

# PTEN Loss Compromises Homologous Recombination Repair in Astrocytes: Implications for Glioblastoma Therapy with Temozolomide or Poly(ADP-Ribose) Polymerase Inhibitors

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## Abstract

Glioblastomas (GBM) are lethal brain tumors that are highly resistant to therapy. The only meaningful improvement in therapeutic response came from use of the S<sub>N</sub>1-type alkylating agent temozolomide in combination with ionizing radiation. However, no genetic markers that might predict a better response to DNA alkylating agents have been identified in GBMs, except for loss of O<sup>6</sup>-methylguanine-DNA methyltransferase via promoter methylation. In this study, using genetically defined primary murine astrocytes as well as human glioma lines, we show that loss of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) confers sensitivity to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a functional analogue of temozolomide. We find that MNNG induces replication-associated DNA double-strand breaks (DSB), which are inefficiently repaired in PTEN-deficient astrocytes and trigger apoptosis. Mechanistically, this is because PTEN-null astrocytes are compromised in homologous recombination (HR), which is important for the repair of replication-associated DSBs. Our results suggest that reduced levels of Rad51 paralogs in PTEN-null astrocytes might underlie the HR deficiency of these cells. Importantly, the HR deficiency of PTEN-null cells renders them sensitive to the poly(ADP-ribose) polymerase (PARP) inhibitor ABT-888 due to synthetic lethality. In sum, our results tentatively suggest that patients with PTEN-null GBMs (about 36%) may especially benefit from treatment with DNA alkylating agents such as temozolomide. Significantly, our results also provide a rational basis for treating the subgroup of patients who are PTEN deficient with PARP inhibitors in addition to the current treatment regimen of radiation and temozolomide. *Cancer Res*; 70(13); 5457–64. ©2010 AACR.

## Introduction

Despite considerable work in recent years elucidating the molecular underpinnings of glioblastoma (GBM), the most deadly of brain cancers, little progress has been made in improving clinical outcomes. The most significant breakthrough in patient response to date emerged from the use of the S<sub>N</sub>1-type alkylating agent temozolomide in combination with ionizing radiation (IR), which increased the overall median survival from approximately 12 to 15 months (1–3). However, no genetic markers that might predict a better response to DNA alkylating agents have

been identified for GBMs except for O<sup>6</sup>-methylguanine-DNA methyltransferase (*MGMT*) promoter methylation (3, 4). The past year has seen unprecedented advances in the genomic analyses of adult GBM tumors by the Cancer Genome Atlas Network and other groups which reveal that these tumors have radically altered genomes with many mutations, gene copy number gains and losses, and methylation changes (5–7). Among the myriad of genetic alterations that populate the GBM genomic landscape, five genetic changes dominate: loss of *Ink4a*, *Arf*, *p53*, or phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) and amplification of epidermal growth factor receptor (*EGFR*). How these genetic aberrations confer therapeutic resistance remains unclear. Understanding the contribution of these lesions, singly and in combination, to GBM therapy resistance along with the underlying mechanism(s) will be of paramount importance in developing more effective therapeutic modalities.

In a previous study, we showed that amplification of *EGFRvIII* confers radioresistance to GBM-relevant cells and tumors by promoting the repair of radiation-induced DNA double-strand breaks (DSB) by nonhomologous end joining (NHEJ; ref. 8). In this study, using genetically defined primary murine astrocytes as well as human glioma lines, we focused on the role of PTEN in modulating sensitivity

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to the  $S_N1$ -type alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG; ref. 9). Loss of PTEN is a very prominent event during gliomagenesis, occurring in about 36% of GBMs (5, 6, 10). PTEN is a lipid phosphatase with a canonical role in dampening the phosphatidylinositol 3-kinase (PI3K)/Akt-1 signaling pathway; hence, loss of PTEN has oncogenic consequences during gliomagenesis (11). In addition, it is becoming increasingly clear that PTEN has novel nuclear functions, including transcriptional regulation of the *Rad51* gene, whose product is essential for homologous recombination (HR) repair of DNA breaks (12, 13). We report here that loss of PTEN in astrocytes results in increased sensitivity to MNNG. We show that MNNG induces secondary DSBs that are poorly repaired in PTEN-null astrocytes due to compromised HR. The increased sensitivity of PTEN-null astrocytes to MNNG tentatively suggests that patients with PTEN-null GBMs may especially benefit from treatment with temozolomide. More importantly, the HR deficiency of PTEN-null astrocytes opens up the possibility of treating PTEN-deficient GBMs with poly(ADP-ribose) polymerase (PARP) inhibitors that are currently in clinical trials for treating HR-deficient breast and ovarian cancers (14–16).

## Materials and Methods

### Cell culture

Astrocytes were isolated from 5-day-old pups, as described (17), from littermates of an *Ink4a/Arf*<sup>-/-</sup> *PTEN*<sup>f/+</sup> × *Ink4a/Arf*<sup>-/-</sup> *PTEN*<sup>f/+</sup> cross (18, 19). Primary mouse astrocytes were maintained in DMEM containing 10% fetal bovine serum in a humidified 37°C incubator with 5% CO<sub>2</sub>. The floxed *PTEN* allele was deleted using an adenovirus expressing Cre. All cells were mycoplasma-free.

### Irradiation and drug treatment

A <sup>137</sup>Cs source (JL Shepherd and Associates) was used for  $\gamma$ -ray irradiation of cells. MNNG (Sigma) and camptothecin (CPT; Sigma) were dissolved in DMSO and stored at -20°C in aliquots of 100 mmol/L. ABT-888 (Alexis Biochemicals) was dissolved in cell culture grade water and stored at -20°C. MNNG treatments were given as a 1-hour pulse, whereas CPT and ABT-888 were added continuously at the indicated concentrations.

### Colony formation assays

Cells were plated in triplicate onto 60-mm dishes (300 cells per dish) and irradiated with graded doses of radiation or treated with increasing concentrations of MNNG, CPT, or ABT-888. Surviving colonies were stained with crystal violet about 7 days later as described (20).

### Immunofluorescence staining and Western blot analyses

Immunofluorescence staining of cells and Western blot analyses of whole-cell extracts were done as described (21). Antibodies used were anti-Rad51 (Santa Cruz), anti- $\gamma$ H2AX (Upstate), anti-53BP1 (Cell Signaling), anti-actin (Sigma),

anti-Akt, anti-phospho-Akt(Ser473) (Cell Signaling), anti-MGMT (Santa Cruz), rhodamine red-conjugated goat anti-rabbit, and FITC-conjugated goat anti-mouse (Molecular Probes).

### Metaphase chromosome preparations and sister chromatid exchange assay

To examine chromosome aberrations, astrocytes were treated with MNNG, and 24 hours later, 1  $\mu$ g/mL colcemid (Sigma) was added for 3 hours. Metaphase chromosome spreads were then prepared using standard procedures. Aberrations were counted and categorized as breaks or asymmetrical exchanges (triradials and quadriradials). To visualize sister chromatid exchanges (SCE), cells were incubated in the presence of bromodeoxyuridine (BrdUrd) (BD Biosciences; 10  $\mu$ mol/L) for two cell divisions, after which metaphases were prepared according to the above protocol. For drug treatments, MNNG was added as a 1-hour pulse immediately before BrdUrd, whereas CPT was added concurrently. Aberrations and SCEs were quantified by analyzing 100 metaphase spreads and differences were statistically analyzed as described below.

### Statistical analyses

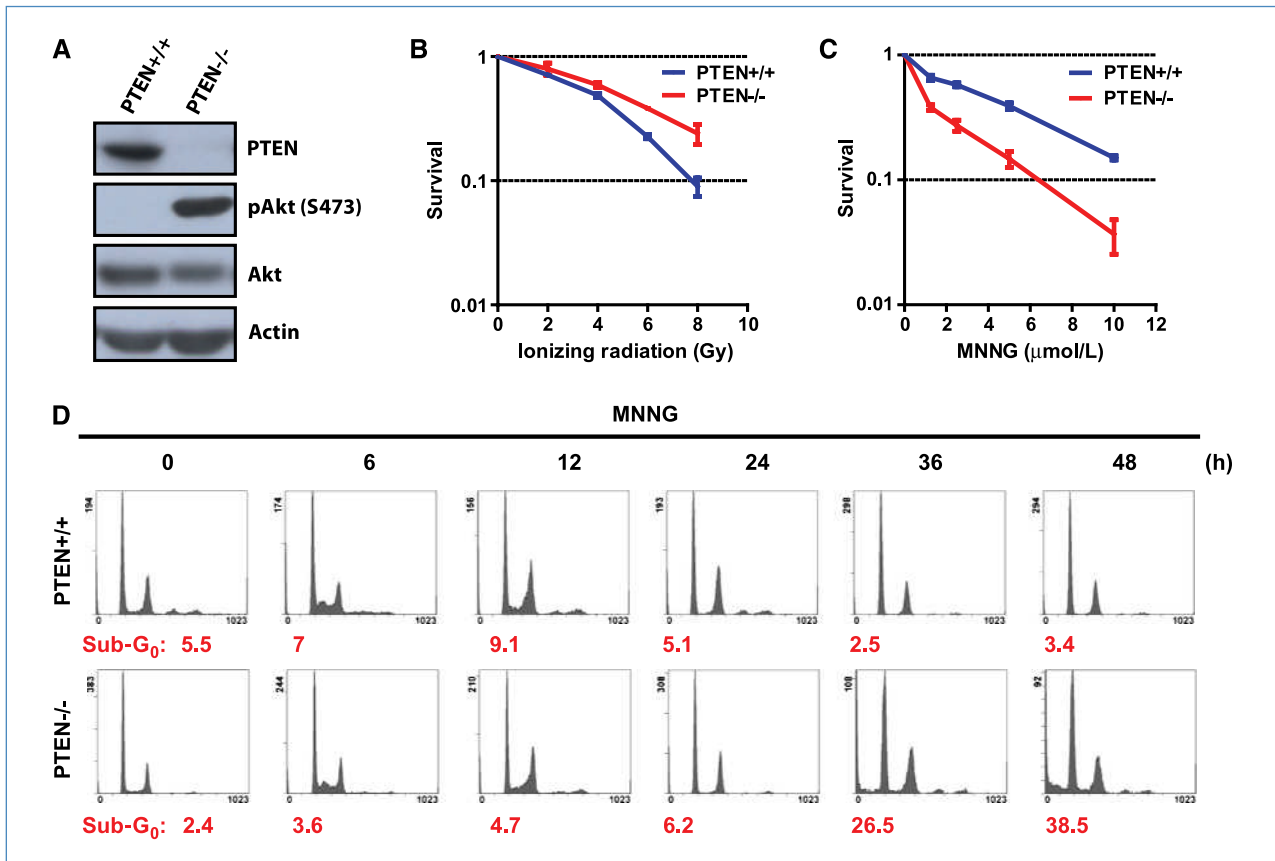
*P* values for experiments were calculated using GraphPad Prism. SCE and chromosome aberration data were analyzed by a two-tailed *t* test, whereas quantitative real-time PCR (qRT-PCR) data and repair kinetics were analyzed using two-way ANOVA.

### Additional methods

See Supplementary Data.

## Results

For this study, primary astrocytes were generated from *Ink4a/Arf*<sup>-/-</sup>*PTEN*<sup>f/+</sup> or *Ink4a/Arf*<sup>-/-</sup>*PTEN*<sup>f/f</sup> transgenic littermates. Once in culture, floxed *PTEN* alleles were deleted by adenoviral expression of Cre recombinase, generating a set of matched astrocytes with the following genotypes: *Ink4a/Arf*<sup>-/-</sup>*PTEN*<sup>f/+</sup> and *Ink4a/Arf*<sup>-/-</sup>*PTEN*<sup>-/-</sup>. Because primary mouse astrocytes senesce within a couple of passages after extraction, the *Ink4a/Arf*<sup>-/-</sup> background ensures that the astrocytes are immortal and provides a tumor-suppressor background that is very relevant to GBMs (5, 6). *PTEN* deletion on adenovirus infection was confirmed by Western blotting (Fig. 1A), as well as by PCR analysis (Supplementary Fig. S1). As expected, *PTEN* loss strongly activated PI3K signaling as evidenced by increased levels of phosphorylated Akt-1 (phospho-Ser473; ref. 11). In accordance with previous reports (22, 23), we found that loss of *PTEN* resulted in increased resistance to IR as assayed by colony survival (Fig. 1B). In contrast, *PTEN* loss resulted in sensitization to MNNG (Fig. 1C). Flow cytometric analyses revealed a significant increase in the sub-G<sub>0</sub> population in MNNG-treated *PTEN*-deficient cultures, indicating that the sensitivity of these astrocytes to MNNG was due to an increase in cell death (Fig. 1D).



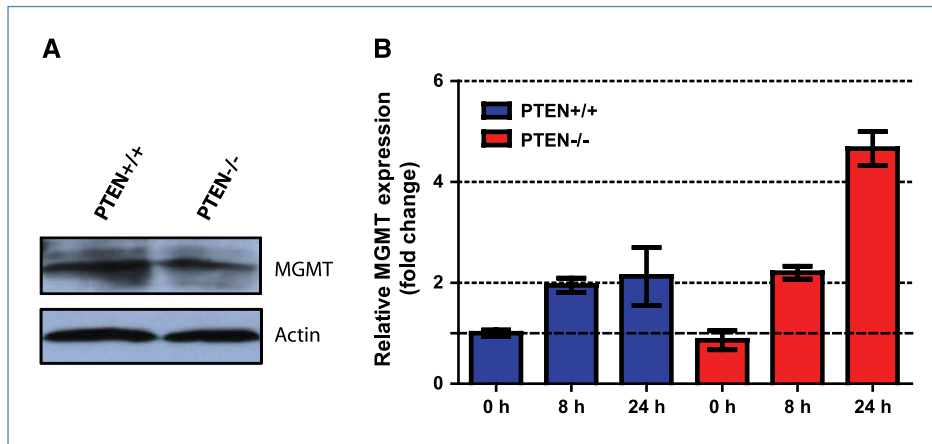
**Figure 1.** PTEN loss sensitizes astrocytes to MNNG. A, loss of PTEN and activation of Akt in PTEN<sup>+/+</sup> and PTEN<sup>-/-</sup> astrocytes were analyzed by Western blotting with  $\alpha$ -PTEN and  $\alpha$ -phospho-Akt(ser473) antibodies. B, the radiation survival of astrocytes was quantified by colony formation assays. The fraction of surviving colonies (y-axis) was plotted against corresponding radiation dose (x-axis). C, the sensitivity of astrocytes to MNNG was quantified by colony formation assays. Note increased sensitivity of PTEN-null cells to MNNG. Bars, SEM of experiments done three or more times. D, induction of cell death by MNNG was assessed by quantifying the sub-G<sub>0</sub> population in MNNG-treated cultures by flow cytometry (percentages in red).

Whereas the cytoplasmic role of PTEN in squelching the PI3K/Akt-1 pathway is an established concept, recent reports clearly indicate novel nuclear functions for this protein, including roles in transcription regulation (11–13). The sensitivity of PTEN-null cells to MNNG was probably not due to hyperactivation of Akt-1 because astrocytes expressing constitutively active myristylated Akt-1 (8) did not display an increased sensitivity to MNNG compared with parental cells (Supplementary Fig. S2). Therefore, it is plausible that the sensitivity to MNNG observed in PTEN<sup>-/-</sup> astrocytes was due to loss of a nuclear function of PTEN.

Toxicity from S<sub>N</sub>1-type alkylating agents results mainly from a specific type of DNA lesion, methylation of the O<sup>6</sup> position of guanine (O<sup>6</sup>meG; ref. 24), which can be reversed by the suicide repair enzyme MGMT (25). It has been previously reported that MGMT promoter silencing by methylation corresponds to a better therapeutic response to temozolomide (3, 4). Therefore, we investigated whether a decrease in MGMT levels due to PTEN loss might underlie the sensitivity of these cell lines to MNNG. However, Western blot analyses of basal MGMT protein levels did not show a significant difference between PTEN<sup>+/+</sup> and PTEN<sup>-/-</sup> astrocytes (Fig. 2A).

MGMT transcription is induced in response to various DNA-damaging agents, including MNNG (24, 26). However, qRT-PCR analyses revealed that both lines were capable of inducing MGMT transcription on MNNG treatment (Fig. 2B). These data strongly suggest that the sensitivity of PTEN-null cells to MNNG was not due to attenuation of MNGG transcript or protein levels.

The cytotoxicity of O<sup>6</sup>meG lesions is attributed to the recognition of O<sup>6</sup>meG/C or O<sup>6</sup>meG/T mismatches by the mismatch repair system (27), with two opposing models proposed: (a) DNA damage signal transduction by the mismatch repair complex engaged at the mismatch sites directly triggering apoptosis (direct signaling model) or (b) reiterative and futile repair attempts by mismatch repair resulting in single-strand and double-strand DNA breaks (futile cycle model; ref. 28). Because DNA DSBs are the most lethal of DNA lesions, we investigated whether such breaks were induced in MNNG-treated astrocytes and whether these breaks were more persistent in PTEN-deficient cells. We analyzed the formation and dissolution of  $\gamma$ H2AX and 53BP1 foci on pulse treatment with MNNG (8, 20), with these foci being bona fide surrogate markers



**Figure 2.** MGMT regulation is not affected by PTEN loss. A, MGMT protein levels in PTEN<sup>+/+</sup> and PTEN<sup>-/-</sup> astrocytes were analyzed by Western blotting with  $\alpha$ -MGMT antibody. B, MGMT transcript levels in mock-treated and MNNG-treated astrocytes were quantified by qRT-PCR. Values were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and expressed as fold change relative to mock-treated PTEN<sup>+/+</sup> cells. Bars, SEM.

for DSBs (29, 30). Interestingly, MNNG induced equivalent levels of DSBs in both lines; however, PTEN-deficient astrocytes exhibited higher levels of DSBs at 24 hours post-treatment, indicating a deficiency in repair of these breaks (Fig. 3A).

DSBs are repaired by NHEJ or HR in mammalian cells. Whereas NHEJ is operative in all phases of the cell cycle, HR is limited to S/G<sub>2</sub> and is particularly important for resolving replication-associated breaks (31). MNNG-induced breaks are presumed to occur in the S/G<sub>2</sub> phases because DNA replication is required for mispairing, and this is borne out by our observations indicating that MNNG-induced H2AX phosphorylation occurs only in S/G<sub>2</sub> cells (Supplementary Fig. S3). Therefore, it is likely that these breaks may be resolved by HR rather than by NHEJ. Indeed, we observed no further sensitization on treating these cells with NU7026, a potent inhibitor of the major NHEJ repair enzyme DNA-PKcs (ref. 32; Supplementary Fig. S4), thereby implicating HR in repair. In support of this idea, a recent report showed that MNNG induces DSBs and that cells defective in HR (XRCC2 and Brca2 mutants), but not cells defective in NHEJ (Ku80 and DNA-PKcs mutants), were sensitive to MNNG, similar to our PTEN-null cells (33).

Cells deficient in various HR components show a decrease in the number of SCEs after treatment with DNA-damaging agents (34), especially agents that induce replication-associated DSBs such as CPT (35). Also, HR-deficient cells are sensitive to CPT (35), and we found that PTEN-deficient astrocytes were more sensitive to this drug compared with their PTEN-proficient counterparts (Fig. 3B). We quantified the number of SCEs in PTEN<sup>+/+</sup> and PTEN<sup>-/-</sup> astrocytes after treatment with CPT or MNNG to determine the relative HR proficiencies of these lines. A statistically significant reduction in SCE events was observed in PTEN<sup>-/-</sup> astrocytes relative to PTEN<sup>+/+</sup> astrocytes, indicating a defect in HR (Fig. 3C). Consequently, PTEN-null cells surviving MNNG treatment exhibited greater numbers of chromosome breaks and radial chromosomes (Fig. 3D), similar to that seen in HR-deficient cells, particularly those deficient in Brca1 or Brca2 (36). These aberrations are indicative of a diminished capacity to repair MNNG-induced

DSBs by error-free HR and subsequent repair of these lesions by error-prone pathways such as NHEJ.

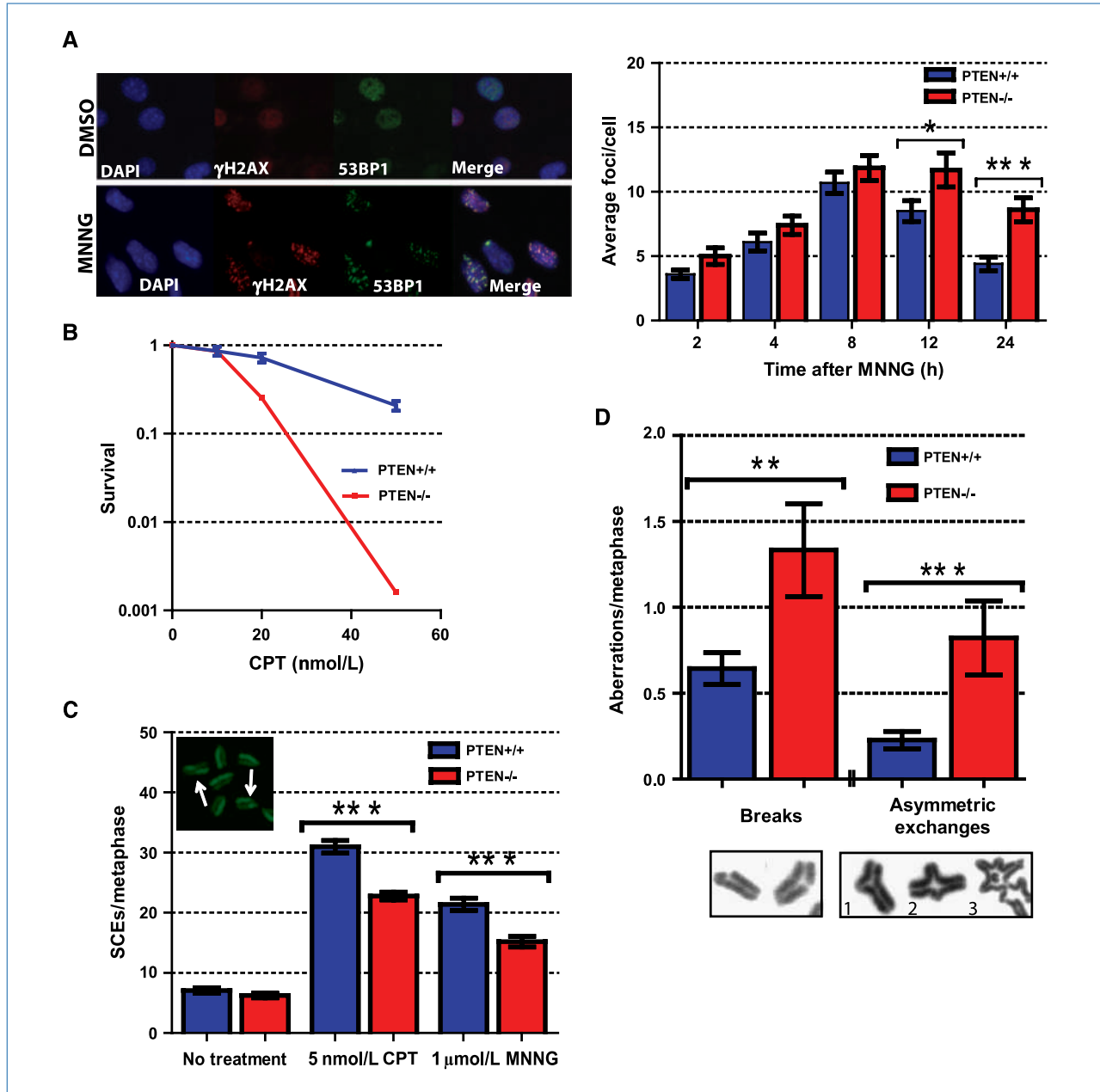
Interestingly, Shen and colleagues recently showed that PTEN is important for maintaining basal levels of transcription of the *Rad51* gene in mouse embryonic fibroblasts (12), providing a potential mechanism to explain the reduced HR capability of PTEN-null astrocytes. However, no significant changes in Rad51 protein or mRNA levels in mouse astrocytes on PTEN loss were noted (Fig. 4A and B). Whereas Rad51 forms a presynaptic nucleofilament that is critical for HR, ancillary proteins such as BRCA1, BRCA2, Rad52, and the Rad51 paralogs (Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3) facilitate multiple steps during the repair process (37). Because there are numerous recent reports of PTEN acting as a transcriptional regulator (13), we screened several of these "recombination mediators" for expression changes on PTEN loss by qRT-PCR and observed decreases in the transcript levels of Rad51B, Rad51C, and Rad51D (Fig. 4B). As these proteins are known to exist in complexes facilitating Rad51 nucleofilament formation (37), it is plausible that reduced levels of these proteins could result in attenuated HR on PTEN loss.

A very important prediction from the observed deficiency in HR is that PTEN-null astrocytes should be sensitive to PARP inhibitors that induce replication-associated DSBs. This phenomenon of "synthetic lethality" was originally identified in the context of BRCA1 and BRCA2 mutations in breast cancer (14, 15), and PARP inhibitors are now in clinical trials for treating HR-deficient breast and ovarian cancers (16). We found that PTEN-null cells were significantly more sensitive to the PARP inhibitor ABT-888 (38) compared with PTEN-proficient cells (Fig. 4C). The sensitivity to ABT-888 is consistent with a HR deficiency in PTEN-null cells, and suggests that it might be logical to treat PTEN-deficient GBMs with PARP inhibitors in the future.

The isogenic murine astrocytes used in this study are ideal for analyzing the effect of a single genetic change (PTEN loss) on MNNG sensitivity. However, in the context of human GBMs, the effect of a single genetic lesion could be modulated by innumerable background genetic changes (5–7). To

explore the relevance of our findings in human GBMs, we compared two commonly used glioma lines (U87MG and U251MG) with a normal human astrocyte line (NHA) that had been immortalized by expression of human telomerase

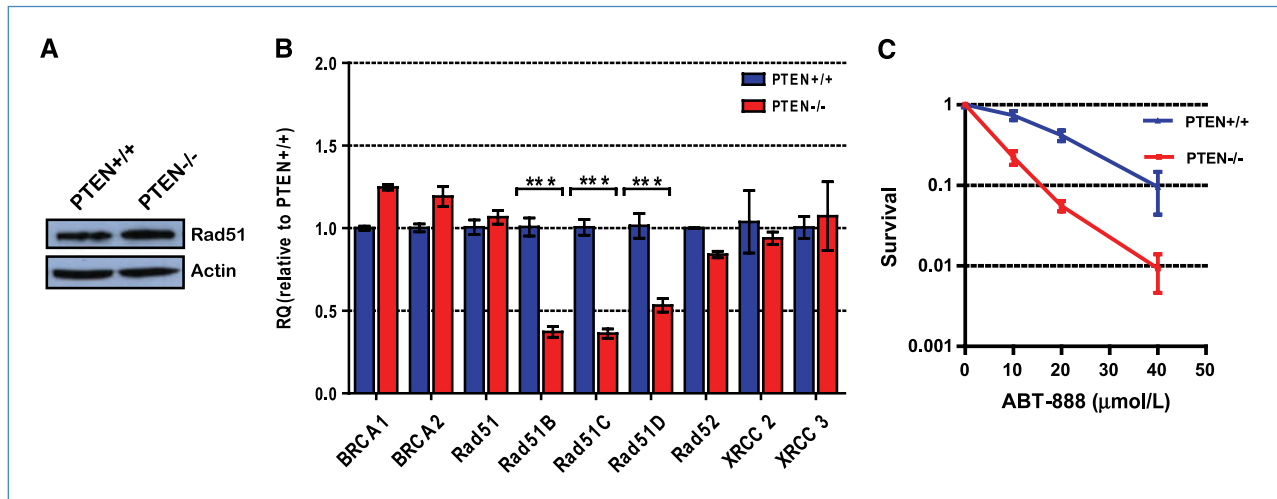
catalytic component (hTERT) and human papillomavirus 16 E6/E7 proteins (39). Both glioma lines are PTEN null (40) and were more sensitive to MNNG compared with the NHA line, which has an intact *PTEN* gene (Supplementary Fig. S5).



**Figure 3.** PTEN loss compromises HR repair. A, induction and repair of DSBs in astrocytes pulsed for 1 h with 5 μmol/L MNNG. Cells were co-immunostained for γH2AX (red) and 53BP1 (green) foci at various times after MNNG treatment. Representative pictures are shown. Foci were scored at the indicated times (average of 100 nuclei) and, after subtracting background (number of foci in untreated nuclei), average foci per nucleus was plotted against time. \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$  (two-way ANOVA with a Bonferroni posttest). B, the sensitivity of astrocytes to CPT was quantified by colony formation assays. Note increased sensitivity of PTEN-null cells to CPT. C, to quantify SCEs, metaphase spreads were prepared from astrocytes treated with MNNG or CPT as indicated. Reciprocal exchange events (see arrows, inset) were counted and plotted as average number of SCEs per metaphase. Approximately 100 metaphases were counted per treatment. \*\*\*,  $P < 0.0001$  (two-tailed  $t$  test). D, metaphase spreads from astrocytes treated with 10 μmol/L MNNG were analyzed for chromatid breaks and asymmetrical exchanges (1, triradials; 2, quadriradials; and 3, complex exchanges). Approximately 100 metaphases were counted per treatment and average aberrations per metaphase were plotted. Representative pictures of aberrations are shown. \*\*,  $P = 0.0027$ ; \*\*\*,  $P = 0.0004$  (two-tailed  $t$  test). Bars, SEM (for all plots).

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**Figure 4.** PTEN-null astrocytes express lower levels of Rad51 paralogs and are sensitive to PARP inhibitors. A, Rad51 levels in PTEN<sup>+/+</sup> and PTEN<sup>-/-</sup> astrocytes were analyzed by Western blotting with  $\alpha$ -Rad51 antibody. B, transcript levels of critical HR genes were analyzed by qRT-PCR. Values were normalized to GAPDH levels and expressed as fold change relative to PTEN<sup>+/+</sup> astrocytes. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (two-way ANOVA with a Bonferroni posttest). C, the sensitivity of astrocytes to the PARP inhibitor ABT-888 was quantified by colony formation assays. Note increased sensitivity of PTEN-null cells to ABT-888. Bars, SEM (for all plots).

These results tentatively suggest that PTEN-deficient glioblastoma cells may be more sensitive to DNA alkylating agents compared with PTEN-proficient normal human astrocytes, and this could confer a selective advantage to DNA alkylating agents for the treatment of PTEN-null GBMs. Importantly, siRNA-mediated depletion of PTEN rendered the NHA line sensitive to MNNG as quantified by the colony formation assay (Supplementary Fig. S6). This was possibly due to attenuated HR because we observed a reduced induction of SCEs on PTEN depletion. Interestingly, SCE induction in PTEN-null U87 cells was also reduced compared with the PTEN-proficient NHA line. More importantly, PTEN depletion could also sensitize transformed, gliomagenic human astrocytes (expressing E6, E7, hTERT, H-Ras, and myristylated Akt-1; ref. 41), indicating that PTEN loss might result in sensitivity to DNA alkylating agents in the context of human gliomas. In sum, these results confirm that, as observed in murine astrocytes, PTEN loss plays an important role in modulating the MNNG sensitivity of normal human astrocytes and gliomagenic derivatives.

## Discussion

The data presented in this article provide a basis for exciting new therapeutic options for GBMs. Thus far, the only meaningful improvement in GBM therapy came from the addition of temozolomide to radiation, with *MGMT* promoter methylation associated with a better outcome (3, 42). However, *MGMT* promoter methylation was also associated with an improvement in survival with radiation alone (4), indicating that this may be a general prognostic marker rather than a specific predictive marker for temozolomide treatment. We show here that PTEN-deficient astrocytes are impaired in HR

and, therefore, sensitive to replication-associated DSBs generated by DNA alkylating agents or by PARP inhibitors. This has novel therapeutic implications: GBM patients with PTEN loss (about 36%) may (a) specially benefit from temozolomide treatment and (b) also benefit from the addition of PARP inhibitors to therapeutic regimens. At the time when this paper was in preparation, a report from the Ashworth group showed that PTEN-null cells and tumors are indeed sensitive to PARP inhibitors (43) thereby bolstering the conclusions drawn from our study. Interestingly, a recent report indicates that the acquired resistance of cancer cells to combinatorial treatment with temozolomide and PARP inhibitors is linked to upregulation of HR (44). These results complement our data showing that downregulation of HR due to PTEN loss results in sensitivity to DNA alkylating agents or PARP inhibitors. Both temozolomide and PARP inhibitors are in clinical trials to treat GBMs and have been used in combination to treat other cancers (45). In light of our results and other recent reports, we posit that (a) PARP inhibitors may have a broader applicability outside of *Brc1/2*-null breast and ovarian cancers and could be used to target other tumors with HR deficiencies due to specific mutations such as PTEN loss, and (b) GBM patients may perhaps be stratified for therapy with temozolomide or PARP inhibitors based not just on their *MGMT* status but also on their PTEN status.

Although our results with genetically defined models suggest that PTEN-null tumors may be more responsive to temozolomide treatment, it is important to point out the caveat that glioblastomas are genetically very heterogeneous (5, 6, 10). Therefore, it is plausible that treatment with DNA alkylating agents could actually select for resistant clones, ultimately precipitating tumor resurgence and therapeutic failure. This possibility is tentatively indicated by *in vitro* studies

where we find that U87 or U251 clones surviving MNNG treatment are more MNNG resistant (to varying degrees) compared with the parental lines (Supplementary Fig. S7). This possibility is also borne out by the Cancer Genome Atlas Research Network study, which suggests that treatment of *MGMT*-deficient glioblastomas with alkylating agents may result in the selection of mutations in mismatch repair genes, leading to therapy resistance (5). It would be interesting to determine if the HR defect due to PTEN loss may actually increase the likelihood of development of resistant clones due to increased genomic instability.

Although the results described in this article come from proof-of-principle experiments carried out with cells growing in monolayer, it will be important to extend these results to organotypic three-dimensional models to verify whether our conclusions are valid in a more "tumor-like" setting. In preliminary studies, we find that both monolayer cultures as well as "spheroids" of U87 cells [generated by the "hanging-drop" method (46)] are similarly sensitive to MNNG (Supplementary Fig. S8). Eventually, extensive research with pre-clinical mouse models, especially orthotopic glioblastoma

models, will be required to firmly establish the nascent but highly novel concepts formulated by this study.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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