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A Critical Role of the PTEN/PDGF Signaling Network for the Regulation of Radiosensitivity in Adenocarcinoma of the Prostate

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Abstract

Purpose—Loss or mutation of the phosphate and tensin homologue (PTEN) is a common genetic abnormality in prostate cancer (PCa) and induces platelet-derived growth factor D (PDGF D) signaling. We examined the role of the PTEN/PDGF axis on radioresponse using a murine PTEN null prostate epithelial cell model.

Methods and Materials—PTEN wild-type ($PTEN^{+/+}$) and PTEN knockout ($PTEN^{-/-}$) murine prostate epithelial cell lines were used to examine the relationship between the PTEN status and radiosensitivity and also to modulate the PDGF D expression levels. $PTEN^{-/-}$ cells were transduced with a small hairpin RNA (shRNA) lentiviral vector containing either scrambled nucleotides (SCRM) or sequences targeted to PDGF D (shPDGF D). Tumorigenesis and morphogenesis of these cell lines were evaluated in vivo via subcutaneous injection of male nude mice and in vitro using Matrigel 3-dimensional (3D) culture. Effects of irradiation on clonogenic survival, cell migration, and invasion were measured with respect to the PTEN status and the PDGF D expression level. In addition, apoptosis and cell cycle redistribution were examined as potential mechanisms for differences seen.

Results—$PTEN^{-/-}$ cells were highly tumorigenic in animals and effectively formed foci in 3D culture. Importantly, loss of PDGF D in these cell lines drastically diminished these phenotypes. Furthermore, $PTEN^{-/-}$ cells demonstrated increased clonogenic survival in vitro compared to $PTEN^{+/+}$, and attenuation of PDGF D significantly reversed this radioresistant phenotype. $PTEN^{-/-}$ cells displayed greater migratory and invasive potential at baseline as well as after irradiation. Both the basal and radiation-induced migratory and invasive phenotypes in $PTEN^{-/-}$ cells required PDGF D expression. Interestingly, these differences were independent of apoptosis and cell cycle redistribution, as they showed no significant difference.

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Conclusions—We propose that PDGF D represents a potentially promising target for PCa treatment resistance in the absence of PTEN function, and warrants further laboratory evaluation and clinical study.

Introduction

Prostate cancer (PCa) represents the second leading cause of cancer death in American men (1). Low-risk disease can typically be treated effectively with radical surgery or local radiation therapy. When adverse risk factors are present, radiation therapy as definitive or adjuvant treatment is often preferred. Despite advances in radiation treatment, a subset of men will not be cured of their local disease.

The loss or mutation of the tumor suppressor phosphatase and tensin homologue (PTEN) is thought to be an important driving force in the pathogenesis of many tumors (2), with an estimated frequency of monoallelic loss or mutation of 50% to 80% in primary PCa (3, 4). Evidence suggests that complete loss of PTEN function is associated with increased aggressiveness and distant metastatic potential (2). In mice with prostate-specific PTEN homozygous deletion, 100% developed mouse prostate intraepithelial neoplasia (mPIN) lesions at the age of 6 weeks and progressed to invasive adenocarcinoma by the 9 weeks, mirroring the typical disease progression from PIN to invasive adenocarcinoma seen in human beings (5).

The platelet-derived growth factor (PDGF) signaling network has likewise been suggested to play a role in the development and progression of PCa (6). The PDGF family consists of 4 ligands, namely, PDGFA, B, C, and D, which form either homodimers and/or a single heterodimer AB. The α-PDGFR can be activated by PDGF A, B, and C, whereas the β-PDGFR is activated by PDGF B and D. Through activation of their receptors, α- and β-PDGFR, PDGF ligands regulate cellular processes such as; cellular proliferation, migration, differentiation, and phenotypic transformation (7). Immunohistochemical analysis has shown that β-PDGFR is upregulated in most primary and metastatic PCa cells (8). In addition, microarray analysis has identified expression of β-PDGFR to be part of a 5-gene model that predicts for clinical PCa recurrence (9). Despite the seeming importance of β-PDGFR, what was once thought to be its sole ligand (PDGF B) was not found to be elevated in PCa tissues (6). Recently, PDGF D was identified as a clinically relevant ligand for β-PDGFR in PCa, and its expression was associated with higher tumor stage and Gleason score in PCa patients (10). Functionally, PDGF D induces PCa cell motility via autocrine signaling, and serves as a chemoattractant for fibroblasts via paracrine stromal interaction (11). In a murine model, PDGF D expression accelerated early onset of prostate tumor growth while enhancing prostate carcinoma cell invasion and interaction with surrounding stromal cells (11).

PTEN loss results in increased β-PDGFR expression and a ligand switch from PDGF B to PDGF D in prostate epithelial cells (12). Although little is known about PDGF isoform–specific β-PDGFR signaling pathways and their cellular effects during cancer development and progression, evidence suggests that, in terms of oncogenic effects, PDGF D/β-PDGFR signaling differs from that of PDGF B/β-PDGFR (13). These differing oncogenic effects

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may be responsible for the aggressiveness and resistance to therapy seen in PCa with PTEN loss. Herein, we developed a murine cell model to examine the role of the PTEN/PDGF axis plays in radioresponsiveness of murine prostate epithelial cells.

Methods and Materials

Cell culture

Cell lines were cultured at 37°C in a humidified incubator with 5% CO_2. Establishment and characterization of murine and PTEN$^{-/-}$ prostate cell lines has been described previously (12, 14). Briefly, the cells are spontaneously immortalized prostate cells obtained from wild-type PTEN mice, and matching littermates with a prostate-specific deletion of PTEN. PTEN$^{-/-}$ cells are highly tumorigenic whereas PTEN$^{+/+}$ cells are nonmalignant. This model allows homogenous genetic background between lines for controlled study of the interaction of PTEN and radiosensitivity. Cells were maintained in advanced Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin (Invitrogen).

In vitro downregulation of PDGF D in mouse PTEN$^{-/-}$ prostate epithelial cell line

Scrambled shRNA sequence (shSCRM; catalog no. RHs4346), shRNA against PDGF D (shPDGF D; catalog no. RMM4431-98978525) were obtained from Open Biosystems (Huntsville, AL). PTEN$^{-/-}$ cells were grown to subconfluence then infected with shSCRM or shPDGF D lentivirus at 3 multiplicity of infection (MOI) per the manufacturer's protocol. Cells were selected with 3 μg/mL of puromycin, and the resulting pooled population was used for experimentation. Downregulation of PDGF D expression was confirmed by immunoblot assays.

Immunoblot analysis

Cells were grown to confluence in 100-mm tissue culture plates, then washed twice with warm phosphate-buffered saline solution (PBS) to remove serum. Cells were then cultured in 5 mL serum-free media for 48 hours. Conditioned medium (CM) was collected, and cells were washed with PBS and scraped for collection of cell lysates. Collected CM was concentrated 50-fold per the manufacturer's recommendation using an Amicon centrifugal filter unit (Millipore, Billerica, MA). A 30-μL quantity of concentrated CM was used for immunoblot analysis.

For whole-cell lysate protein analysis cells were lysed in RIPA lysis buffer (Millipore) containing 1 mmol/L PMSF, 2 mmol/L sodium orthovanidate, 1 mmol/L sodium fluoride, and a complete protease inhibitor cocktail (Roche, Indianapolis, IN) for 30 minutes, followed by centrifugation for 20 minutes. Lysate was quantified using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL), and 30 μg of whole cell lysate was used for immunoblot analysis.

In vivo tumorigenicity in a SCID mouse model

Either shSCRM or shPDGF D PTEN$^{-/-}$ cells at 1×10^6 cells per 100 μL in a 1:1 mixture of phenol red free Matrigel (BD Biosciences, San Jose, CA): serum-free medium were injected

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subcutaneously into both flanks of 6- to 8-week-old male nude (nu/nu) mice (Charles River, Wilmington, MA). Five mice (10 injections) per group were used per experiment, and in vivo analysis was repeated twice. Tumor volume was monitored for 2 weeks by external caliper measurements and calculated as $V = (L^2 \times l)/2$, where L and l represent small and large tumor diameter, respectively.

In vitro 3-dimensional cell culture

Cells were trypsinized into single cell suspension. A 20-μL quantity of growth factor-reduced Matrigel (BD Biosciences) was layered on a 1.0-mm coverslip and allowed to dry for 20 minutes at 37°C. Two thousand cells of each cell line were diluted in 80 μL of medium, and applied to each solidified Matrigel layer in a dropwise fashion. After 1 hour of incubation, cells were supplemented with 2 mL of medium containing 2% Matrigel. Medium was changed twice weekly, and cultures were grown for 14 days. Photography was performed at 2-day intervals, until distinct foci had grown.

Clonogenic cell survival assay upon irradiation

Cells were incubated in a 100-mm plate for 72 hours until approximately 80% confluent. Subsequently, cells were irradiated with 0, 2, and 6 Gy using a gantry-mounted Theratron Cobalt-60 machine with a dose rate of 1 Gy/min. Irradiation was performed at room temperature under atmospheric oxygen conditions. Dose delivered was confirmed with the use of a Farmer chamber. After irradiation, 1000 cells were seeded in triplicate into new 100-mm plates and grown for 7 or 10 days. Colonies were then fixed in methanol, stained with Crystal Violet, and colonies of more than 50 cells were counted. Survival curves were generated after normalization of plating efficiency of the sham-irradiated cells was calculated.

Migration and invasion assays

Transwell chambers (8-μm) were used for both migration and invasion. For invasion, Matrigel-coated transwells were used (BD Biosciences). After irradiation (as described above), cells were trypsinized and brought into single-cell suspension. Next, 7500 cells were resuspended in serum-free medium and placed in the well of each chamber. Serum containing growth medium was used as a chemoattractant. After 24 hours of incubation, each well was fixed, rinsed, and stained with Crystal Violet. After serial washes and drying, the number of migrating or invading cells per high-power field were counted using a Nikon TMS-F inverted microscope at ×100 magnification. Using the surviving fraction of cells, as determined by the concomitant clonogenic survival assay described above, the percentage of surviving cells with migratory and/or invasive potential was calculated.

Cell cycle analysis

Cell cycle distribution was analyzed 24 hours after irradiation by flow cytometry. One million cells were fixed in a 70% ethanol/PBS solution on ice for 1 hour. Cells were then treated with 10 mg/mL RNase A (Sigma, St. Louis, MO) for 1 hour at 37°C before staining with 1 mg/mL propidium iodide (Sigma). Fluorescence activated cell sorting (FACS) analysis at 488 nm was performed on a BD LSR II controlled by Diva software (BD

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Biosciences, San Jose, CA) and analyzed with ModFit LT software (Verity Software House, Topsham, ME).

Apoptosis

The DEVDase analysis for apoptosis was used. Briefly, 24 hours after irradiation, cells were lysed with a 0.5% NP40 lysis buffer, and 50 μL of lysate was incubated with 10 mmol/L Ac-DEVD-AMC substrate (Sigma) at 37°C for 1, 2, 3 and 4 hours. Substrate fluorescence was detected using a SpectraMax Gemini (Molecular Probes, Carlsbad, CA) at 380-nm excitation, 460 nm emission, and normalized to protein concentration.

Statistical analysis

Statistical analysis was performed using an unpaired Student *t* test. A *P* value of <.05 was considered to indicate a statistically significant difference between studied groups.

Results

A role for PDGF D in PTEN loss-mediated phenotypic transformation

When PTEN$^{+/+}$ and PTEN$^{-/-}$ prostate epithelial cells were plated into single cells, PTEN$^{+/+}$ cells grew into clusters by cell–cell adhesion after cell division, whereas PTEN$^{-/-}$ cells displayed scattered morphological characteristics similar to those of fibroblasts (Fig. 1A). In a 3D Matrigel morphogenesis assay (Fig. 1B), PTEN$^{+/+}$ cells formed smooth, rounded acini, whereas the PTEN$^{-/-}$ cells manifested as an invasive and nonpolarized morphotype. In agreement with a previous report (14), control PTEN$^{-/-}$ murine prostate epithelial cells were tumorigenic (Fig. 1C), whereas PTEN$^{+/+}$ cells fail to grow in animals (data not shown). These results confirmed PTEN loss-mediated phenotypic transformation in prostate epithelial cells.

PTEN loss led to increased PDGF D expression (Fig. 2). Immunoblot analysis confirmed significant downregulation of PDGF D expression in the population of PTEN$^{-/-}$ cells engineered to express PDGF D shRNA vector compared to the control shRNA vector (Fig. 2A). Loss of PDGF D did not affect PTEN expression levels (Fig. 2B). As shown in Figure 1, PDGF D attenuation in PTEN$^{-/-}$ cells reversed their fibroblast-like morphological characteristics in 2D culture (Fig. 1A) and the invasive morphology in a 3D Matrigel culture assay (Fig. 1B), reverted PTEN$^{-/-}$ cell phenotype into a PTEN$^{+/+}$-like appearance. Importantly, the tumorigenic potential of PTEN$^{-/-}$ cells was effectively abrogated, as shown in Figure 1C. These results demonstrate a critical role for PDGF D in PTEN loss-mediated phenotypic transformation and tumorigenicity in vivo.

A role for PDGF D in radioresistant phenotype in PTEN$^{-/-}$ cells

Inactivation of PTEN function and its effects on PCa cell survival and growth upon irradiation have not been well characterized at the molecular level. Thus, we sought to examine the effect of irradiation on the clonogenic survival of prostate epithelial cells in relation to PTEN status. We found that PTEN$^{-/-}$ cells have increased clonogenic survival in vitro compared to PTEN$^{+/+}$ cells after irradiation of 2 and 6 Gy (Fig. 3A). Importantly, PDGF D knockdown significantly reduced clonogenic survival in PTEN$^{-/-}$ cells (Fig. 3B).

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Irradiation did not affect PDGF D expression (Supplemental Fig. 1). These results suggest that increased PDGF D expression in PTEN$^{-/-}$ cells contributes to the radioresistant phenotype observed in PTEN$^{-/-}$ cells.

PDGF D is essential for PTEN loss-mediated cell motility and invasive phenotype before and after irradiation

PTEN$^{-/-}$ cells demonstrated enhanced migratory (Fig. 4A and B) and invasive (Fig. 5A and B) phenotypes in comparison to PTEN$^{+/+}$ cells. After irradiation, this difference was even more pronounced, as a significantly greater migratory and invasive potential was observed in the PTEN$^{-/-}$ line, particularly at the 6-Gy dose level (Figs. 4 and 5A and B). Both the basal and radiation-induced migratory and invasive phenotypes in PTEN$^{-/-}$ cells required PDGF D expression, as observed in the knockdown cell line (Figs. 4 and 5C and D).

Difference in radioresponse is likely independent of cell cycle or apoptosis

Next, we examined whether radiosensitivity regulated by the PTEN/PDGF D signaling axis involves regulation of cell cycle redistribution and/or apoptosis. As shown in Figure 6A, we observed little difference in cycle redistribution between the PTEN$^{+/+}$ and PTEN$^{-/-}$ cell lines or between the control and PDGF D knockdown PTEN$^{-/-}$ cell lines after irradiation (Fig. 6A). In addition, there was no increase in caspase-3-like activity in either PTEN$^{+/+}$ or PTEN$^{-/-}$ cells before or after irradiation. Interestingly, it was noted that PTEN$^{-/-}$ cells displayed higher levels of basal caspase-3 activity (Fig. 6B). The expression levels of PDGF D also had little effect on caspase-3-like activity in PTEN$^{-/-}$ cells before or after irradiation (Fig. 6C).

Discussion

The present study identifies PDGF D as a critical component for PTEN loss-mediated phenotypic transformation of prostate epithelial cells. PTEN is a non-redundant, plasma-membrane lipid phosphatase that hydrolyzes the 3-phosphate on phosphatidylinositol 3,4,5-triphosphate (PIP3), thereby negatively regulating PIP3-mediated signal transduction pathways such as the PI3K/Akt pathway (2). By regulating the pathways of Akt, PTEN plays a critical role in the regulation of many cellular processes including cell cycle, cell motility/invasion, cell adhesion, protein synthesis, and glucose metabolism. Loss of PTEN upregulates Akt, especially Akt3, in prostate epithelial cells, which is essential for PDGF D expression (12). Kwon et al demonstrated reactive oxygen species produced by stimulation by growth factors transiently inactivate PTEN, allowing for such downstream signaling (15). The effect of ionizing radiation on this loop is unexplored. The present study demonstrates that the PTEN/PDGF D signaling axis is critical for prostate epithelial cell motility/invasion and cell adhesion.

Evidence suggests that PTEN is critical for the maintenance of chromosomal integrity (16, 17). Loss of PTEN's ability to guard the genomic integrity is likely to result in the accumulation of genetic defects for the acquisition of aggressive tumor phenotypes. We made an interesting observation that the steady-state level of caspase activity is higher in PTEN$^{-/-}$ cells compared to the control PTEN$^{+/+}$ cells. Increased caspase activity in

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PTEN$^{-/-}$ cells may be a reflection of increased stress signaling in the absence of PTEN-mediated checkpoint regulation of cell cycle and DNA damage.

Studies show that PTEN is a critical determinant for radiosensitivity in human cancer cells (18, 19). Although the molecular mechanisms by which PTEN modulates radiosensitivity are not clear, recent studies suggest that increased cell survival in the absence of functional PTEN is unlikely to be associated with its ability to regulate DNA repair machinery involving RAD51 (18, 19). In this study, we demonstrated that tumorigenic murine cell lines with PTEN loss require PDGF D expression to maintain their radioresistant and highly migratory/invasive phenotype. Murine PTEN$^{-/-}$ prostate epithelial cells have a greater survival to the radiation doses specified in this study, as compared to PTEN$^{+/+}$ cells. Importantly, this increased survival is suppressed via the knockdown of PDGF D. Interestingly, this increased survival seems to be independent of cell cycle factors or apoptosis. Our observations demonstrate enhanced cell migration, invasion, and tumorigenesis in the absence of PTEN, and these phenotypes are abrogated by PDGF D downregulation. It should be noted that PDGF D knocked-down PTEN$^{-/-}$ cells were compared to the scrambled shRNA-transduced PTEN$^{-/-}$ cells rather than the parental PTEN$^{-/-}$ cells to properly control unanticipated potential effects associated with viral transduction, vector integration, or drug selection. Importantly, PTEN$^{-/-}$ cells in the presence of PDGF D signaling that survived after irradiation displayed an even more invasive phenotype. To our knowledge, this represents the first report of the effect of PDGF D expression and radioresponse in prostate cancer.

The mechanism by which the PTEN/PDGF signaling axis mediates radioresistance is unknown, warranting further investigation. It is possible that PDGF signaling regulates a set of gene expression critical for the radioresistant phenotype, such as androgen receptor (AR). AR has long been implicated in the carcinogenesis of PCa by maintaining cell proliferation and survival. Furthermore, loss of PTEN function mediates the development of ligand-independent activation of AR with a suggested mechanism of crosstalk between AR and the PI3K/AKT/mTOR pathway further increasing proliferation and survival (20). We hypothesize that PDGF D may be necessary for cells with the loss of PTEN to manifest in such cross-talk. We observed reversal of the radioresistant phenotype, which could have implications regarding potential targets for directed therapy used in conjunction with radiation as primary treatment.

Given the totality of our data, we conclude that PDGF D represents a potentially promising target for PCa treatment resistance that warrants further laboratory evaluation and clinical study.

Supplementary Material

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Summary

β-PDGFR is frequently upregulated and activated in prostate cancer. Loss of PTEN results in β-PDGFR ligand switch from PDGF B to PDGF D in prostate epithelial cells, which is associated with increased Gleason score and tumor stage in prostate cancer. The present manuscript identified PDGF D as a critical component for PTEN loss-mediated phenotypic transformation and radioresistance of prostate epithelial cells. We propose that PDGF D represents a potentially promising target for prostate cancer treatment resistance.

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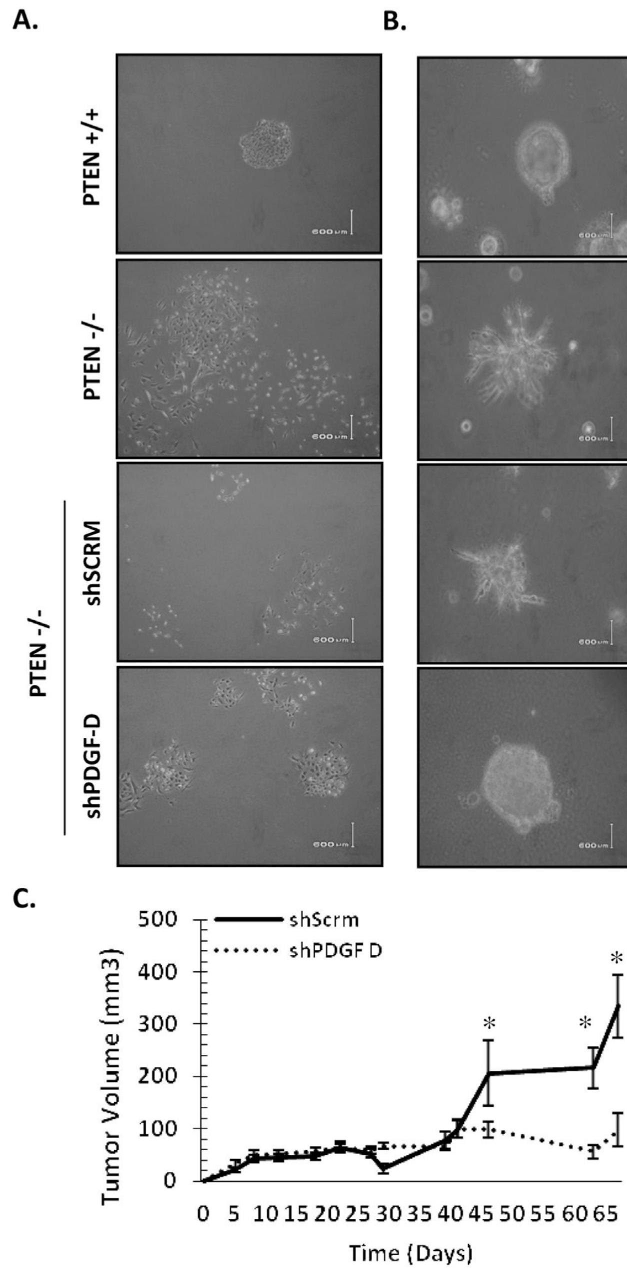

Fig. 1.

PDGF D mediates in vitro transformation and in vivo tumorigenesis. Morphology of PTEN$^{+/+}$, PTEN$^{-/-}$, and PTEN$^{-/-}$ expressing shSCRM and shPDGF D constructs in 2D culture (A) and 3D culture (B). In vivo tumorigenesis using PTEN$^{-/-}$ shSCRM and shPDGF D (C). shSCRM = scrambled shRNA. $^{*}P$<.05.

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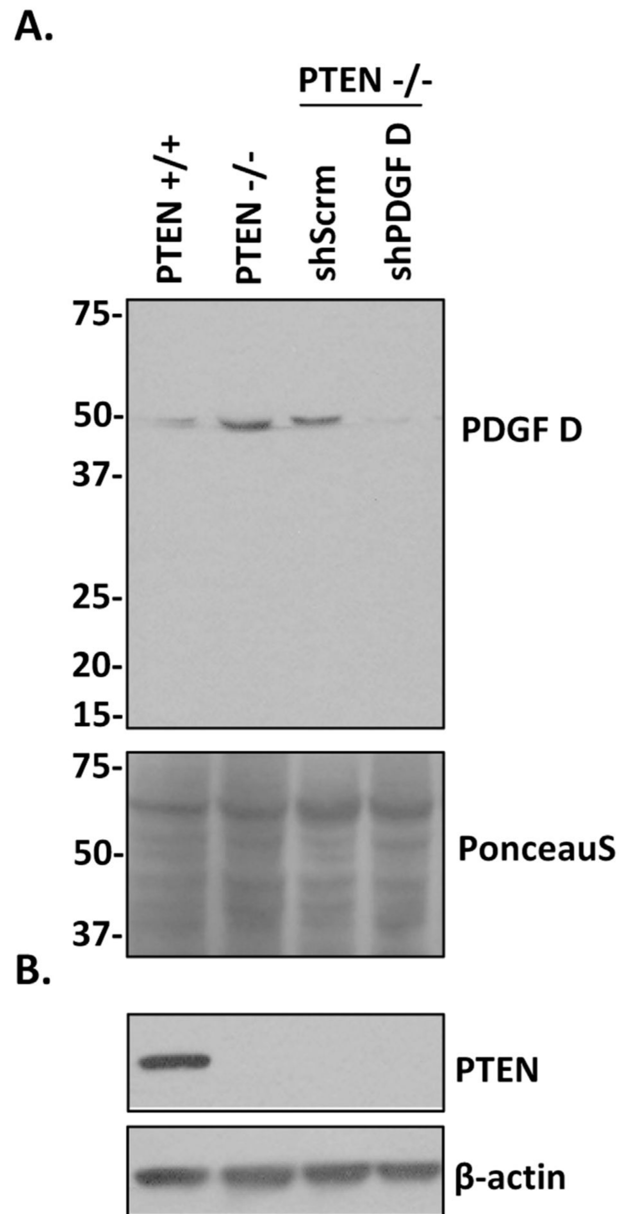

Fig. 2.

PDGF D expression profile in murine prostate cancer cell lines. Immunoblot analysis of concentrated conditioned media (A) and whole-cell lysate (B) from $PTEN^{+/+}$, $PTEN^{-/-}$, and $PTEN^{-/-}$ derivative cell lines. shSCRM = scrambled shRNA.

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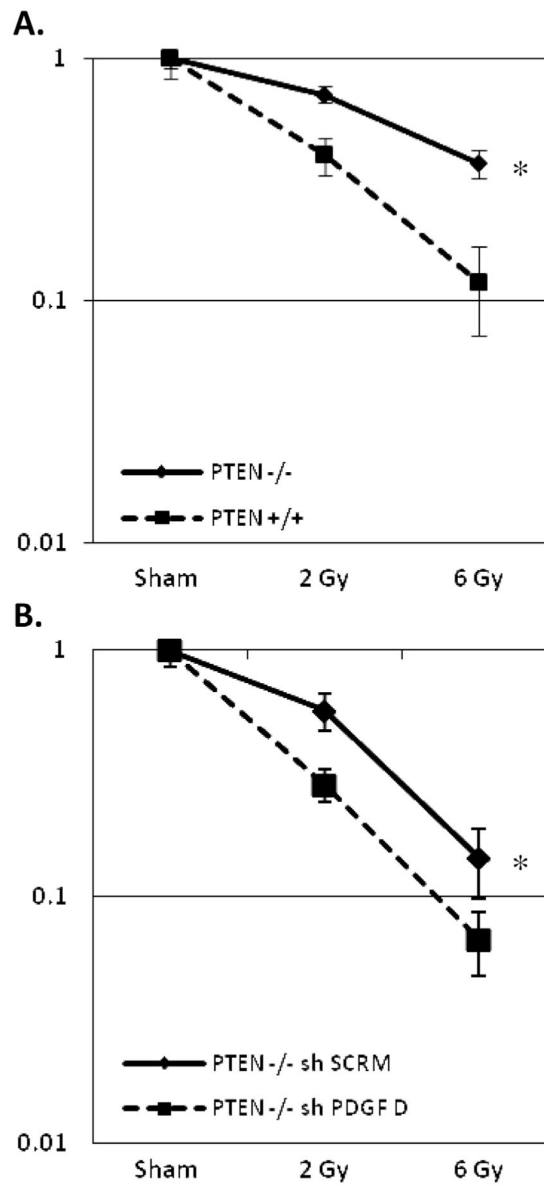

Fig. 3.
PTEN radioresistance is mediated by PDGF D. $PTEN^{-/-}$ cells have increased clonogenic survival in vitro compared to $PTEN^{+/+}$ at the radiation doses of null, 2, and 6 Gy (A). Importantly, PDGF D knockdown significantly reversed radioresistant phenotype in $PTEN^{-/-}$ cells (B). shSCRM = scrambled shRNA. $^{*}P<.05$.

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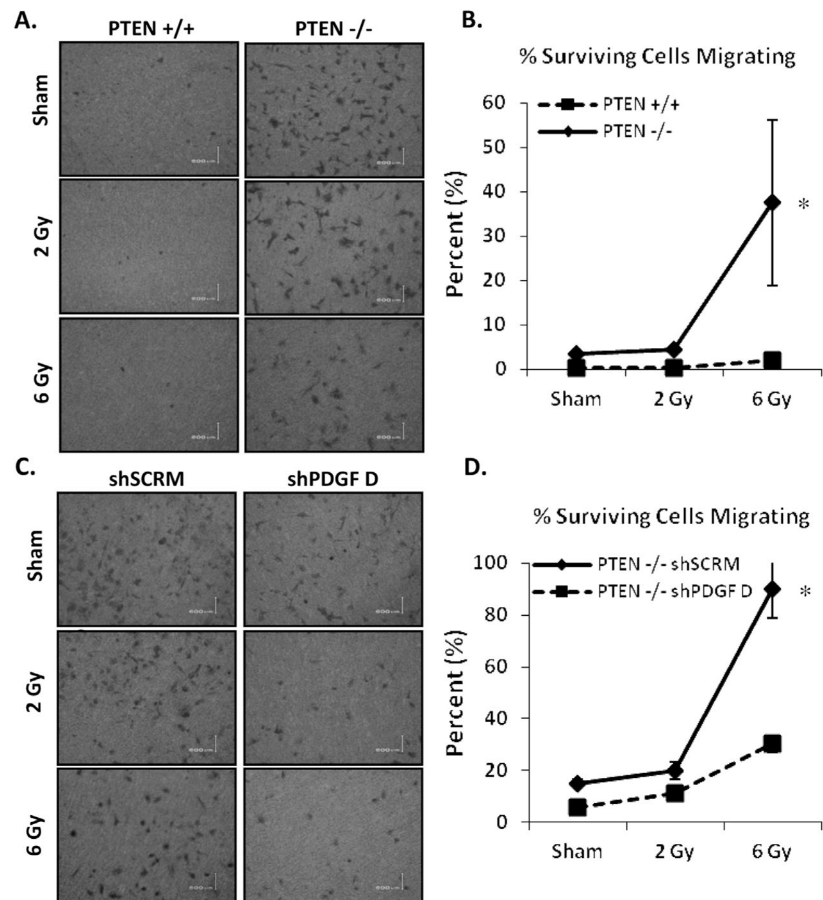

Fig. 4.

PDGF D plays a critical role in prostate cancer cell migration. Transwell assays revealed migratory phenotypes in $PTEN^{-/-}$ cells, whereas $PTEN^{+/+}$ cells were barely motile, particularly after irradiation (A and B). Both the basal and radiation-induced migratory phenotypes in $PTEN^{-/-}$ cells required PDGF D expression (C and D). shSCRM = scrambled shRNA. $^{*}P<.05$.

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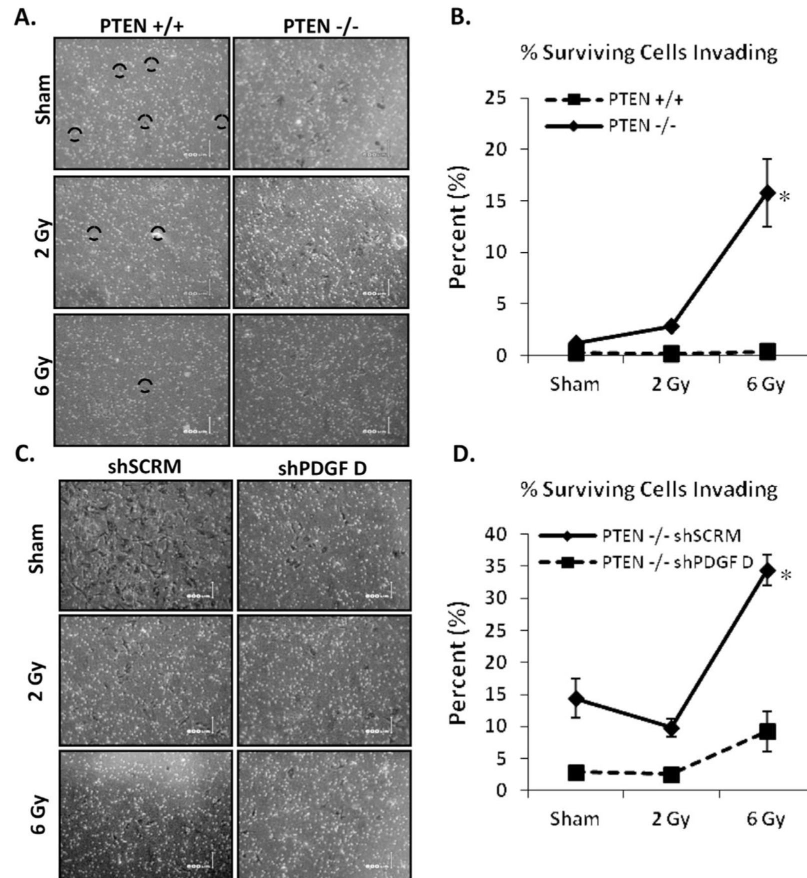

Fig. 5.

The invasive phenotype of PTEN$^{-/-}$ prostate cancer cell lines is PDGF D dependent. Greater invasive potential was observed in PTEN$^{-/-}$ cells than in PTEN$^{+/+}$ cells (A and B). Invasive phenotype required PDGF D expression (C and D). shSCRM = scrambled shRNA. $^{*}P$<.05.

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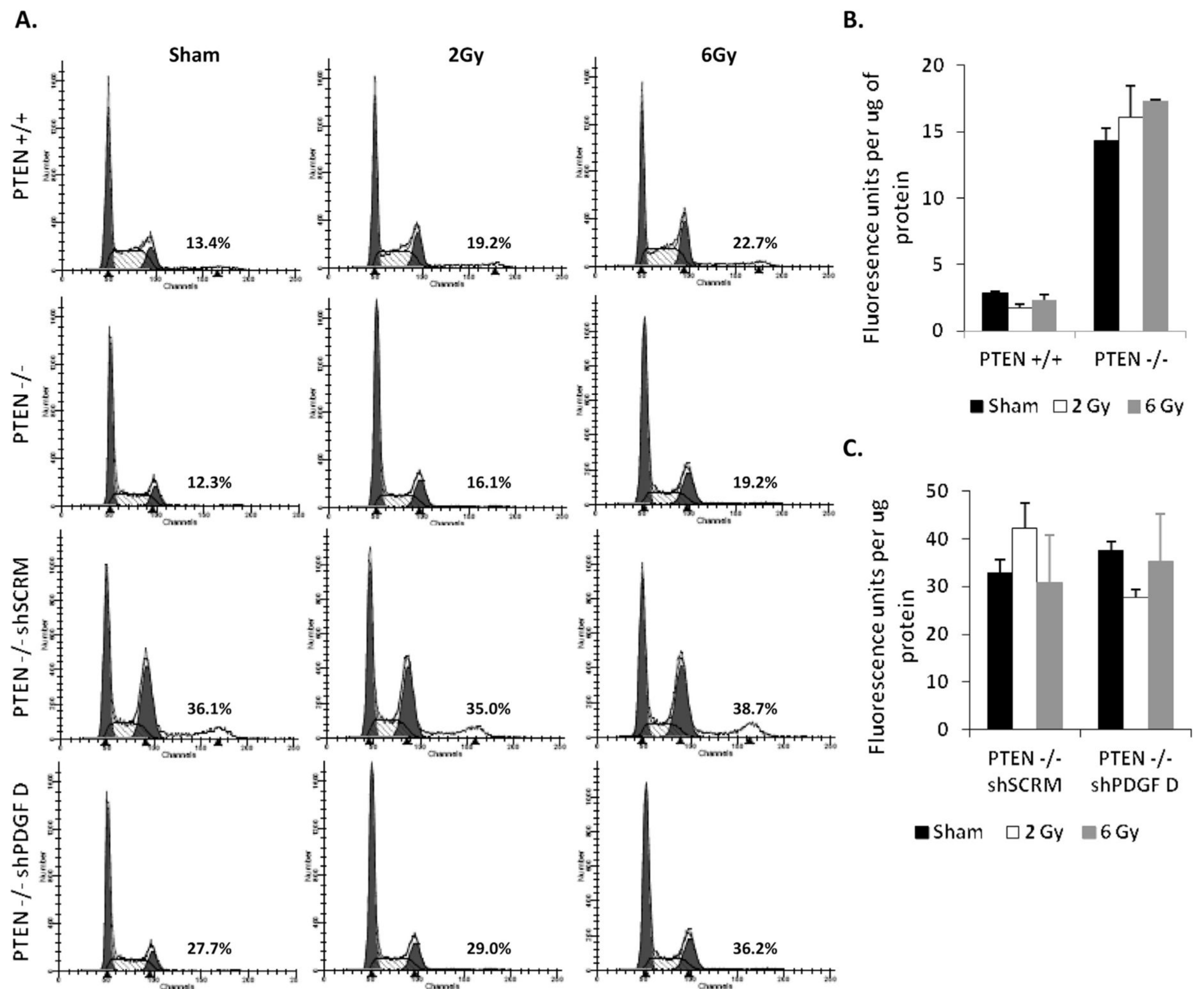

Fig. 6.

Cell cycle and apoptosis analysis in PTEN prostate cancer cell lines. Difference in cycle redistribution after irradiation was minimal when comparing either the parental cells or the transformed lines (A). Little caspase-3-like activity in $PTEN^{+/+}$ cells before or after irradiation, $PTEN^{-/-}$ cells displayed much higher levels of caspase-3 activity when the assay was run 24 hours after irradiation (B). PDGF D expression had little effect on caspase-3-like activity in $PTEN^{-/-}$ cells before or after irradiation (C). shSCRM = scrambled shRNA.