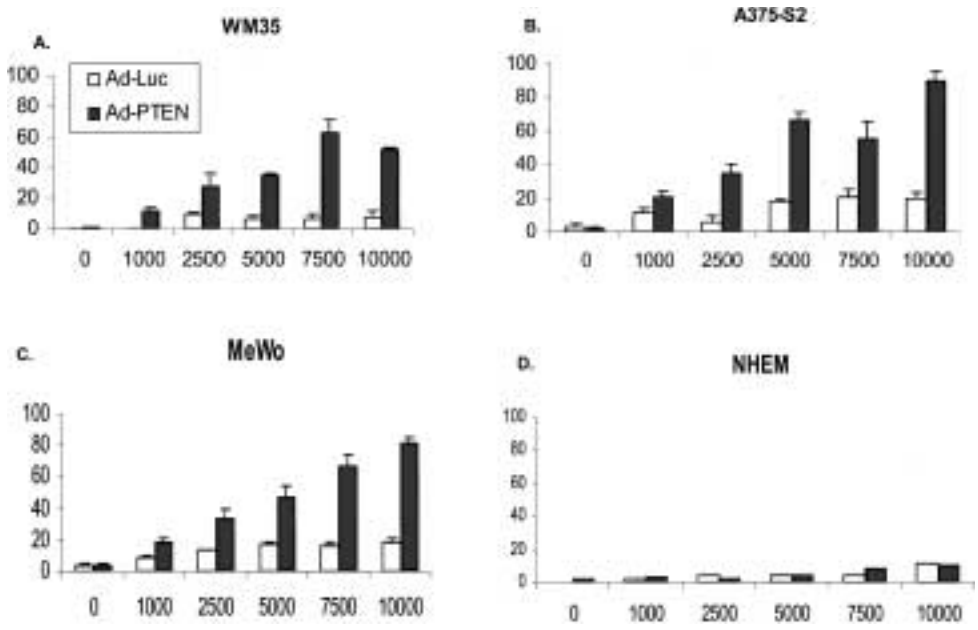

Fig. 2. Ad-PTEN, but not Ad-Luc, causes cell death in melanoma cells. Increasing doses of viruses (0, 1000, 2500, 5000, 7500, and 10,000 vp/cell) were used to treat cells and cells were harvested 3 days after infection, treated with Trypan blue and counted. The percentage of dead cells is displayed on the *y* axis. **(A)** WM35 cells. **(B)** A375-S2 cells. **(C)** MeWo cells. **(D)** NHEM cells. All graphs are the average of three experiments, except that of NHEM, which is the average of two experiments. The error bars represent the standard error of the mean.

A375-S2, and MeWo cells. Negligible staining was seen in the nucleus. Furthermore, no staining was observed in the untreated or Ad-Luc–infected cells, demonstrating the specificity of antibody binding. The expression or subcellular localization of PTEN did not appear to change across the cell cycle as asynchronous cells were used in these staining experiments.

PTEN Blocks PI3K Signaling and Causes Apoptosis in Melanoma Cell Lines

RGP and metastatic melanoma cell lines (WM35, A375-S2, and MeWo) and NHEM (Normal Human Epithelial Melanocytes) were infected with increasing MOI of Ad-PTEN or Ad-Luc as control. The percentage of dead cells was measured by Trypan blue exclusion assay after 3 days of infection. As shown in Figure 2A–C, dose-dependent cell death was observed in both early- and late-stage melanoma lines after transduction with Ad-PTEN, but not with Ad-Luc. Furthermore, NHEM cells were resistant to cell death by Ad-PTEN infection, indicating that the effect of Ad-PTEN is tumor cell-specific (Fig. 2D). To confirm the cell death was caused by apoptosis, the Annexin V staining assay was used to identify apoptotic cells after 3 days of infection with either Ad-PTEN or Ad-Luc (Fig. 3A). All Ad-PTEN–sensitive cell lines displayed elevated levels of apoptosis as measured by the percentage of Annexin V–positive cells. This indicates that Ad-PTEN triggers the onset of apoptosis in melanoma cells even when they carry wild-type PTEN alleles.

To gain insight into how Ad-PTEN triggers apoptosis in these cells, three melanoma lines, WM35, A375-S2, and MeWo were infected with Ad-Luc or Ad-PTEN. Total cell lysates were prepared 2 days after infection and resolved by SDS-PAGE. As shown in Figure 4A high levels of ectopic expression of PTEN were seen in all Ad-PTEN transduced cells but not in Ad-Luc transduced cells. The overexpression of PTEN triggers the dephosphorylation of AKT/PKB in all melanoma cell lines, consistent with the role of PTEN in interfering with the PI3K-dependent cell survival pathway. Furthermore, the kinase activity of AKT/PKB was also inhibited upon Ad-PTEN infection, but not Ad-Luc infection (Fig. 4B). MeWo cells were treated with Ad-PTEN and the kinetics of phospho-AKT (p-AKT) evaluated. p-AKT levels strongly decreased within 24 hr after Ad-PTEN treatment and remained decreased at 48 hr (Fig. 4C). The decrement of p-AKT induced by Ad-PTEN treatment was comparable to that observed with the PI3K inhibitor, LY 294002 (20 μM), demonstrating that Ad-PTEN is a potent inhibitor of PI3K and AKT/PKB kinase.

Ad-PTEN transduction did not result in significant change in the steady state levels of AKT/PKB, or p27, an inhibitor of the cyclin E-CDK2 kinase (Fig. 4A). The level of Apaf-1, a key player in the execution of mitochondria-mediated apoptosis, also remained constant in these cells. Caspase activation was also examined in these cells. Activation of caspase-3 was observed in Ad-PTEN transduced MeWo and WM35 cells and, to a lesser extent, in A375-S2 cells (data not shown). Caspase-3 activation was specific for Ad-PTEN treatment; no activation was observed in Ad-Luc treated cells. No activation of caspase-8 or caspase-9 was observed by Western blotting (data not shown). These data suggest that PI3K inhibition by Ad-PTEN directly or indirectly participates in the regulation of the onset of apoptosis in melanoma cells.

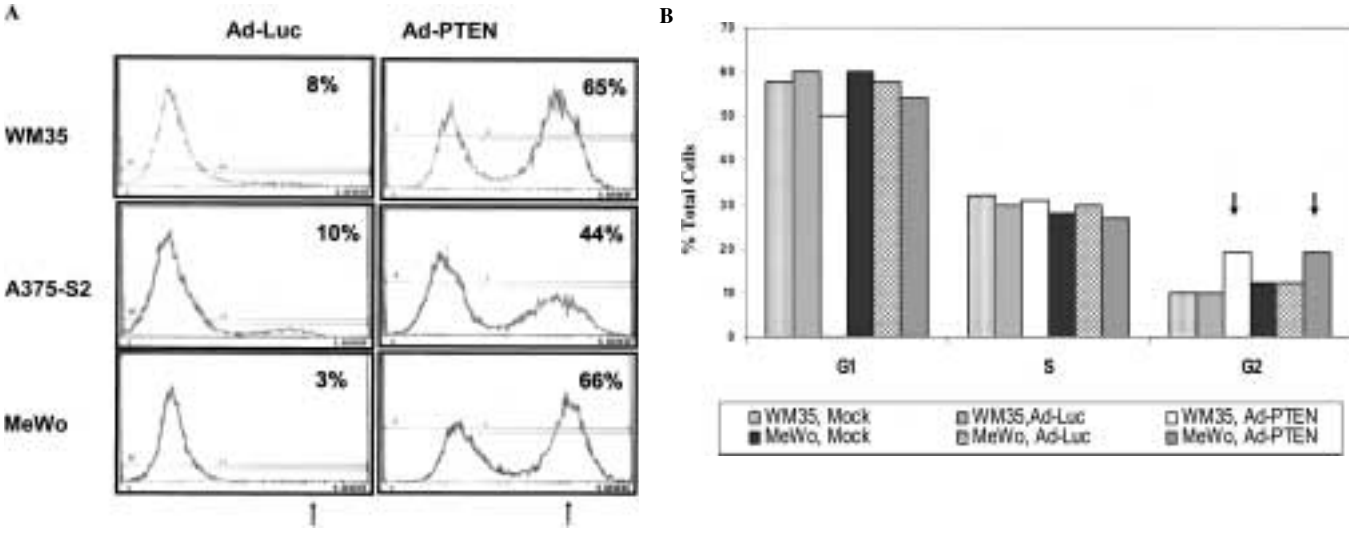

Fig. 3. Melanoma cells undergo apoptosis following Ad-PTEN, but not Ad-Luc, treatment. (A) WM35, A375-S2, and MeWo cells were treated with 5000 vp/cell viruses, harvested 4 days after infection, the Annexin V assay performed, and the cells analyzed by flow cytometry. The peak signal of apoptotic cells are shown with arrows. The percentage of apoptotic cells is indicated. **(B)** Ad-PTEN regulates cell-cycle progression in melanoma cells. WM35 and MeWo cells were treated with 2500 vp/cell of Ad-PTEN or Ad-Luc and 3 days postinfection; cells were harvested and stained with PI for cell-cycle analysis using a FACScan. Ad-PTEN treatment causes a G2/M block in tumor cells as indicated by arrows.

PTEN Causes G2/M Cell-Cycle Block

WM35 or MeWo cells were treated with Ad-luc or Ad-PTEN and 48 hr later, the cells were fixed and cell-cycle analysis was performed. In both RGP and metastatic melanoma cells, Ad-PTEN caused a significant increase in the G2/M population of cells, with an attendant decrease in G1 phase cells (Fig. 3B). Ad-luc treatment had only minor effects on the cell cycle compared to mock treatment.

PTEN Suppresses Tumor Cell Invasion

Cell invasion and migration are two critical parameters for tumor metastasis. Melanoma is characterized by its rapid propensity to metastasize. To investigate

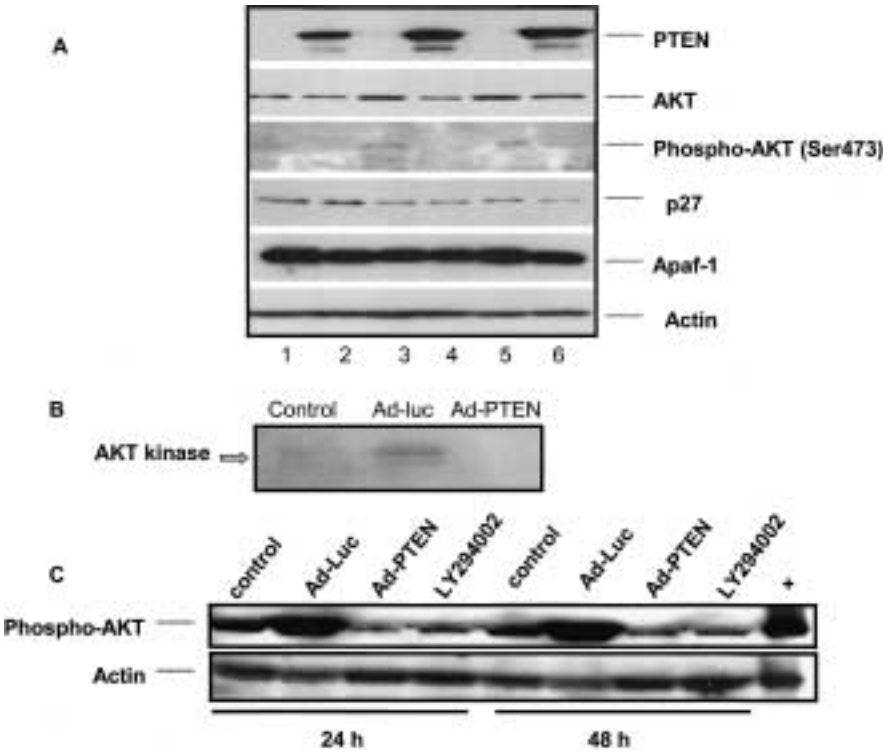

Fig. 4. Molecular targets of PTEN. (A) WM35 (lanes 1 and 2), A375-S2 (lanes 3 and 4), and MeWo (lanes 5 and 6) cells were transduced with either Ad-Luc (odd lanes) or Ad-PTEN (even lanes). Twenty-four hours postinfection, cells were harvested, lysed, and resolved by SDS-PAGE. Proteins were transferred to nitrocellulose and probed with antibodies against PTEN, AKT/PKB, Phospho-AKT, p27, Apaf-1, and actin. **(B)** AKT kinase activity is inhibited by Ad-PTEN. Mock treated, Ad-Luc–, or Ad-PTEN–treated MeWo cells were extracted and their AKT kinase activity was measured by ^{32}P-labeling using GS3K as substrate. **(C)** The level of phospho-specific AKT/PKB decreased following Ad-PTEN transduction. MeWo cells were mock infected or infected with Ad-PTEN or Ad-Luc and harvested after 24 or 48 hr following infection. Total cell lysates were prepared and probed for p-AKT. The treatment of cells with LY 294002 was used as a positive control for AKT/PKB dephosphorylation.

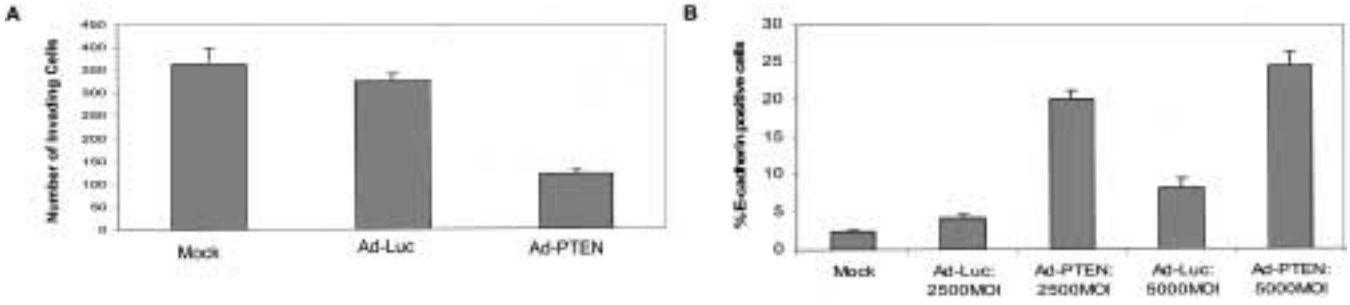

Fig. 5. **(A)** Cell invasion was inhibited by Ad-PTEN in MeWo cells. Mock, Ad-Luc, or Ad-PTEN treated MeWo cells were used. The number of invading cells is indicated on the *y* axis. Error bars indicate the standard deviation. **(B)** Cell-surface E-cadherin levels are increased by Ad-PTEN transduction in MeWo cells. Cells were mock, Ad-Luc, or Ad-PTEN treated (2500 or 5000 vp/cell), harvested, and subjected to E-cadherin surface labeling and analyzed by FACS. Error bars indicate the standard deviation.

the effects of Ad-PTEN on tumor metastasis, metastatic MeWo cells were transduced with Ad-PTEN or Ad-Luc and 2 days later were evaluated for invasion/migration using the Boyden chamber assay. As shown in Figure 5A, treatment of MeWo cells with Ad-PTEN led to a significant decrease in invading cells compared to Ad-Luc treated or mock treated cells. Similarly, cell migration in monolayer culture was also inhibited by Ad-PTEN in the same cells (data not shown). These observations suggest that Ad-PTEN might be a potent anti-metastatic agent in melanoma. We then evaluated the effect of Ad-PTEN on expression of a molecule causally implicated in metastatic spread. E-cadherin is a molecule involved in homotypic cell–cell interactions and is altered in the majority of epithelial cancers (24,35). Forced expression of E-cadherin in cultured cancer cells and transgenic mice harboring tumors reduced the invasive and metastatic phenotypes in these cells. Cell-surface labeling of E-cadherin was evaluated in cells infected with Ad-PTEN or Ad-Luc at 3 days postinfection. The levels of E-cadherin (Fig. 5B) increased approximately 3- to 4-fold following Ad-PTEN transduction, as compared to Ad-Luc transduction.

Cellular Localization of PTEN in Melanoma Cells

Due to the sequence similarity of PTEN to tensin and auxilin, it has been proposed that PTEN might be a cytoplasmic protein with the potential to bind cell membranes. However, published studies evaluating subcellular localization of PTEN have produced conflicting results. Immunohistochemical analyses of Ad-PTEN treated melanoma cells showed PTEN staining in the cytoplasm and the membrane (Fig. 1). Confocal microscopy was used to further evaluate the subcellular localization of PTEN (Fig. 6). Specific staining was observed only in Ad-PTEN treated cells. As shown above by conventional immunohistochemistry (Fig. 1), cytoplasmic staining was observed; however, confocal evaluation demonstrated high levels of PTEN protein concentrated in discrete regions of the plasma membrane (Fig. 6). These regions of enriched PTEN localization are consistent with adhesion plaques. PTEN also was enriched in cell–cell junctions in A375-S2 cells.

Ad-PTEN Blocks Angiogenesis by In Vitro Inhibition of Endothelial Differentiation

The tumor suppressor PTEN down-regulates PI3K signaling (36), and PI3K is important in controlling angiogenesis via regulation of VEGF expression. We thus evaluated the effects of Ad-PTEN on angiogenesis using an *in vitro* endothelial differentiation assay as a correlate for angiogenesis. When primary HUVECs are plated on Matrigel, they differentiate into tubelike structures; this is believed to be an *in vitro* model for neo-angiogenesis. As shown in Figure 7, Ad-PTEN specifically inhibited VEGF-mediated tube formation. HUVECs treated with Ad-Luc as control were indistinguishable from media-treated control cultures. Ad-PTEN at 3000 vp/cell was as effective at blocking tube formation as the positive control suramin, a known inhibitor of endothelial differentiation. Note that Ad-PTEN does not kill HUVECs (data not shown), but blocks their differentiation. This finding demonstrates that Ad-PTEN potently modulates VEGF-mediated signaling and function, and that PTEN may be a target in therapeutic approaches to inhibit angiogenesis.

Discussion

To investigate the therapeutic potential of inhibiting PI3K signaling via PTEN in the treatment of melanoma, we constructed a replication-impaired adenovirus vector carrying the PTEN tumor suppressor gene under the control of the CMV promoter. Our study indicated that Ad-PTEN infection into melanoma cell lines resulted in high levels of exogenous PTEN expression (Figs. 1 and 4A). Ectopic PTEN expression led to growth inhibition and apoptotic cell death in all melanoma lines *in vitro* (Figs. 2 and 3). The ability to induce apoptosis by adenovirus-mediated PTEN gene transfer was evident in cell lines derived from both early-stage RGP melanoma as well as highly metastatic

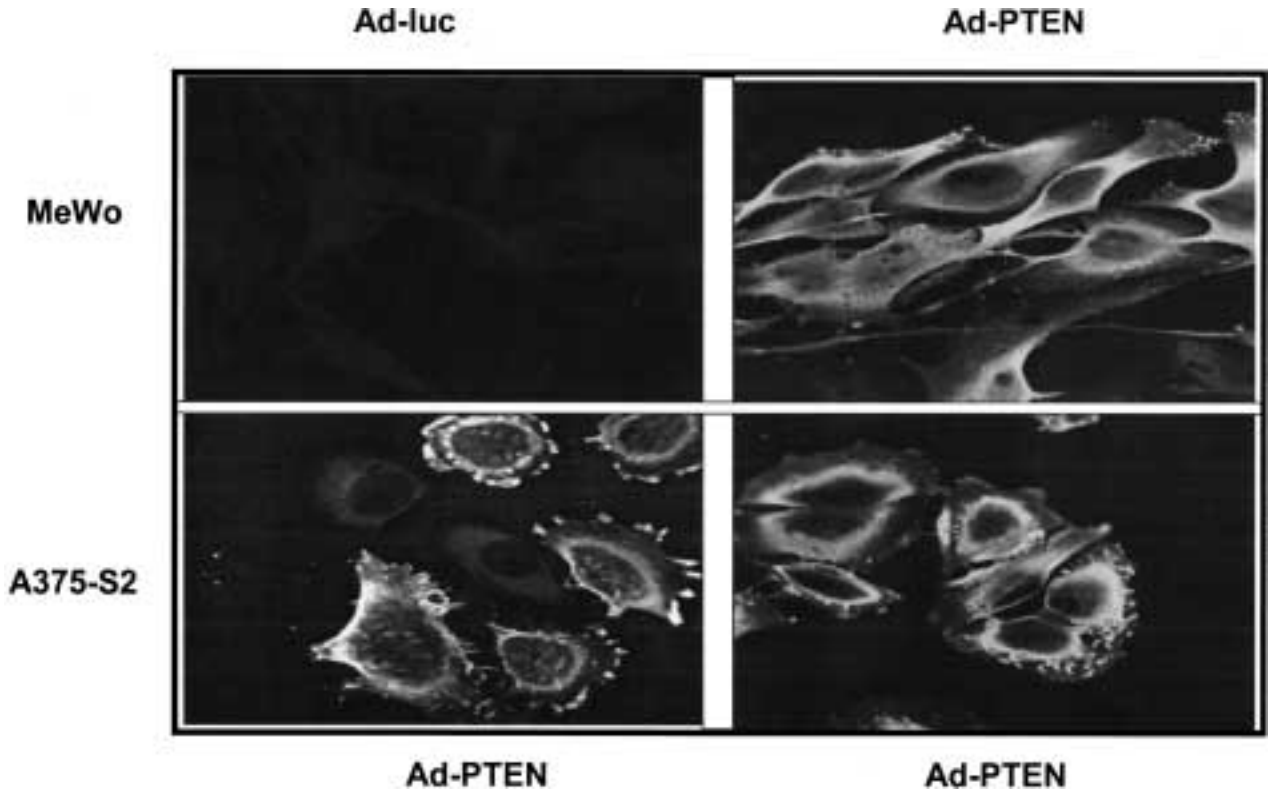

Fig. 6. Subcellular localization of PTEN in melanoma cells. A375-S2 or MeWo cells were transduced with Ad-Luc or Ad-PTEN for 24 hr and monitored by confocal microscopy. Ad-PTEN treated cells exhibited high levels of PTEN expression in cytoplasm and cell periphery. Note concentration of PTEN in specific regions of membrane consistent with adhesion plaques and intercellular structures.

melanomas (Fig. 4). Furthermore, the killing induced by Ad-PTEN was independent of PTEN mutational status because all cell lines evaluated have wild-type PTEN alleles (Table 1). The latter result is different from earlier reports that introduction of wild-type PTEN into tumor cell lines caused growth arrest, anoikis, or apoptosis only in cells containing mutated PTEN alleles, especially with studies involved glial cells (37–41). However, more recent studies conducted in breast (32), ovarian (42), and

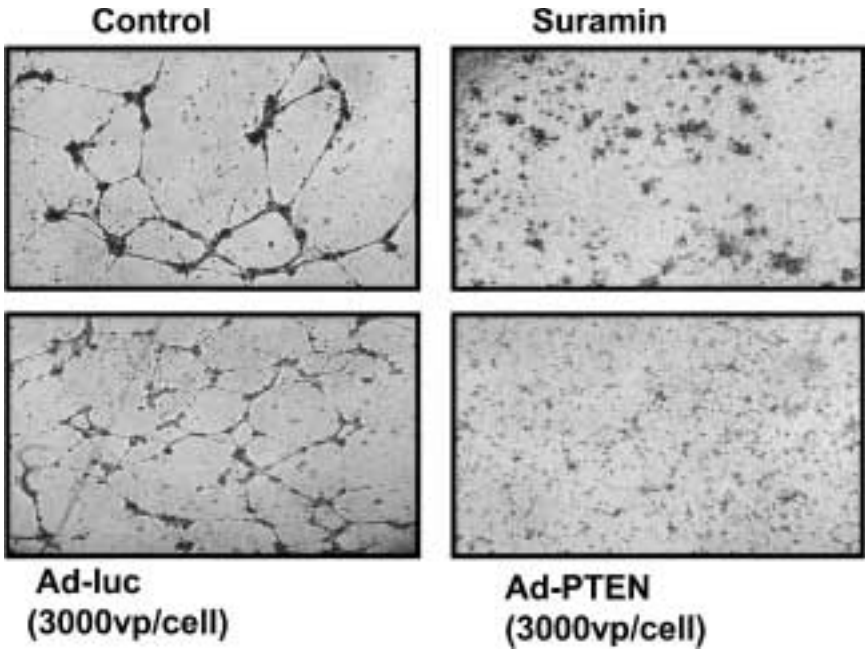

Fig. 7. Ad-PTEN inhibits angiogenesis *in vitro*. HUVEC cells were transduced with Ad-Luc or Ad-PTEN (3000 vp/cell) or treated with Suramin or media for 24 hr. The cells were then plated onto Matrigel and incubated at 37°C. Control HUVEC exhibited mature tube formation by 12–16 hr after plating whereas Suramin and Ad-PTEN treatment blocked tube structure formation.

endometrial (43) cancer cell lines using adenovirus vector–mediated gene transfer also indicated that genotypically wild-type PTEN cancer cells can be growth inhibited with or without induction of apoptosis upon reintroduction of high levels of exogenous PTEN protein. We suspect the difference between these studies could be attributed to different tumor types and/or experimental systems. Because tumors and tumor cell lines contain multiple, and often different mutations, it is possible that the mutational status of the PTEN gene is not the only rate-limiting factor in apoptosis induction. In addition, although the cell lines we tested here contain wild-type PTEN alleles, the endogenous levels of PTEN protein remain extremely low or undetectable. Another possibility for the widespread apoptosis we observe compared to the limited apoptosis and growth arrest observed by others may be due to the differences in the PTEN delivery efficiency. Note that the PTEN gene in our vector is under the control of the CMV promoter and is therefore capable of expressing very high supraphysiologic levels of PTEN (Figs. 4 and 6) as compared to other gene transfer systems. Others have reported limited apoptosis and primarily growth arrest in glial cells after PTEN transfection or retroviral transduction (37). It is also interesting to note that although growth of endometrial carcinoma cell lines with endogenous wild-type PTEN was mildly suppressed in one study using adenovirus-mediated PTEN gene delivery (43), growth of the same endometrial carcinoma cell line was completely unaffected in a separate study using a retrovirus-mediated gene transfer system (44). In addition, PTEN-induced cell-cycle arrest was observed in the latter study instead of apoptosis observed in the earlier study. Alternatively, the degree of cell growth inhibition and ability to undergo apoptosis might also be associated with the transduction efficiency of particular cell lines as observed in studies involving ovarian cancer cells (42).

In this study, the exogenous PTEN protein is functional because the AKT/PKB kinase activity and phospho-AKT/PKB levels are reduced following Ad-PTEN treatment. It is possible that overcoming cellular survival signals to induce AKT-dependent apoptosis may only occur when very high levels of PTEN are expressed. On the other hand, the status of other oncogenic factors and cell growth condition may also attribute to PTEN-induced apoptosis in our assay system. This hypothesis is further supported by studies that PTEN reintroduction into different cell lines or the same cell lines under different culture conditions (45–47) resulted in either cell-cycle arrest or apoptosis. Note that the Ad-PTEN vector used here is capable of inducing cell death in cells containing wild-type PTEN alleles. This finding may be very significant in a clinical setting; only 30–40% of melanomas contain PTEN mutations and our findings suggest that the remaining 60–70% of wild-type PTEN-expressing tumors might also be responsive to Ad-PTEN treatment.

We showed that the cell killing by Ad-PTEN is tumor selective; apoptosis or growth suppression was not observed in normal melanocytes transduced with Ad-PTEN (Fig. 2). In addition, Ad-PTEN did not induce significant growth suppression in other normal human cell lines, including fibroblast and endothelial cells (data not shown). The mechanism underlying the tumor selectivity of Ad-PTEN is not clear. Our cell-cycle analyses demonstrate that Ad-PTEN treatment causes G2/M block in melanoma cells (Fig. 3B). Previous reports evaluating PTEN gene transfer into tumor cells have reported G1 cell-cycle arrest with concomitant increased levels of p27 expression (31,37). In melanoma cells, we do not see alterations in p27 expression and no evidence of G1 arrest. One difference between our results and the previously published data may be that we have used cell lines containing wt PTEN, whereas other studies have evaluated PTEN mutated or deleted cell lines (31,33,37,44).

Consistent with earlier experiments using glial (41), breast (32), bladder (48), prostate (39), thyroid (47), and endometrial (44) cancer lines, we also showed that the introduction of PTEN results in efficient dephosphorylation of AKT/PKB without a decrease in total AKT/PKB protein. The PTEN protein is a protein and lipid phosphatase, and exerts its effects by altering the phosphorylation state of multiple signaling pathways. It has been reported that PTEN dephosphorylates FAK, which leads to inhibition of integrin-mediated cell spreading, migration, and focal adhesion formation. Previous studies evaluating the effect of PTEN transfection into murine 3T3 cells or U87 glioblastoma cells demonstrated inhibition of cell invasion and migration. Here, we show for the first time that Ad-PTEN inhibits cell invasion and migration in metastatic melanoma cells (Fig. 5A). The combination of pro-apoptotic and anti-metastatic activities suggests that Ad-PTEN may be a promising agent in treating tumors that are prone to metastasis.

We have shown that Ad-PTEN inhibits endothelial cell differentiation and migration (data not shown) in a dose-dependent and transgene-specific manner *in vitro* (Fig. 7). This further supports the notion that Ad-PTEN may play an important role in inhibiting the tumor metastasis pathway. As a molecular correlate for metastatic potential, we evaluated the levels of cell surface E-cadherin in response to Ad-PTEN. Cadherins are a large group of cell adhesion molecules located at intercellular junctions called adherens junctions. They play important roles in embryogenesis and morphogenesis in animals and humans due to their adhesive and cell-signaling functions. Disturbances of the expression or function of cadherins and their associated proteins called catenins are crucial for the initiation

and development of many pathologic states. E-cadherin is an epithelium-specific cadherin required for the development and maintenance of the normal function of all epithelial cells in tissues. The loss or down-regulation of E-cadherin is a key event in the process of tumor invasion and metastasis. The levels of E-cadherin on the cell surface of MeWo cells increased substantially in a dose-dependent manner following Ad-PTEN transduction, but not Ad-Luc transduction (Fig. 5B). Similar results were obtained in other melanoma cell lines (data not shown). These results indicate that Ad-PTEN regulates cadherin-mediated cell–cell adhesion and migration processes. The exact mechanism by which PTEN causes up-regulation of E-cadherin is currently under investigation.

Ad-PTEN is able to inhibit endothelial differentiation in the tube formation assay (Fig. 7). PTEN inactivation is associated with increased angiogenesis in different tumors (49). Several studies have evaluated endogenous or ectopic PTEN localization (10,49–52); epitope-tagged PTEN was transfected into several mammalian cell lines by transient transfection and was found to be localized primarily to the cytoplasm (10,37). Immunohistochemical studies of endogenous PTEN in esophageal squamous cell carcinoma and endocrine pancreatic tumors also suggested that PTEN is localized predominantly in the cytoplasm (50,51). In contrast, predominantly nuclear or perinuclear staining was observed in normal islet cells, neurons and endothelial cells (51,52). Differential nuclear and cytoplasmic expression of endogenous PTEN was observed in normal thyroid tissue and epithelial thyroid tumors (49). In our initial immunohistochemical analyses, PTEN protein was found in the cytoplasm and appeared enriched around the membrane of Ad-PTEN treated cells (Fig. 1). The subcellular localization of PTEN did not change in cells in different cell-cycle compartments. Confocal imaging of ectopically expressed PTEN in A375-S2 and MeWo cells supported this pattern of expression, but also revealed that in metastatic melanoma cells, PTEN was enriched in membrane processes that appeared to be adhesion plaques (Fig. 6). In A375-S2 cells, PTEN was mostly perinuclear in location, and also appeared to be enriched in cell–cell junctions (see Fig. 6).

In summary, we constructed an Ad-PTEN vector, which causes the expression of high levels of PTEN protein and blocks PI3K signaling pathways. Dysregulation of the PI3K pathway is involved in controlling tumor cell survival and also appears to mediate resistance to conventional chemo- and radiotherapeutic approaches in a broad spectrum of tumors. We have tested the ability of Ad-PTEN to inhibit the proliferation of melanoma cell lines. Our results indicate that Ad-PTEN has anti-proliferative and pro-apoptotic activity in melanoma cells but not in normal melanocytes. Although the sample size is currently small, our data indicate that the sensitivity of melanoma lines to Ad-PTEN treatment does not correlate with PTEN gene mutational status. In this same set of cell lines, we have also evaluated p53 mutational status (data not shown) and do not find a correlation between p53 mutation and Ad-PTEN apoptotic activity. Melanoma is a very aggressive tumor with a propensity to metastasize rapidly. PI3K blockade via Ad-PTEN inhibited cell invasion and migration in metastatic melanoma cells, and also inhibited angiogenesis, *in vitro,* suggesting Ad-PTEN might be useful in treating both early- and late-stage tumors.

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