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Synthetic Lethal Targeting of PTEN-Deficient Cancer Cells Using Selective Disruption of Polynucleotide Kinase/Phosphatase

Todd R. Mereniuk¹, Mohamed A.M. El Gendy^{1,3}, Ana M. Mendes-Pereira⁴, Christopher J. Lord⁴, Sunita Ghosh¹, Edan Foley², Alan Ashworth^{4,5}, and Michael Weinfeld¹

Abstract

A recent screen of 6,961 siRNAs to discover possible synthetic lethal partners of the DNA repair protein polynucleotide kinase/phosphatase (PNKP) led to the identification of the potent tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*). Here, we have confirmed the PNKP/*PTEN* synthetic lethal partnership in a variety of different cell lines including the PC3 prostate cancer cell line, which is naturally deficient in *PTEN*. We provide evidence that codepletion of *PTEN* and PNKP induces apoptosis. In HCT116 colon cancer cells, the loss of *PTEN* is accompanied by an increased background level of DNA double-strand breaks, which accumulate in the presence of an inhibitor of PNKP DNA 3'-phosphatase activity. Complementation of PC3 cells with several well-characterized mutated *PTEN* cDNAs indicated that the critical function of *PTEN* required to prevent toxicity induced by an inhibitor of PNKP is most likely associated with its cytoplasmic lipid phosphatase activity. Finally, we show that modest inhibition of PNKP in a *PTEN* knockout background enhances cellular radiosensitivity, suggesting that such a "synthetic sickness" approach involving the combination of PNKP inhibition with radiotherapy may be applicable to *PTEN*-deficient tumors. *Mol Cancer Ther*; 12(10); 2135–44. ©2013 AACR.

Introduction

Synthetic lethality provides a means to target loss-of-function mutations commonly associated with the formation of neoplastic malignancies because it takes advantage of a cell's propensity to lose tumor suppressor function during its stepwise progression to cancer cell formation by targeting a second, distinct protein not essential for cell survival. Codisruption of both of these nonessential proteins or the genes encoding them in the same cell causes lethality, whereas each corresponding single disruption is compatible with survival (1–4). In this way, it is possible to selectively kill only those cells in which both of these proteins are disrupted, that is, cancer cells, whereas the effect on normal cells is considerably less detrimental.

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Therapeutic advantage can also be gained through the related concept of "synthetic sickness," in which codisruption of the genes/proteins severely weakens cells and increases their sensitivity to radiation or cytotoxic drugs (5–7).

To date, most of the focus on synthetic lethality has centered on the use of inhibitors of PARP and cancers that have lost function of the breast cancer susceptibility loci, *BRCA1* and *BRCA2* (8–11). However, increasing evidence shows that PARP inhibitors may benefit not only *BRCA*-deficient cancers but also cancers lacking noncanonical DNA repair proteins such as the important tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*; ref. 12). *PTEN* is inactive in a broad spectrum of hereditary and sporadic human cancers and is the second most frequently lost tumor suppressor behind only *p53* (13, 14). It participates in regulating cell growth and the cell cycle and plays a critical role in antiapoptotic pathways (13).

We have recently broadened the scope of synthetic lethality to include polynucleotide kinase/phosphatase (PNKP) as another viable therapeutic target (15). Like PARP, PNKP is an enzyme involved in the repair of DNA strand breaks. It possesses two activities, a 3'-DNA phosphatase and 5'-DNA kinase, which are required to restore the chemical composition of strand break termini to forms suitable for the subsequent action of DNA polymerases and ligases, i.e., 3'-hydroxyl and 5'-phosphate termini (16, 17). PNKP participates in several repair

pathways including base excision repair and single- and double-strand break (DSB) repair (18, 19). Depletion of PNKP activity by short hairpin RNA (shRNA), siRNA, or a small-molecule inhibitor of its phosphatase activity sensitizes cells to ionizing radiation and the topoisomerase I poison camptothecin (20, 21). Importantly, reduction of PNKP increases the spontaneous mutation frequency, indicating that it is required for the repair of endogenous DNA damage induced by reactive oxygen species (ROS; ref. 20).

To identify potential synthetic lethal partners of PNKP, we previously conducted an siRNA-library based screen of the "druggable" genome and discussed our findings about the partnership with the tumor suppressor SHP-1 (15). Among the other potential synthetic lethal partners of PNKP, we identified *PTEN*. Here, we report the validation of our initial findings confirming that indeed PNKP and *PTEN* act in a synthetic lethal partnership and describe initial experiments to uncover the potential mechanism. We also show that loss of *PTEN* coupled with partial inhibition of PNKP significantly sensitizes cells to ionizing radiation. Our data suggest that the clinical usefulness of PNKP disruption may be extended to include *PTEN*^{-/-} cancers.

Materials and Methods

Cell lines

A549 cells were purchased from the American Type Culture Collection (ATCC). A549δPNKP (A549 cells stably depleted of PNKP using shRNA) and A549-SC (A549 cells stably expressing a scrambled shRNA) have been previously described (15). The PC3 human prostate cancer parental cell line and its variants were previously described (12). PC3 cells were sourced from ATCC and maintained according to the supplier's instructions in a mycoplasma-free environment. To confirm the identity of PC3 cells (both in their original and modified forms), we used short tandem repeat typing of DNA derived from PC3 cultures as described in the work of Dirks and Drexler (22). The correct identity of PC3 cells was last confirmed on February 8, 2013. The HCT116 human colon cancer parental cell line and its *PTEN* knockout variants and G418-resistant control (23) were generously provided by Dr. Todd Waldman (Georgetown University, Washington DC). The presence or absence of *PTEN* expression was confirmed by Western blot analysis but no further authentication was carried out by the authors.

Cell lines were cultured at 37°C and 5% CO₂ in a humidified incubator in a 1:1 mixture of Dulbecco's modified Eagle medium and F12 (DMEM/F12) supplemented with 10% FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 2 mmol/L L-glutamine, 0.1 mmol/L nonessential amino acids, and 1 mmol/L sodium pyruvate. All culture supplements were purchased from Invitrogen.

Vectors and siRNA

pSUPER.neo vectors (Oligoengine) contained either an shRNA directed against nucleotides 1,391 to 1,410 of

PNKP (20) to stably deplete PNKP in A549 cells or an shRNA to no known gene target (scrambled shRNA) to generate the control cell line A549-SC.

The preparation of pBABE.puro (Addgene) vectors containing wild-type (WT) RAD51 cDNA or WT or mutated *PTEN* cDNA used to generate the PC3-reconstituted cell lines: WT *PTEN* (full-length, WT *PTEN*), p.K289E (*PTEN* mutant with reduced nuclear shuttling), p.R55fs*1 (truncation mutant found in PC3), and p.C124S (a phosphatase inactive *PTEN* mutant), was reported previously (12).

All siRNAs were purchased from Qiagen with the exception of PNKP siRNA (Ambion).

Cell transfection

Twenty-thousand cells were plated and allowed to adhere overnight in a 24-well dish at 37°C and 5% CO₂. The transfection mixture was prepared from two separate solutions, one containing 1 µg of plasmid DNA dissolved in 50 µL total of Opti-MEM (Invitrogen) and the other 3 µL of Lipofectamine 2000 (Invitrogen) in 50 µL total Opti-MEM. The solutions were incubated at room temperature for 5 minutes before combination, mixed, and then held at room temperature for 20 minutes. The media from the preplated cells was removed and replaced with the transfection mixture and the cells were incubated for 24 hours at 37°C and 5% CO₂. The cells were then trypsinized and expanded as previously described (15). Transient transfections were conducted using 4,000 cells per well and a final concentration of 16 nmol/L of siRNAs as previously described (15).

Cell proliferation assay

Assays were conducted using the transient transfection technique described above; however, after incubation with siRNA for 72 hours, 10% v/v of 440 µmol/L Alamar Blue (Sigma-Aldrich) was added to each well and the cells were incubated for 50 to 90 minutes, after which the fluorescence in each well was determined using an EnVision 2104 Multilabel Reader (PerkinElmer) with an excitation wavelength of 563 nm and emission wavelength of 587 nm. HCT116-based cell lines were subjected to a 10.7% v/v 440 µmol/L Alamar Blue solution per well.

Clonogenic survival assay

Cells, seeded in 60 mm dishes 24 hours in advance, were treated with the PNKP inhibitor A12B4C3 (kindly provided by Dr. Dennis Hall, University of Alberta; refs. 21, 24) for 9 to 14 consecutive days at 0, 0.1, 1, and 10 µmol/L final concentration. 100 cells were plated for the 0, 0.1, and 1 µmol/L concentration groups and 300 cells in the 10 µmol/L concentration group. Colonies were then stained with a crystal violet stain containing 20% methanol for one hour, after which the plates were washed in warm water and left to dry overnight. Colonies of 50 cells or more were counted using an automated colony counter (Oxford Optronix).

To determine the radiation response, cells were treated with 0, 1, 2, 4, 6, or 8 Gy γ -radiation (⁶⁰Co Gammacell,

AECL) in the absence of PNKP inhibitor or under continuous PNKP inhibition using 2 $\mu\text{mol/L}$ A12B4C3. Cells subjected to continuous PNKP inhibition were pretreated with 2 $\mu\text{mol/L}$ A12B4C3 for 24 hours before irradiation.

Statistical analysis

Reported P values were generated using a two-sided Student t test. Z-scores were generated from an average of 24 to 96 individual wells of data per assay (conducted at least in triplicate). The high number of replicates allowed us to achieve robust statistical data. A Z-score is a dimensionless quantity denoting the number of SDs a sample is above or below the mean of a control. It is defined as:

$$z = \frac{x - \mu}{\sigma}$$

z = Z-score

x = the raw score to be standardized

μ = population mean

σ = SD of the population

Z-scores can therefore be positive or negative depending on whether the sample is more or less than the mean of controls. We were interested in a negative Z-score as this showed that the experimental condition was lethal in comparison with control conditions. A Z-score of 3 or less is significantly different than control as it is at least three SDs less than the control average.

To analyze for additivity and synergy, we used the median effect principle as proposed by Chou (25, 26) to calculate the median effect and the combination index (CI) based on the multiple drug effect equation provided by Chou and Talalay (27, 28). The CI value of 1 was considered for additive effect, less than 1 was considered for synergism effect, and more than 1 was considered for antagonism effect. The median effect and CI were calculated using CALCUSYN software version 1.2 (Biosoft).

Determination of mode of cell death

A549-SC or A549 δ PNKP cells were grown on coverslips in complete DMEM/F12 and transfected with ASN or PTEN siRNA. As a positive control, the cells were treated with 100 $\mu\text{mol/L}$ 5-(p-bromobenzylidene)- α -isopropyl-4-oxo-2-thioxo-3-thiazolidineacetic acid (BH3I-1, Sigma-Aldrich), which induces apoptosis. The cells were triple-stained after the indicated length of time with Hoechst 33342, Ethidium Homodimer III, and Annexin V-fluorescein isothiocyanate (FITC) as previously described (15).

Monitoring DSB repair

DSBs were monitored on the basis of the level of phosphorylated histone H2AX (γ -H2AX). Two experiments were conducted. First, we monitored the level of γ -H2AX before and after γ -irradiation. Cells were seeded on coverslips (10^5 cells/well) in 6-well plates with 2 mL DMEM/F12 without antibiotics and left overnight to adhere. The cells were irradiated (5 Gy) and allowed to repair for the indicated time points, after which γ -H2AX

was detected as previously described (29). Fluorescence was normalized against background fluorescence and quantified using MetaXpress software (version 5.0.0.21, Molecular Devices). Second, we tested the effect of PNKP inhibition on the accumulation of DSBs in the absence of irradiation. Cells were incubated with 5 $\mu\text{mol/L}$ of A12B4C3 and γ -H2AX was detected and quantified at different time points.

Results

Confirmation of PTEN as a possible synthetic lethal partner of PNKP

We previously sought to discover synthetic lethal partnerships of PNKP, as an alternative to PARP, without necessarily limiting our search to partner proteins directly involved in DNA repair (15). We conducted a forward transfection screen with a library of siRNAs targeting 6,961 genes using pooled samples of four distinct siRNAs targeting each gene. The screen was conducted in duplicate using A549 lung cancer cells stably depleted of PNKP (A549 δ PNKP) and again using cells expressing a scrambled shRNA (A549-SC) under identical conditions. Cells were exposed to siRNA transfection complexes continuously for 72 hours allowing for at least two cell cycles to occur at a concentration known to be effective at knocking down target proteins. Among the potential synthetic lethal partners of PNKP was the major tumor suppressor PTEN. Figure 1A shows the difference between five selected tumor suppressors, including the previously characterized PNKP synthetic lethal partner SHP-1 (15), compared with two proteins not synthetically lethal when codisrupted with PNKP.

To confirm the synthetic lethal relationship between PNKP and PTEN, we repeated the transfections, but reduced the concentration of siRNA previously used in the screen by 3.5-fold. At this concentration, the siRNA still showed synthetic lethality in PNKP knockdown cells but not in A549-SC control cells (Fig. 1B). We then sought to use each of the four originally pooled siRNAs separately to further minimize the potential for off-target effects (Fig. 1C). When the distinct siRNAs directed against PTEN were assayed, two displayed selective killing of A549 δ PNKP cells and no toxicity in control cells, siRNA #6 Z-factor = -9.0 , $P < 0.001$; siRNA #8 Z-factor = -9.1 , $P < 0.001$. Because more than one siRNA showed synthetic lethality with PNKP, the effect was most likely attributable to the double knockdown of PNKP and PTEN and not due to off-target effects. Activation of the RNAi pathway using AllStars negative control siRNA (ASN) is also not responsible for the lethality seen, indicating this is a true synthetic lethal partnership. The greater cytotoxicity seen with the PTEN/PNKP double knockdown using PTEN #6 siRNA than when using PTEN #8 siRNA probably reflected the efficiency of the two siRNAs to knockdown PTEN expression (Fig. 1E). This would imply a dose-response effect that may have implications regarding natural levels of active PTEN found in tumors.

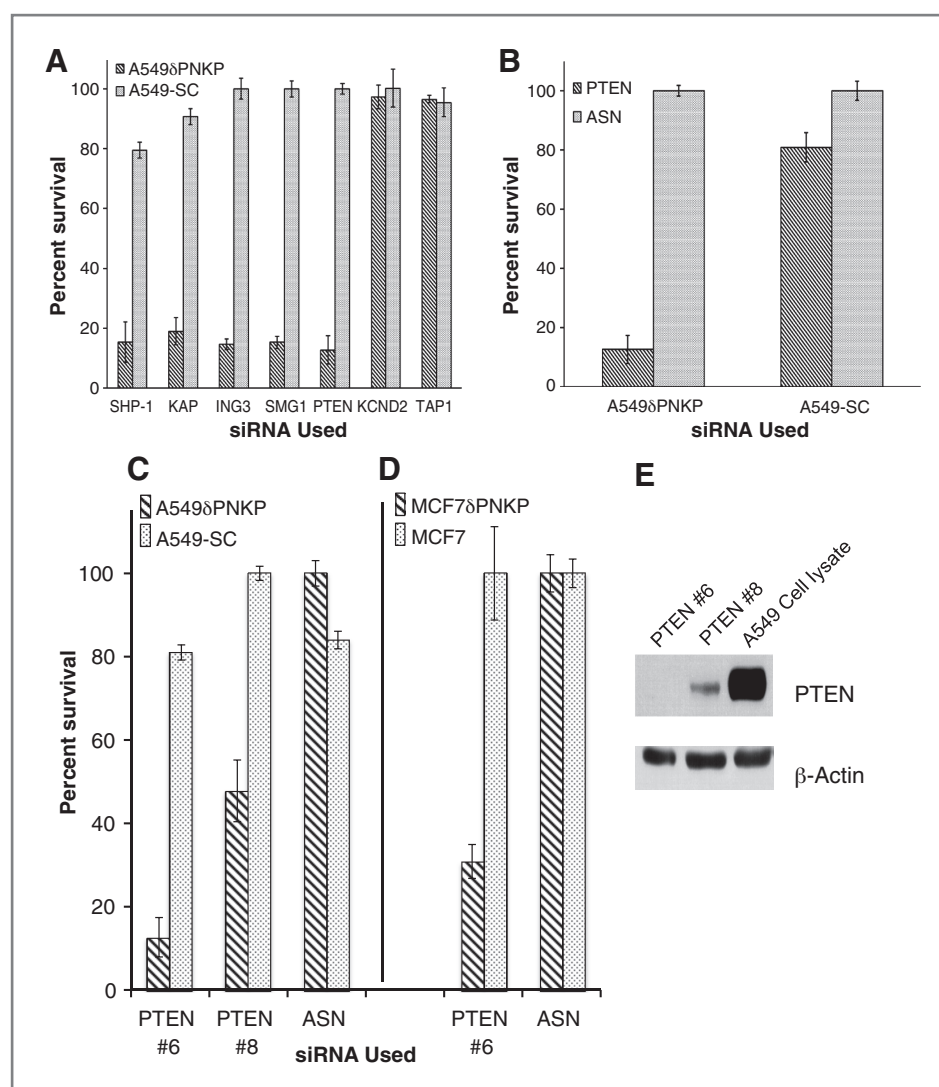


Figure 1. Tumor suppressors, including PTEN, identified by an siRNA-based screen of cell proliferation (15) as potentially synthetic lethal with PNKP. Two cell lines were used for the screen: A549 cells stably depleted of PNKP by shRNA (A549 δ PNKP) and A549 cells expressing a scrambled shRNA (A549-SC). In addition to the five tumor suppressor genes showing potential synthetic lethality, two randomly selected genes (*KCND2* and *TAP1*) are shown to identify the difference between potential hits and non-hits. B, further confirmation of PTEN–PNKP synthetic lethality. To limit the off-target effects, 16 nmol/L of siRNA (3.5-fold less than the concentrations used for screening) was used to transiently transfected A549 δ PNKP and A549-SC cells with the PTEN siRNA pool and a scrambled control siRNA (ASN). Only when PTEN was knocked down in combination with PNKP did we see significant lethality (Z-factor = -9.01 ; $P < 0.001$). Error bars represent \pm SEM from at least three independent determinations. C, validation of the PTEN–PNKP synthetic lethal partnership. The four siRNAs that were pooled for screening purposes were tested independently to assess their effectiveness at killing A549 δ PNKP cells as determined by the cell proliferation assay. Only two of the four siRNAs caused lethality in combination with PNKP disruption, with PTEN #6 siRNA displaying greater cytotoxicity than #8 siRNA (PTEN #6 Z-factor = -9.01 ; $P < 0.001$, PTEN #8 Z-factor = -9.06 ; $P < 0.001$). D, WT MCF7 cells and MCF7 cells stably depleted of PNKP (MCF7 δ PNKP) were transiently transfected with PTEN #6 siRNA or control scramble siRNA (ASN). Error bars represent \pm SEM from at least three independent determinations. E, Western blot analysis of A549 cells transiently transfected with PTEN #6 and #8 siRNAs.

To further substantiate that a synthetic lethal partnership exists between PTEN and PNKP, we carried out a similar analysis with the MCF7 breast cancer cell line and #6 siRNA. As seen with A549 cells, the combined disruption of both PTEN and PNKP was responsible for lethality, as the depletion of PNKP or PTEN individually was not lethal (Fig. 1D, Z-score for PTEN = -8.0 , $P < 0.001$, Z-score for SHP-1 = -3.4 , $P < 0.001$), nor is the activation of RNAi machinery responsible for lethality. These findings imply

that a true synthetic lethal relationship exists between PTEN and PNKP.

Finally, isogenically matched HCT116 parental, Neo124 vector only (PTEN $^{+/+}$) and PTEN $^{-/-}$ (#22 and #35) cells (23) were subjected to increasing concentrations of the PNKP phosphatase inhibitor A12B4C3 (21). Figure 2 clearly shows that loss of PTEN sensitized the cells to A12B4C3 and underscores the importance of the DNA 3'-phosphatase activity of PNKP to the synthetic lethal process.

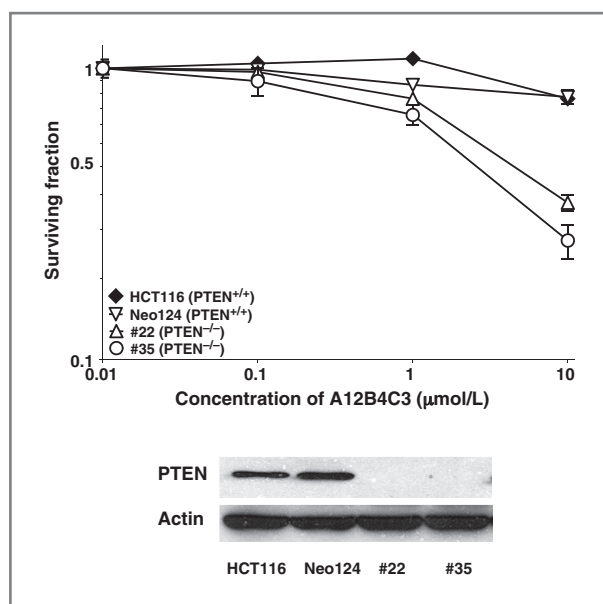


Figure 2. Survival of PTEN-null cells treated with a PNKP phosphatase inhibitor. Isogenically matched PTEN^{+/+} cells (HCT116 parental and Neo124 vector control cells) and two PTEN^{-/-} HCT116 strains, #22 and #35, were subjected to increasing concentrations of the PNKP inhibitor A12B4C3 and survival was assessed by clonogenic survival assay. Error bars represent \pm SEM from at least three independent determinations; each assay was conducted in triplicate for a minimum of nine total assessed plates per point.

Mode of cell death

To identify the mode of cell death responsible for synthetic lethality following codisruption of PTEN and PNKP, A549-SC and A549 δ PNKP cells were grown on glass coverslips and transiently transfected with either PTEN or ASN siRNA. As a positive control, the known apoptosis inducer, BH3I-1 was added to the medium at a concentration of 100 μ mol/L. After the indicated lengths of time, cells were triple stained with Hoechst 33342, Ethidium Homodimer III, and Annexin V-FITC to distinguish between those cells undergoing apoptosis, necrosis, or neither. Figure 3A shows that a small population of both apoptotic and necrotic cells was present at every time point; however, treatment of cells with BH3I-1 dramatically increased the proportion of apoptotic cells relative to necrotic cells beginning at 24 hours posttreatment in both A549-SC and A549 δ PNKP (Fig. 3B). Similarly, when both PTEN and PNKP were disrupted in the same cells, there was a substantial increase in apoptosis (Fig. 3C). Conversely, when PTEN was knocked down in A549-SC cells, there was no increase in the proportion of either apoptotic or necrotic cells (Fig. 3C). Therefore, cells codysfunctional for PTEN and PNKP undergo cell death through apoptotic mechanisms, similar to cells in which both SHP-1 and PNKP are doubly disrupted (15).

DNA damage and repair in PTEN-null cells

To begin to identify a potential mechanism for the PTEN/PNKP synthetic lethal partnership, we examined

the effect of loss of PTEN on DNA DSB repair, as determined by the level of phosphorylation of the histone variant H2AX in the HCT116 cell lines following exposure to ionizing radiation. As shown in Fig. 4A, PTEN^{-/-} HCT116 cells display (i) a higher background level of γ -H2AX in unirradiated cells compared with the control PTEN^{+/+} cells and (ii) slower repair kinetics, especially during the initial 4-hour period postirradiation. The slower repair kinetics may reflect decreased homologous recombination seen previously in PTEN^{-/-} HCT116 cells (12).

We then examined the influence of PNKP inhibition on the background level of DSBs and observed that exposure of the PTEN^{-/-} HCT116 cells to A12B4C3 led to a modest but constant increase in γ -H2AX fluorescence, whereas the control HCT116 cells showed no response to A12B4C3 (Fig. 4B).

Because the elevated background level of DSBs was reminiscent of our earlier observation with SHP-1-depleted cells (15), albeit to a less marked extent, we measured the production of ROS in PTEN-deficient cells. However,

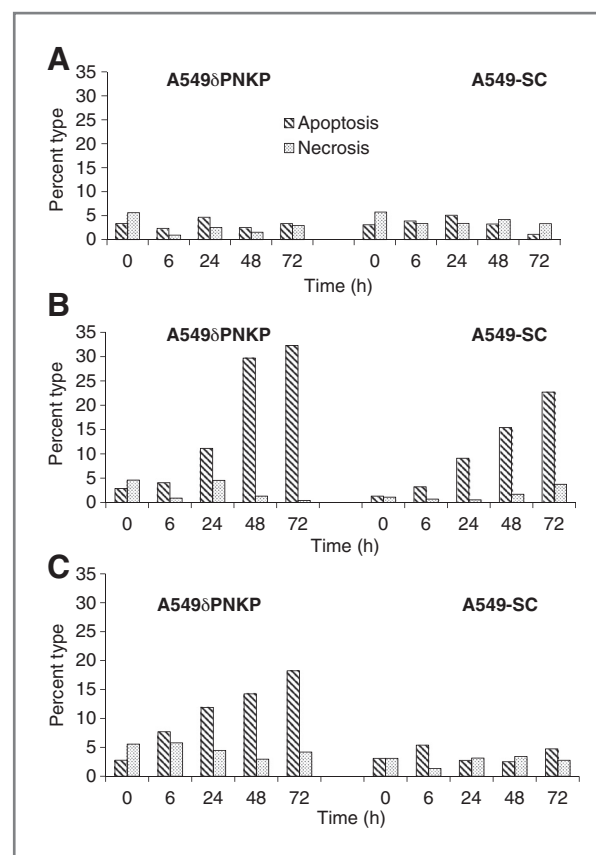


Figure 3. Determination of the mode of cell death of cells undergoing synthetic lethality due to the concurrent ablation of PTEN and PNKP function. A, A549 δ PNKP and A549-SC cells were transiently transfected with scrambled control siRNA (ASN) and the proportion of apoptotic versus necrotic cells was determined at times posttransfection as described in Materials and Methods. B, ASN-treated cells were additionally treated with the apoptosis inducer BH3I-1. C, measurement of apoptotic and necrotic cells at the indicated time points following transient transfection with PTEN siRNA.

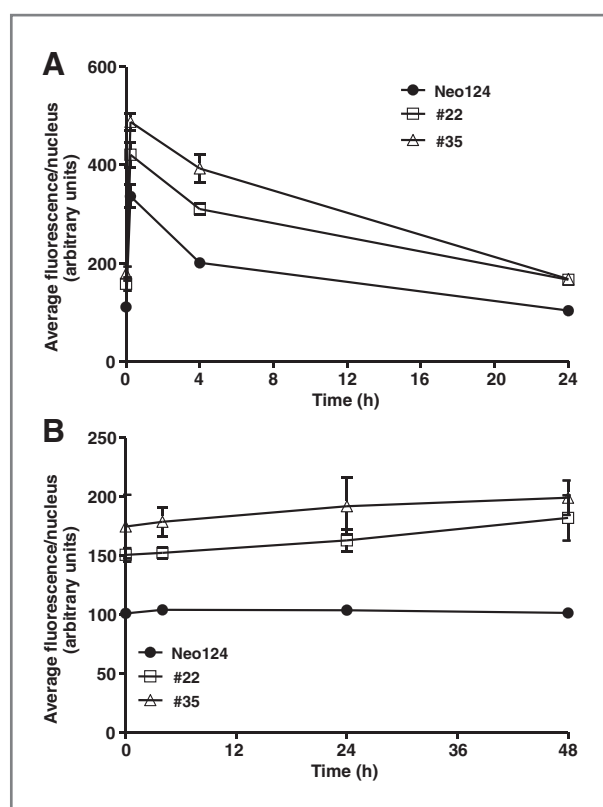


Figure 4. Influence of PTEN depletion on DNA DSB induction and repair. **A**, PTEN^{+/+} HCT116 (Neo 124) and PTEN^{-/-} (#22 and #35) cells were plated 24 hours in advance, after which they were subjected to γ -radiation (5 Gy). The level of DSBs was monitored by quantification of the average integrated fluorescence intensity per nucleus due to phosphorylation of histone H2AX as a function of time after 5-Gy irradiation. **B**, accumulation of DSBs by exposure of the HCT116 cells to 5 μ mol/L A12B4C3. Error bars represent \pm SEM.

examination of the isogenically matched PTEN^{+/+} and PTEN^{-/-} HCT116 cells revealed that these cells produced almost identical levels of peroxynitrite and hydroxyl radicals, indicating that knocking out PTEN, unlike SHP-1 depletion (15, 30), does not cause a significant increase in the production of these ROS (Supplementary Fig. S1).

Survival of naturally occurring PTEN-negative cells in response to PNKP inhibition

The ability to effectively and efficiently target one member of a synthetic lethal partnership will determine synthetic lethality's clinical usefulness. We therefore investigated whether we could take advantage of the newly identified partnership between PTEN and PNKP. We subjected the prostate cancer cell line, PC3 (naturally PTEN^{-/-}), to an increasing concentration of the PNKP inhibitor A12B4C3 over a period of 12 to 16 days. The dose-response curve (Fig. 5C) indicates that at A12B4C3 doses ≥ 10 μ mol/L there was a marked decrease in survival of the PC3 parental cell line. We then made use of ectopic expression of WT and various mutant PTEN cDNAs to further analyze the role of key components of PTEN (Fig. 5C). As

anticipated, expression of the empty vector, p.BABE.puro and a vector coding for the PTEN deletion-frameshift mutation found in PC3 cells, p.R55fs*1, did not alter the result seen with the parental PC3 cells. Similarly, expression of the catalytically inactive mutant of PTEN, coding for the C124S-altered protein, also failed to elicit an increase in the survival of A12B4C3-treated cells. In contrast, PC3 cells reconstituted with either WT PTEN (WT) protein or the K289E-modified PTEN, which is phosphatase proficient but undergoes reduced shuttling to the nucleus, restored resistance to PNKP inhibition. Finally, because of a potential association between PTEN and Rad51 (12, 31–33), we examined PC3 cells ectopically expressing RAD51 cDNA, but found no increase in survival in response to treatment with A12B4C3 (Fig. 5D). From these data, we infer that the critical function of PTEN lies in its phosphatase activity and its localization in the cytoplasm.

A cell proliferation assay (Supplementary Fig. S2) indicated that the natural differences in cell growth rate could not account for the observed responses of the PC3-derived cells to A12B4C3.

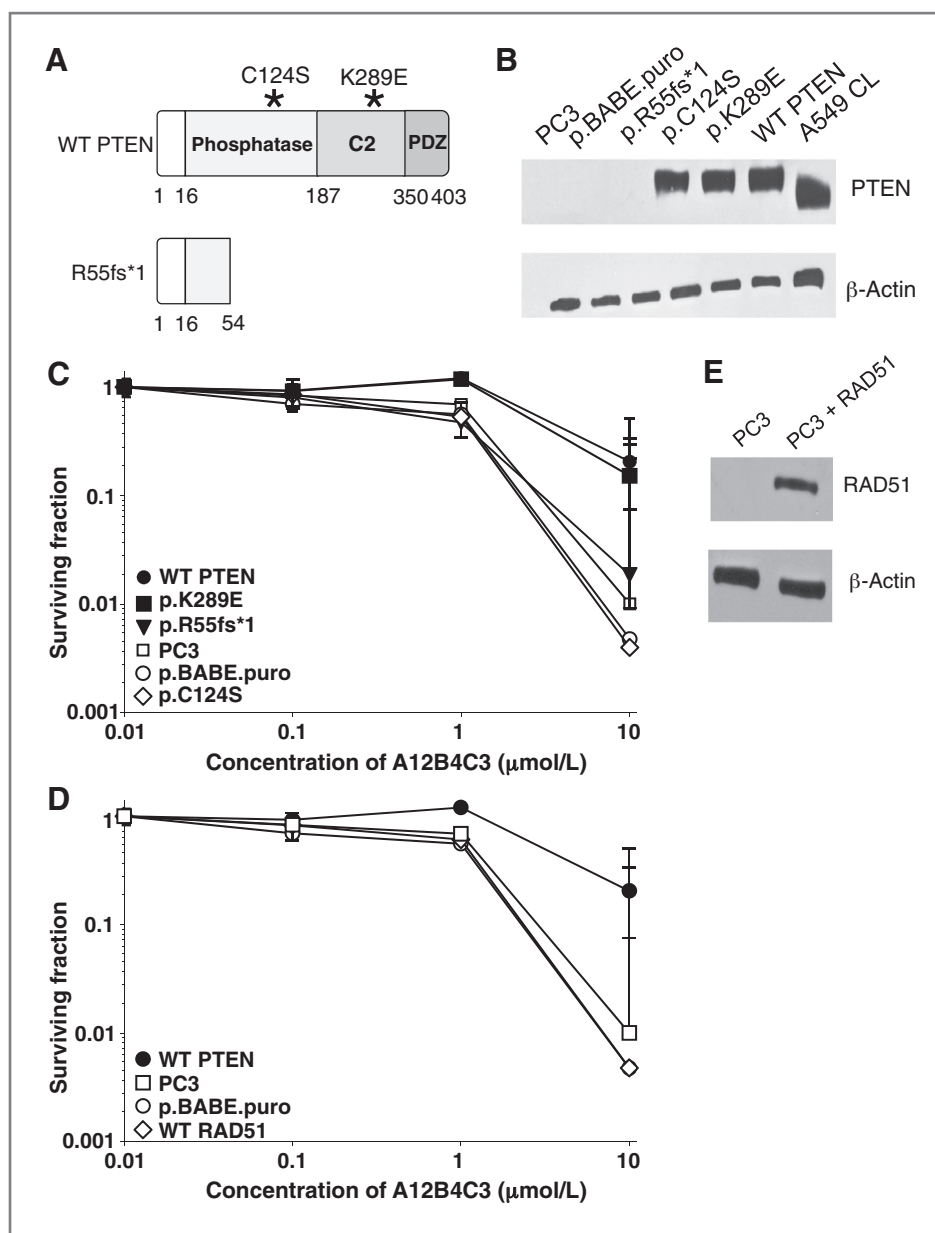
Radiosensitization by combined disruption of PNKP and PTEN

We have previously observed that depletion or inhibition of PNKP sensitizes cells to ionizing radiation (20, 21). We therefore examined whether disruption of PNKP in PTEN-negative cells would hypersensitize cells to ionizing radiation (Fig. 6). WT and the two PTEN^{-/-} HCT116 cell lines were incubated with a nontoxic dose (2 μ mol/L) of A12B4C3 [or just the dimethyl sulfoxide (DMSO) vehicle] for 24 hours before irradiation with doses up to 8 Gy and then maintained in the presence of the PNKP inhibitor until colonies were counted. As expected, A12B4C3 sensitized the control PTEN^{+/+} HCT116 cells to radiation in a similar manner to that previously seen with A549 cells (21). We also observed that the PTEN^{-/-} cell lines were modestly radiosensitive in comparison with the control cell line in accordance with published data (23). However, when PTEN and PNKP were simultaneously disrupted, there was a significant enhancement of radiosensitization. Applying the method of Chou and Talalay to analyze for additivity and synergy (27, 28), we found a synergistic radiation hypersensitization at 8 Gy (PTEN^{-/-} #22 cells) and 6 Gy (PTEN^{-/-} #35 cell line).

Discussion

We have confirmed a synthetic lethal relationship between PTEN and the DNA repair protein PNKP following our initial screen (15). PTEN is recognized as the second most frequently compromised tumor suppressor. Its downregulation or complete loss is implicated in the development and/or progression of many sporadic human cancers (13, 14). For example, PTEN functional mutations or complete protein loss was found to occur frequently in glioblastoma, endometrial cancer, melanoma, and prostate

Figure 5. Identification of the critical function of PTEN for survival under PNKP disruption. **A**, graphical representation of the forms of PTEN used to reconstitute PC3 cells, including the truncation mutation found in PC3 cells (R55fs*1), indicating the location of the other mutations in the phosphatase domain (C124S) and in the C2 structural domain involved in targeting proteins to cell membranes (K289E). The PDZ domain is a structural domain commonly found in signaling proteins, but was not mutated in these experiments (50). **B**, Western blot analyses of PC3 cells transfected with vector only (p.BABE.puro), cDNA for R55fs*1 or the different forms of full-length PTEN. **C**, survival of PC3 cells transfected with expression vectors encoding various forms of PTEN and exposed to increasing concentrations of A12B4C3: p.BABE.puro (vector only); WT PTEN (full-length)–WT PTEN; p.K289E–PTEN mutant with reduced nuclear shuttling; p.R55fs*1 – truncation mutant; p.C124S – phosphatase inactive PTEN mutant. Error bars represent \pm SEM from at least three independent determinations, each experiment carried out in triplicate for a minimum of nine total assessed plates per point. **D**, survival response of PC3 cells transfected with a vector expressing WT RAD51 cDNA (WT RAD51) and exposed to increasing concentrations of A12B4C3. Error bars represent \pm SEM from at least three independent determinations; each experiment was conducted in triplicate for a minimum of nine total assessed plates. **E**, Western blot analysis of RAD51 expression in PC3 cells.



cancer (28.8%, 34.6%, 12.1%, and 11.8%, respectively) (14). PTEN-deficient tumors thus represent an excellent target for synthetic lethal approaches to treatment. Other synthetic lethal partners of PTEN have been discovered including PARP (12) and more recently the TTK protein tyrosine kinase (34).

PTEN plays a critical role as an antagonist of the phosphoinositide 3-kinase (PI3K) pathway in the cytoplasm through its lipid phosphatase function by dephosphorylating the 3 position of the second messenger phosphatidylinositol 3,4,5-triphosphate (PIP3) to form inactive phosphatidylinositol 4,5-bisphosphate (PIP2), thereby suppressing downstream signaling events, including those involving phosphoinositide-dependent kinase-1 (PDK1) and the serine/threonine kinase Akt

(35–41). When PTEN is deficient, there is an accumulation of PIP3, which activates downstream signaling molecules such as the Akt and mTOR complex 1. In addition to its cytoplasmic roles, PTEN also localizes to the nucleus in a cell-cycle-dependent manner with higher levels seen in G_0 – G_1 (42). In the nucleus, the protein phosphatase activity of PTEN regulates MAPK phosphorylation and cyclin D1 (43, 44) and progression of the cell cycle. Several studies have also linked PTEN to genomic stability and homologous recombination primarily via expression of *RAD51* (12, 31–33), although this is an issue of some debate (45, 46).

The synthetic lethality manifested by the treatment of PTEN-deficient tumor cells with PARP inhibitors has been ascribed to a reduction in RAD51 levels and homologous recombination coupled with incomplete single-strand

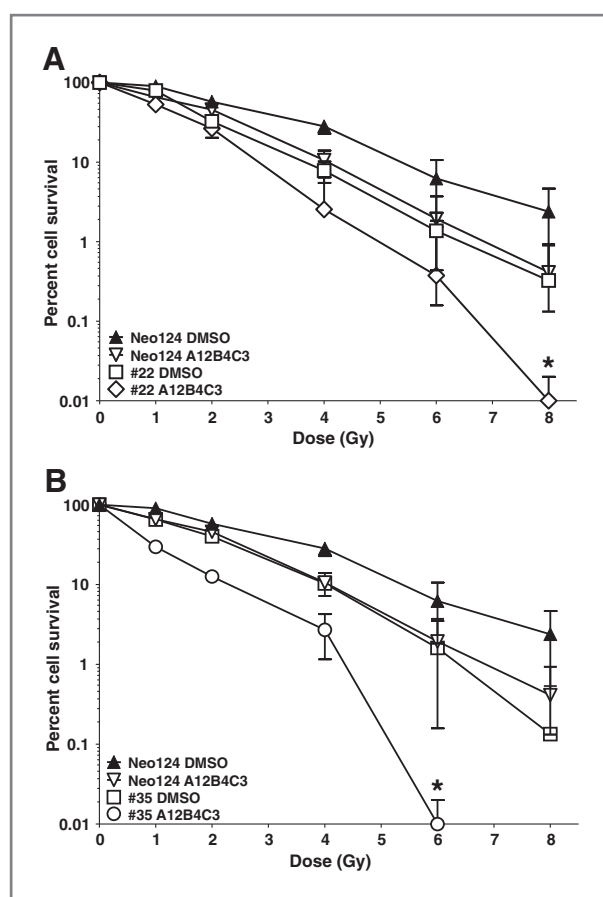


Figure 6. Radiation sensitization by combined inactivation of PTEN and PNKP. A, vector only control HCT116 cells (Neo124), and HCT116 PTEN^{-/-} strains #22 (A) and #35 (B) were grown in the presence of A12B4C3 (2 μ mol/L) or DMSO vehicle for 24 hours and then subjected to increasing doses of γ -radiation and further incubated in the presence of A12B4C3. Survival was measured by clonogenic survival assay. Inhibition of PNKP and loss of PTEN alone modestly sensitized the cells to radiation, but a more marked radiosensitization was observed when the PTEN knockout cells were irradiated and further incubated in the presence of A12B4C3. Asterisks indicate synergy in radiosensitization due to combined inactivation of PTEN and PNKP. Error bars represent \pm SEM from at least three independent determinations; each experiment was conducted in triplicate for a minimum of nine total assessed plates.

break repair (12). In contrast, the synthetic lethality we observed between PTEN and PNKP could not be alleviated by ectopic expression of *RAD51* (Fig. 5D). Furthermore, unlike the response to PARP inhibition, resistance to PNKP inhibition was restored by expression of the phosphatase active and cytoplasmically enriched PTEN K289E protein, but not the catalytically inactive C124S protein that can enter the nucleus. Therefore, the function of PTEN that is critical for survival under PNKP disruption most likely lies in its cytoplasmic function as a lipid phosphatase in signal transduction pathways. Interestingly, in our original screen we identified MAGI3 and MAST2 as potential synthetic lethal partners of PNKP (15), although this will need further confirmation. Because it has been shown that PTEN stability is enhanced through

interaction with the PDZ domains of both of these cytoplasmic proteins (47–49), it is conceivable that a synthetic lethal partnership with PNKP would be mediated by reduced stability and activity of PTEN located in the cytoplasm.

The clear differences between the responses to RAD51 and PTEN isoforms indicate that distinct mechanisms underlie the synthetic lethal pathways between PTEN and PARP and between PTEN and PNKP despite the fact that both PARP and PNKP play significant roles in DNA strand break repair. Our observation that PTEN-deficient cells show an increase in the baseline level of DSB and slower DSB repair (Fig. 4) suggests that loss of PTEN leads to increased production of DNA strand breaks with 3'-phosphate termini requiring PNKP for their repair. However, given the relatively modest differences between WT and PTEN^{-/-} cells, coupled with our failure to observe increased ROS production in PTEN^{-/-} cells, this issue requires further investigation.

In addition to using the PTEN/PNKP relationship under purely synthetic lethal conditions, we also examined the possibility of taking advantage of synthetic sickness, that is, weakening the cell to other therapeutic agents. From a clinical standpoint, the use of a repair protein inhibitor in a synthetic sickness approach offers two advantages—either augmenting cell killing for a given dose of the primary genotoxic anticancer agent or allowing the use of a lower dose of the primary agent to achieve the same level of cancer cell killing but reducing the likelihood of normal tissue damage. The potential of such an approach was shown by the increased radiosensitization afforded by cotreatment with the PNKP inhibitor. This provides a possible therapeutic modality in which PTEN-depleted tumors would first be sensitized by inhibition of PNKP and then targeted by focused radiation. Because PNKP disruption is well tolerated by PTEN-proficient normal cells, there would be little damage to normal tissues and thus side effects should be minimized.

Disclosure of Potential Conflicts of Interest

T.R. Mereniuk, E. Foley, and M. Weinfeld have ownership interest in a provisional US patent application—UNAB 017 Synthetic lethality in cancer. A. Ashworth may benefit financially from the development of PARP inhibitors through patents held jointly with AstraZeneca through the Institute of Cancer Research's rewards to inventors scheme. No potential conflicts of interest were disclosed by the other authors.

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