

# Human Methyl Purine DNA Glycosylase and DNA Polymerase $\beta$ Expression Collectively Predict Sensitivity to Temozolomide

Ram N. Trivedi, Xiao-hong Wang, Elena Jelezcova, Eva M. Goellner, Jiang-bo Tang, and Robert W. Sobol

*Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine and University of Pittsburgh Cancer Institute, Hillman Cancer Center, Pittsburgh, Pennsylvania*

Received January 9, 2008; accepted May 13, 2008

## ABSTRACT

Overexpression of *N*-methylpurine DNA glycosylase (MPG) has been suggested as a possible gene therapy approach to sensitize tumor cells to the cell-killing effects of temozolomide, an imidazotetrazine-class chemotherapeutic alkylating agent. In the present study, we show that both elevated MPG expression and short hairpin RNA-mediated loss of DNA polymerase  $\beta$  (Pol  $\beta$ ) expression in human breast cancer cells increases cellular sensitivity to temozolomide. Resistance to temozolomide is restored by complementation of either wild-type human Pol  $\beta$  or human Pol  $\beta$  with an inactivating mutation specific to the polymerase active site yet functional for 5'-deoxyribose-phosphate (5'dRP) lyase activity. These genetic and cellular studies uniquely demonstrate that overexpression of MPG causes an imbalance in base excision repair (BER), leading to an accumulation of cytotoxic 5'dRP lesions, and that the 5'dRP

lyase activity of Pol  $\beta$  is required to restore resistance to temozolomide. These results imply that Pol  $\beta$ -dependent 5'dRP lyase activity is the rate-limiting step in BER in these cells and suggests that BER is a tightly balanced pathway for the repair of alkylated bases such as N7-methylguanine and N3-methyladenine. Furthermore, we find that 5'dRP-mediated cell death is independent of caspase-3 activation and does not induce the formation of autophagosomes, as measured by green fluorescent protein-light chain 3 localization. The experiments presented herein suggest that it will be important to investigate whether an active BER pathway could be partially responsible for the temozolomide-mediated resistance seen in some tumors and that balanced BER protein expression and overall BER capacity may help predict sensitivity to temozolomide.

Base excision repair (BER) is the predominant pathway for the repair of base damage mediated by endogenous and exogenous stressors (Lindahl and Wood, 1999; Almeida and Sobol, 2007). The repair of DNA bases damaged by alkylation is initiated in mammalian cells by *N*-methylpurine DNA glycosylase (MPG), also known as alkyladenine DNA glycosylase (Wood et al., 2001). The majority of repair that is

initiated by MPG occurs via short-patch BER, a mechanism whereby only one nucleotide is replaced. Once the modified base is removed by MPG, the resulting abasic site is hydrolyzed by AP endonuclease (APE1) (Wood et al., 2001), catalyzing the incision of the damaged strand, leaving a 3'OH and a 5'-deoxyribose-phosphate moiety (5'dRP) at the margins of the repair site. DNA polymerase  $\beta$  (Pol  $\beta$ ) subsequently hydrolyzes the 5'dRP moiety and fills the single nucleotide gap, preparing the strand for ligation by either DNA ligase I or a complex of DNA ligase III $\alpha$  and XRCC1.

As with many DNA repair processes, BER functions via a series of repair complexes that assemble at the site of the DNA lesion. For the repair of DNA damaged by alkylation, MPG, APE1, Pol  $\beta$ , and XRCC1 are essential, with little evidence of effective complementary repair capacity (Almeida and Sobol, 2007). This would suggest that inhibition of or a

This research was supported by research scholar grant RSG-05-246-01-GMC from the American Cancer Society and grants from the Susan G. Komen Breast Cancer Foundation (grant BCTR0403276), National Institutes of Health (1R01-AG24364-01, P20-CA103730, 1P20-CA132385-01, and 1P50-CA097190-01A1), the Brain Tumor Society, the UPMC Health System Competitive Medical Research Fund, and the University of Pittsburgh Cancer Institute (to R.W.S.). This project is also funded, in part, by a grant from the Pennsylvania Department of Health.

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.108.045112.

**ABBREVIATIONS:** BER, base excision repair; MPG, *N*-methylpurine DNA glycosylase; APE1, AP endonuclease 1; Pol  $\beta$ , polymerase  $\beta$ ; 5'dRP, 5'-deoxyribose-phosphate; TMZ, temozolomide; shRNA, small hairpin RNA; RNAi, RNA interference; GFP-LC3, green fluorescent protein-light chain 3; mAb, monoclonal antibody; PCNA, proliferating cell nuclear antigen; FLAG-Pol  $\beta$  (D256A), polymerase defective mutant of human Pol  $\beta$ ; Z-VAD-FMK, benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone; WT, wild type; FIV, feline immunodeficiency virus; KD, knockdown; ETO, etoposide; 3-MA, 3-methyladenine; IP, immunoprecipitation; IB, immunoblot; PAGE, polyacrylamide gel electrophoresis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ORF, open reading frame; UTR, untranslated region.

deficiency in one or more of these essential BER proteins will lead to DNA repair-intermediate induced cell death (e.g., alkylated bases, abasic sites, 5'dRP-containing lesions or DNA single-strand breaks). When cells, or in some cases mice, are deficient in Mpg, Xrcc1, or Pol  $\beta$ , they are hypersensitive to alkylating agents (Engelward et al., 1996; Sobol et al., 1996; Elder et al., 1998; Paik et al., 2005; Horton et al., 2008). In addition, decreased APE1 expression can also lead to an increase in sensitivity to alkylating agents (Ono et al., 1994; Walker et al., 1994). We have extended our characterization of the role of Pol  $\beta$  in the repair of alkylation damage in mouse cells, demonstrating that Pol  $\beta$  provides cellular resistance to the clinical alkylating agent temozolomide (TMZ; an imidazotetrazine-class chemotherapeutic alkylating agent) by repairing lesions that ultimately trigger activation of the DNA damage response checkpoint (Trivedi et al., 2005). Interestingly, both MPG knockdown (Paik et al., 2005) and increased expression of MPG have been found to sensitize cells to alkylators (Rinne et al., 2004; Fishel et al., 2007). To avoid alkylation damage-induced mutations that accumulate in the absence of MPG expression, forced overexpression of MPG has been suggested as a strategic and viable gene therapy approach to sensitize tumor cells to TMZ.

To directly evaluate the role of MPG and Pol  $\beta$  in human tumor cells with regard to the cellular response to alkylation damage, we developed human Pol  $\beta$ -specific shRNA-expressing lentiviruses to completely deplete human tumor cells of Pol  $\beta$ , as well as vectors for ectopic expression of MPG and RNAi-resistant human Pol  $\beta$  transgenes so as to define the enzymatic activity of Pol  $\beta$  (5'dRP lyase or DNA polymerase activity) that confers TMZ resistance. We show that both elevated MPG expression and shRNA-mediated loss of Pol  $\beta$  expression increase cellular sensitivity to TMZ in human breast cancer cells. In both cases, resistance to TMZ is re-

stored by complementation of either wild-type human Pol  $\beta$  or human Pol  $\beta$  with an inactivating mutation specific to the polymerase active site yet functional for 5'dRP lyase activity. These genetic and cellular studies uniquely demonstrate that overexpression of MPG causes an imbalance in BER by saturating the Pol  $\beta$ -dependent removal of the cytotoxic 5'dRP lesion. Failure to remove this cytotoxic lesion does not induce caspase-3 activation and does not induce the formation of autophagosomes, as measured by GFP-LC3 localization. These studies support the possibility that the lyase activity of Pol  $\beta$  is the rate-limiting step in BER in human cells and suggest that BER is a tightly balanced pathway for the repair of alkylated bases such as N7-methylguanine and N3-methyladenine.

## Materials and Methods

**Chemicals and Reagents.** RPMI 1640 medium and heat-inactivated fetal bovine serum were from Lonza Walkersville (Walkersville, MD) and Invitrogen (Carlsbad, CA). Temozolomide (NSC 362856; IUPAC name, 3-methyl-2-oxo-1,3,4,5,8-pentazabicyclo[4.3.0]nona-4,6,8-triene-7-carbo oxamide; CAS no. 85622-93-1) (Sobol, 2008b) was from the National Cancer Institute Developmental Therapeutics Program (Bethesda, MD) and prepared as 100 mM stock in dimethyl sulfoxide. We used the following primary antibodies: anti-Pol  $\beta$  (mAb clone 61; Thermo Fisher Scientific, Waltham, MA); anti-human MPG (mAb clone 506-3D; kindly provided by Dr. S. J. Kennel, Oak Ridge National Laboratory, Oak Ridge, TN); anti-APE1 (EMD Biosciences, San Diego, CA); anti-PCNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); and anti-FLAG (M2 mAb; Sigma-Aldrich, St. Louis, MO). All electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). Neomycin and Dynabeads protein G were purchased from Invitrogen. Puromycin, gentamicin sulfate (10 mg/ml), and 3 $\times$  FLAG peptide were from Clontech (Mountain View, CA), Irvine Scientific (Santa Ana, CA), and Sigma-Aldrich, respectively. Etoposide (ETO) and 3-methyladenine (3-MA) were obtained from Sigma-Aldrich. Z-VAD-FMK was

TABLE 1  
Cell lines developed and used in this study

Cell Line Name and Clone No.	TMZ IC <sub>50</sub>	Cell Line Description	Growth Media
MDA-MB-231	2.0	Human, Caucasian, breast, adenocarcinoma, epithelial cell	RPMI 1640 medium (450 ml), heat-inactivated fetal bovine serum (10%), gentamicin (10 $\mu$ g/ml)
MDA-MB-231/Pol $\beta$ -KD			
2	0.8	Human breast cancer cells expressing Pol $\beta$ lentiviral shRNA	Growth media supplemented with puromycin (0.5 $\mu$ g/ml)
10	0.8		
18	0.7		
MDA-MB-231/MPG			
4	0.57	MPG overexpression in human breast cancer cells	Growth media supplemented with G418 (800 $\mu$ g/ml)
5	0.50		
6	0.90		
MDA-MB-231/Pol $\beta$ -KD(10)/MPG			
1	0.27	MPG overexpression in Pol $\beta$ down-regulated human breast cancer cells	Growth media supplemented with puromycin (0.5 $\mu$ g/ml) and G418 (750 $\mu$ g/ml)
2	0.27		
MDA-MB-231/Pol $\beta$ -KD(10)/FLAG-Pol $\beta$ -WT			
2	1.6	WT FLAG-Pol $\beta$ reconstituted in Pol $\beta$ down-regulated human breast cancer cells	Growth media supplemented with puromycin (0.5 $\mu$ g/ml) and G418 (700 $\mu$ g/ml)
3	2.0		
4	1.5		
MDA-MB-231/Pol $\beta$ -KD(10)/FLAG-Pol $\beta$ -D25A			
3	1.63	D256A mutant FLAG-Pol $\beta$ reconstituted in Pol $\beta$ down-regulated human breast cancer cells	Growth media supplemented with puromycin (0.5 $\mu$ g/ml) and G418 (700 $\mu$ g/ml)
4	1.27		
5	1.66		
MDA-MB-231/MPG(4)/FLAG-Pol $\beta$ -WT			
19	1.31	WT FLAG-Pol $\beta$ reconstituted in MPG-overexpressing human breast cancer cells	Growth media supplemented with puromycin (0.5 $\mu$ g/ml) and G418 (700 $\mu$ g/ml)
20	2.0		
21	1.88		

from Calbiochem (San Diego, CA), and the caspase-3 colorimetric activity assay kit was from Millipore Corporation (Billerica, MA). pEGFP-LC3 plasmid was kindly provided by Dr. Tamotsu Yoshimori (National Institute of Genetics, Mishima, Japan).

**Plasmid Expression Vectors and RNAi Development.** We used the following mammalian expression vectors: human MPG: pRS1422 (Sobol et al., 2003); and human Pol  $\beta$ : pIRES-Neo/FLAG-Pol  $\beta$  (WT), pIRES-Puro/FLAG-Pol  $\beta$  (WT), and pIRES-Neo/FLAG-Pol  $\beta$  (D256A) (Sobol et al., 2000). Human Pol  $\beta$ -targeted FIV-based lentiviral shRNA expression vectors (pFIV-H1-Puro-hpol $\beta$ .1, pFIV-H1-Puro-hpol $\beta$ .2, and pFIV-H1-Puro-hpol $\beta$ .3) and pFIV-34N and pVSV-G constructs were from System Biosciences (Mountain View, CA). pSuper-Retro shRNA (pSuper-Retro shRNA-1 and pSuper-Retro shRNA-2) expression vectors were from Oligoengine (Seattle, WA).

**Cell Line Transfection and Viral Transduction Conditions.** Human MPG overexpression, Pol  $\beta$  knockdown (KD), FLAG-Pol  $\beta$  (WT), and FLAG-Pol  $\beta$  (D256A) overexpressing cell lines were prepared by transfection using FuGene 6 transfection reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's instructions. Stable cell lines (Table 1) were selected in G418 [800  $\mu$ g/ml for human MPG expression plasmids; 700  $\mu$ g/ml for pIRES-Neo/FLAG-Pol  $\beta$  (WT) and pIRES-Neo/FLAG-Pol  $\beta$  (D256A)] and puromycin [0.5  $\mu$ g/ml for pIRES-Puro/FLAG-Pol  $\beta$  (WT)] for 2 weeks, individual clones (stably expressing human MPG or Pol  $\beta$ ) were amplified, and 30  $\mu$ g of nuclear extract was analyzed by immunoblotting for the expression of human MPG or human Pol  $\beta$  protein and also probed for expression of APE1 and PCNA.

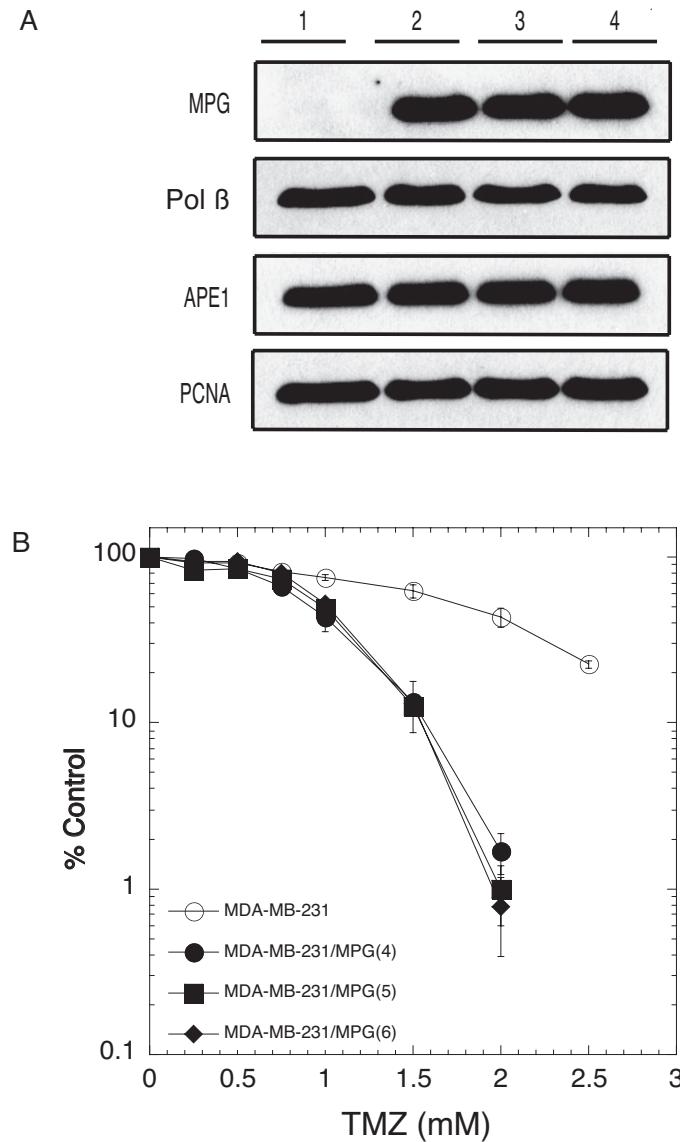
Lentiviral particles were generated by transfection of three plasmids (the expression plasmid, e.g., pFIV-H1-puro-hPOLB.1; plus pFIV-34N and pVSV-G) into 293-FT cells (Poeschla et al., 1998) using FuGene 6. Culture media from transfected cells was collected 48 h after transfection to isolate the viral particles, passed through 0.45- $\mu$ m filters, and used immediately or stored at  $-80^{\circ}$ C in single-use aliquots. Lentiviral transduction was completed as follows: in brief,  $6.0 \times 10^4$  cells were seeded into a six-well plate and incubated for 24 to 30 h at 5% CO<sub>2</sub> at 37°C. Cells were transduced for 18 h with shRNA-expression lentiviral stocks at 32°C and cultured for 72 h at 37°C. Stable cell lines were isolated after transduction as described above, followed by selection in puromycin (0.5  $\mu$ g/ml) for 2 weeks. Individual clones (stable knockdown of human Pol  $\beta$  protein) were amplified, and 30  $\mu$ g of nuclear extract was analyzed by immunoblotting for the expression of endogenous human Pol  $\beta$  protein and also probed for expression of MPG, APE1, and PCNA. A descriptive list of the cell lines developed for and used in this study is detailed in Table 1.

**Culture Conditions and Cell Cytotoxicity Assays.** MDA-MB-231 breast cancer cells (HTB-26) were obtained from American Type Culture Collection (Manassas, VA). Human MPG overexpression, human Pol  $\beta$  knockdown and human Pol  $\beta$  overexpressing MDA-MB-231 cell lines were cultured at 37°C in a humidified incubator with 5% CO<sub>2</sub> in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and gentamicin (10  $\mu$ g/ml). TMZ-, ETO-, or 3-MA-induced cytotoxicity was determined by a modified MTT assay, as we described previously (Trivedi et al., 2005). Results were calculated from the average of four separate experiments and are reported as the percentage of treated cells relative to the cells in control wells (% control).

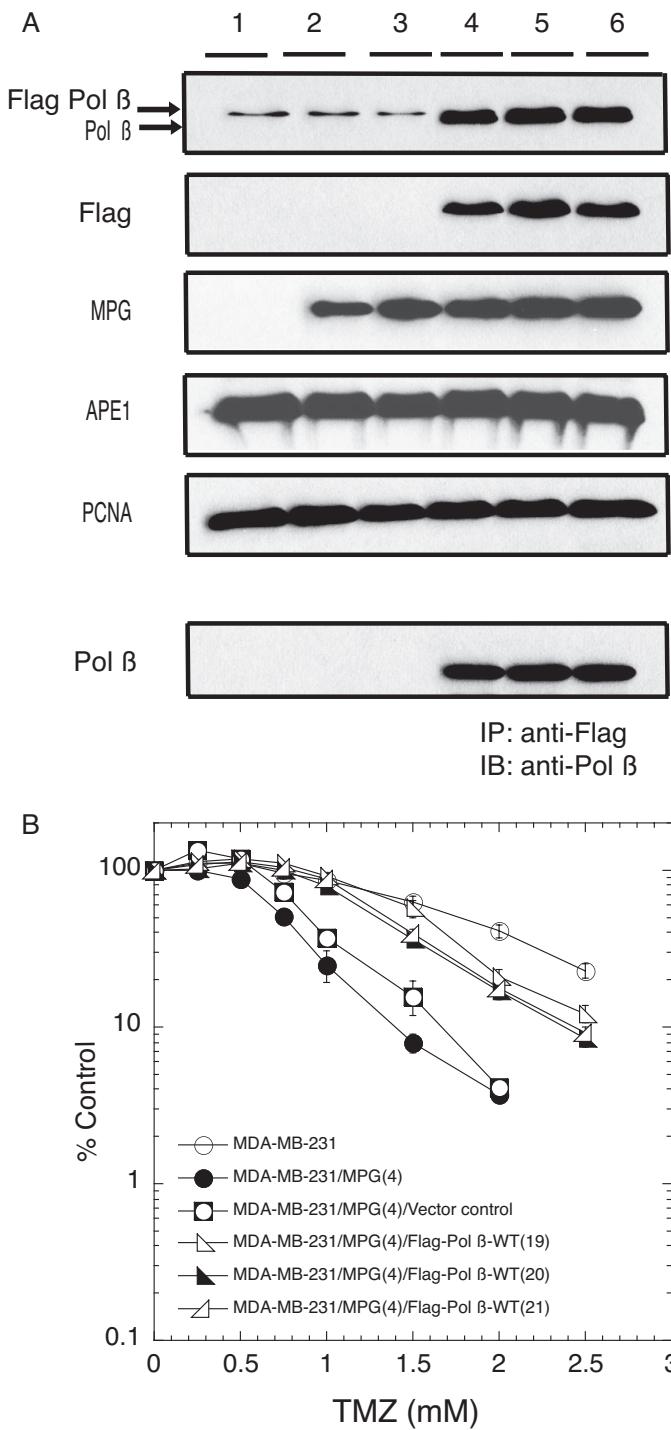
**Cell Extract Preparation, Immunoblot, and Immunoprecipitation Assays.** Nuclear extracts were prepared and protein concentration was determined as we described previously (Trivedi et al., 2005). Nuclear protein (30  $\mu$ g) was separated by electrophoresis in a 4 to 20% Tris-glycine SDS-polyacrylamide gel (Invitrogen) and electrotransferred to a 0.45- $\mu$ m nitrocellulose membrane (Trans-Blot; Bio-Rad Laboratories). Antigens were detected using standard protocols. Primary antibodies (anti-Pol  $\beta$ , 500 $\times$ ; anti-hMPG, 1000 $\times$ ; anti-APE-1, 3000 $\times$ ; anti-FLAG 2000 $\times$ ; and anti-PCNA, 1000 $\times$ ) and the horseradish peroxidase-conjugated secondary antibody (goat an-

ti-mouse horseradish peroxidase; Bio-Rad Laboratories) were diluted in Tris-buffered saline/Tween 20 5% milk.

For immunoprecipitation (IP), cell lysate from the above-mentioned cell lines was prepared in radioimmunoprecipitation assay buffer, incubated overnight with anti-FLAG M2 antibodies at 4°C, followed by 1-h incubation with protein G Dynabeads at 4°C. The immunoprecipitated material was washed with radioimmunoprecipitation assay buffer and eluted with 3 $\times$  FLAG peptide, separated



**Fig. 1.** Overexpression of human MPG in MDA-MB-231 cells shifts the rate-limiting step in the BER pathway and induces a temozolomide-hypersensitive phenotype. A, MPG overexpression as determined by immunoblot analysis of nuclear proteins isolated from the MDA-MB-231 or MPG overexpressing MDA-MB-231 cells. Proteins isolated from three separate MPG-overexpressing clones are shown [MDA-MB-231/MPG(4), MDA-MB-231/MPG(5), and MDA-MB-231/MPG(6); lanes 2–4] compared with proteins isolated from control cells (MDA-MB-231; lane 1). Pol  $\beta$  and APE1 expression as determined by immunoblot. PCNA expression is shown as a loading control (bottom). B, MDA-MB-231 cells (○) or MPG overexpression MDA-MB-231 cells [MDA-MB-231/MPG(4), ●; MDA-MB-231/MPG(5), ■; and MDA-MB-231/MPG(6), ◆] were cultured in 96-well plates for 24 h before exposure to TMZ. Viable cells were determined using a modified MTT assay. Plots show the percentage of viable cells compared with untreated (control) cells. Means are calculated from quadruplicate values in each experiment. Results indicate the mean  $\pm$  S.E. of four independent experiments.



**Fig. 2.** Reconstitution of human FLAG-Pol  $\beta$  in MPG-overexpressing MDA-MB-231 cells restores resistance to temozolomide. **A**, DNA Pol  $\beta$  expression as determined by immunoblot analysis of nuclear proteins isolated from the MDA-MB-231 or MPG overexpressing MDA-MB-231 cells transfected with FLAG-Pol  $\beta$ . Proteins isolated from three separate FLAG-Pol  $\beta$ -expressing clones are shown [MDA-MB-231/MPG(4)/FLAG-Pol  $\beta$ -WT(19), MDA-MB-231/MPG(4)/FLAG-Pol  $\beta$ -WT(20), and MDA-MB-231/MPG(4)/FLAG-Pol  $\beta$ -WT(21); lanes 4–6] compared with proteins isolated from control cells (MDA-MB-231; lane 1), MPG overexpressing/MDA-MB-231 cells (MDA-MB-231/MPG(4); lane 2) or pIREs-Puro transfected in MPG-overexpressing/MDA-MB-231 cells [MDA-MB-231/MPG(4)/Puro(1); lane 3]. FLAG, MPG, and APE1 expression was determined by immunoblot. PCNA expression is shown as a loading control (bottom). For IP, cell lysate from the above-mentioned cell lines was incubated overnight with anti-FLAG antibodies followed by 1-h incubation with protein G Dynabeads. Immunoprecipitates were eluted

on SDS-PAGE, transferred to nitrocellulose filters, and probed with anti-Pol  $\beta$  antibody.

**Induction of Apoptosis and Caspase-3 Activation Assay.** Twenty-four hours after seeding duplicate plates for each cell line (2000 cells/well in a 96-well plate), each plate was pretreated with media alone or media supplemented with Z-VAD-FMK at a final concentration of 50  $\mu$ M. After 1-h Z-VAD-FMK treatment, cells were treated with ETO (15  $\mu$ M) or various doses of TMZ (0.25–2.5 mM) and then incubated at 37°C for 48 h. Cell cytotoxicity was determined by a modified MTT assay, as described above.

Caspase-3 activation was measured using a caspase-3 colorimetric activity assay kit as per the manufacturer's instructions (Millipore Corporation). In brief, the assay is based on the spectrophotometric identification of *p*-nitroaniline after cleavage of a labeled DEVD-*p*-nitroaniline substrate. For the caspase-3 activation assay,  $0.75 \times 10^6$  cells were seeded in 100-mm dishes. Twenty-four hours after seeding, the cells [MDA-MB-231, MDA-MB-231/Pol  $\beta$ -KD(10), MDA-MB-231/MPG(4), and MDA-MB-231/Pol  $\beta$ -KD(10)/MPG(4)] were treated with 2.0, 0.8, 0.6, and 0.3 mM TMZ (the corresponding TMZ IC<sub>50</sub> value for each cell line; Table 1) or media for 6, 12, 24, or 48 h. Cells were treated separately with 15  $\mu$ M ETO or media for 24 h. After induction of apoptosis with TMZ or ETO, the cell pellet (containing approximately  $2 \times 10^6$  cells) was resuspended in 1× cell lysis buffer, and the supernatant (cytosolic extract) was used to measure caspase-3 activation. Samples were mixed with assay mixture in a 96-well plate and incubated for 2 h at 37°C. Absorbance was measured at 405 nm. The -fold increase in caspase-3 activation was calculated by comparing the optical density from the TMZ or ETO samples with the optical density from the untreated control samples.

**Inhibition of Autophagy by 3-Methyladenine.** Twenty-four hours after seeding duplicate plates for each cell line (2000 cells/well in a 96-well plate), each plate was pretreated with media alone or media supplemented with 3-MA at a final concentration of 5 mM. After 30 min of 3-MA treatment, cells were treated with various ranges of TMZ (0.25–2.5 mM) and then incubated at 37°C for 48 h. Cell cytotoxicity was determined by a modified MTT assay, as described above.

**Identification of Autophagosome Formation with GFP-LC3.** MDA-MB-231 and derived cell lines were seeded at 4900 cells per well in eight-well chamber slides and cultured for 36 h at 37°C and 5% CO<sub>2</sub>. Cells were then transfected with pEGFP-LC3 for 36 h, using FuGene as described above. Cells were pretreated with media alone or media supplemented with 5 mM 3-MA for 30 min and then treated with or without the IC<sub>50</sub> dose of TMZ for 24 h (Table 1). Cells were washed twice in phosphate-buffered saline, fixed in 4% formaldehyde for 30 min at room temperature, and counterstained with 4',6-diamidino-2-phenylindole (0.5  $\mu$ g/ml) for 5 min.

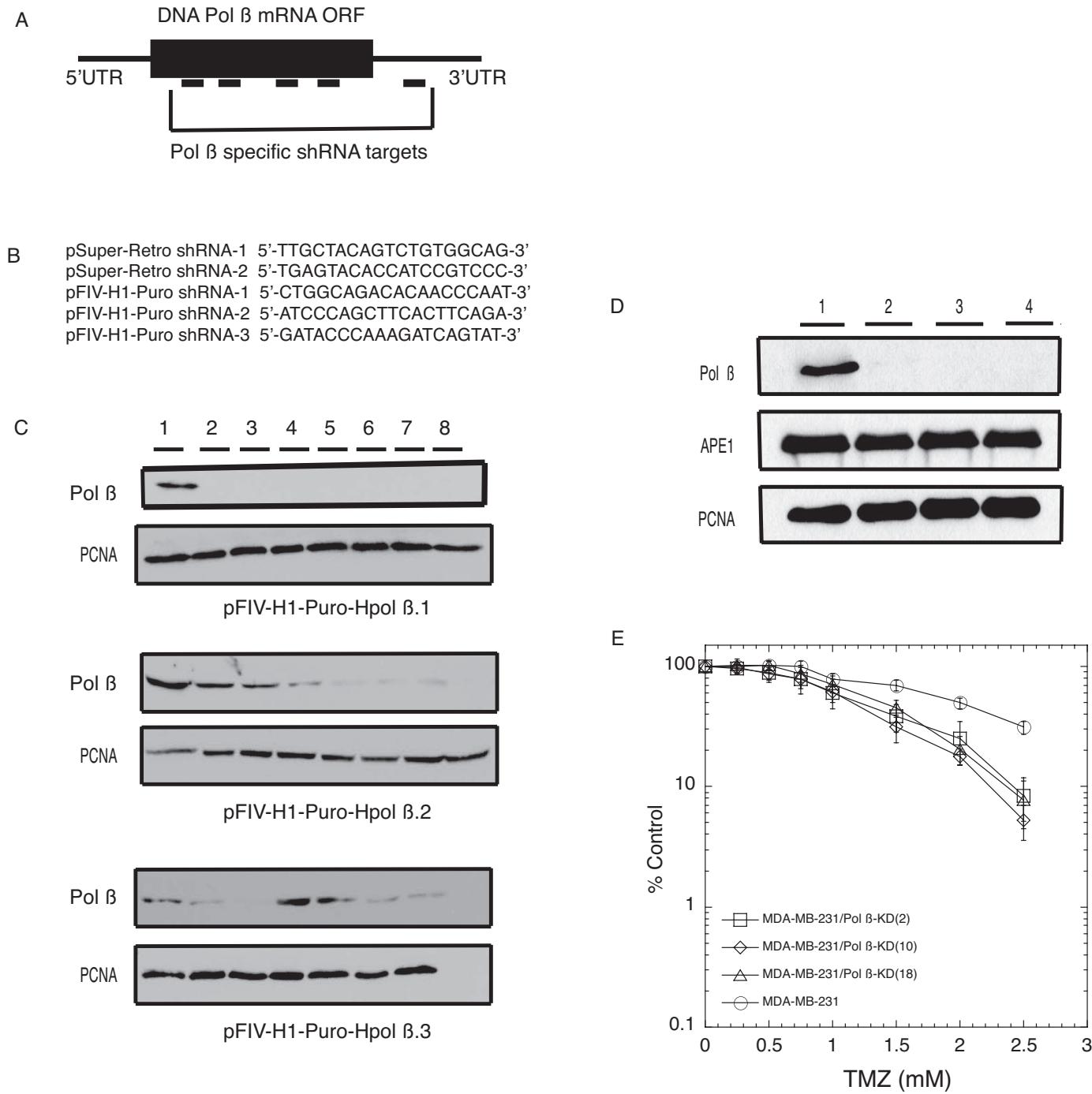
## Results

**Overexpression of Human MPG Shifts the Rate-Limiting Step in the BER Pathway and Induces a TMZ Hypersensitive Phenotype.** In-line with our hypothesis that the BER pathway repairs TMZ-induced lesions (Trivedi et al., 2005) and that therefore variations in BER protein expression will affect TMZ-mediated cell death, we have analyzed the involve-

with 3× FLAG peptide, separated on SDS-PAGE, and transferred to nitrocellulose filters and probed with anti-Pol  $\beta$  antibody. **B**, MDA-MB-231 cells ( $\ominus$ ) or MPG-overexpressing MDA-MB-231 cells [MDA-MB-231/MPG(4);  $\bullet$ ] or pIREs-Puro transfected in MPG-overexpressing/MDA-MB-231 cells [MDA-MB-231/MPG(4)/Puro(1); circles within squares] or MPG overexpressing/MDA-MB-231 cells expressing FLAG-Pol  $\beta$  [MDA-MB-231/MPG(4)/Flag-Pol  $\beta$ -WT(19), half squares; MDA-MB-231/MPG(4)/Flag-Pol  $\beta$ -WT(20), filled half squares; and MDA-MB-231/MPG(4)/Flag-Pol  $\beta$ -WT(21), inverted half squares] were cultured in 96-well plates for 24 h before exposure to TMZ. Viable cells were determined using a modified MTT assay as described in Fig. 1.

ment of MPG in TMZ responsiveness in human tumor cells (Table 1) by increasing MPG expression so as to enhance BER initiation. Our MPG expression system allows us to significantly overexpress MPG in MDA-MB-231 human breast cancer

cells (Fig. 1A), as much as 10-fold, as determined by immunoblot analysis and quantification by NIH Image version 1.62 (<http://rsb.info.nih.gov/nih-image/>) analysis. Note the almost undetectable expression of MPG in the parental cells compared

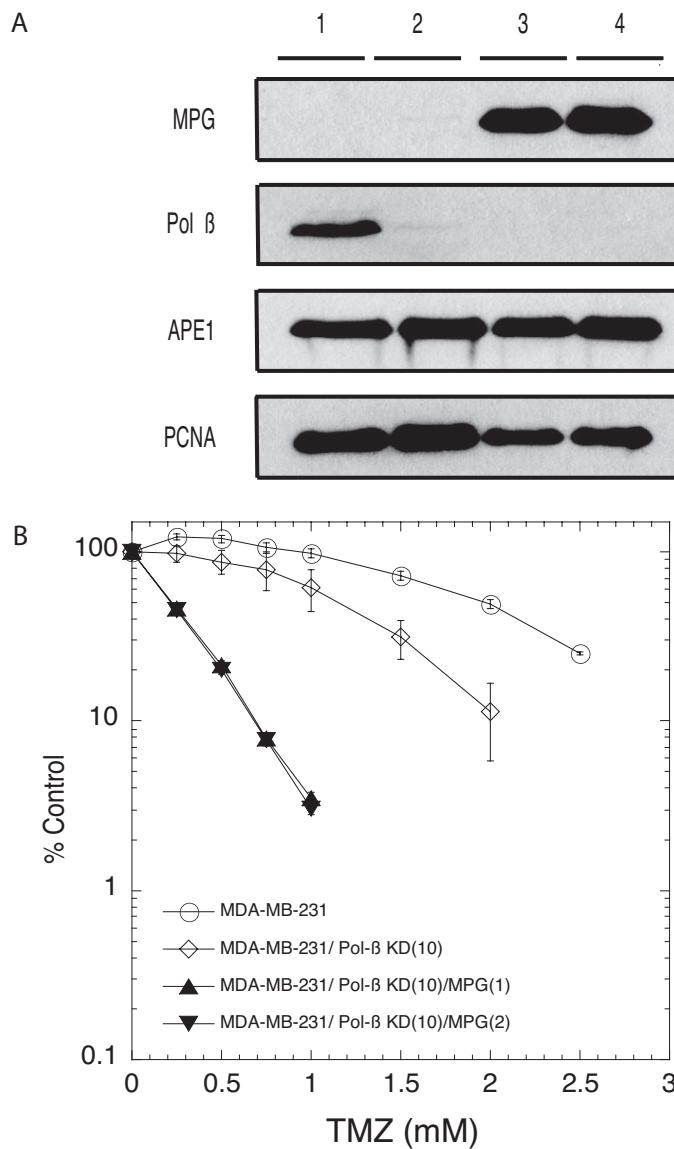


**Fig. 3.** Down-regulation of endogenous human Pol  $\beta$  protein expression in human breast cancer cells. A, diagram depicting the target sequence for five separate shRNA-expressing vectors (plasmid and lentiviral-based), specific for different regions of Pol  $\beta$  mRNA. Four predicted sequences of shRNA targets are within the ORF, and one sequence was specific for a region outside the ORF, within the 3'UTR. B, sequences of the target for five different Pol  $\beta$ -specific shRNA vectors. C, down-regulation of endogenous human Pol  $\beta$  protein expression in human breast cancer cells (MDA-MB-231) after transduction of three different Pol  $\beta$  shRNA-specific lentiviral vectors. DNA Pol  $\beta$  expression as determined by immunoblot analysis of nuclear proteins isolated from seven separate shRNA-expressing clones is shown. PCNA expression is shown as a loading control (bottom). D, DNA Pol  $\beta$  expression as determined by immunoblot analysis of nuclear proteins isolated from the MDA-MB-231 or MDA-MB-231 cells transduced with a human Pol  $\beta$  shRNA lentiviral vector. Proteins isolated from three separate shRNA-expressing clones are shown [MDA-MB-231/Pol  $\beta$ -KD(2), MDA-MB-231/Pol  $\beta$ -KD(10), and MDA-MB-231/Pol  $\beta$ -KD(18); lanes 2–4] compared with proteins isolated from control cells (MDA-MB-231; lane 1). APE1 expression as determined by immunoblot. PCNA expression is shown as a loading control (bottom). E, MDA-MB-231 cells (○) or MDA-MB-231 cells expressing Pol  $\beta$ -specific shRNA [MDA-MB-231/Pol  $\beta$ -KD(2), □; MDA-MB-231/Pol  $\beta$ -KD(10), ◇; and MDA-MB-231/Pol  $\beta$ -KD(18), △] were cultured in 96-well plates for 24 h before exposure to TMZ. Viable cells were determined using a modified MTT assay as described in Fig. 1.

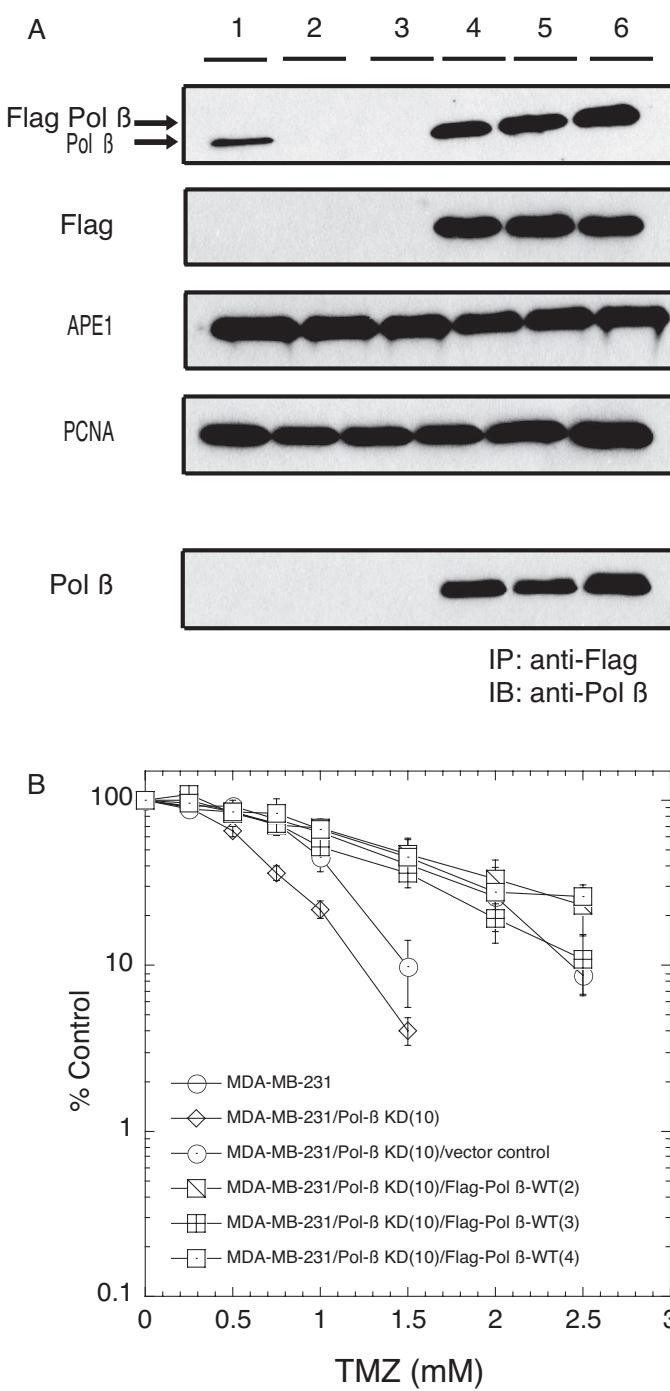
with the overexpression cell lines (Fig. 1A, compare lane 1 with lanes 2–4) and that overexpression of MPG does not alter the expression of the other BER proteins, Pol  $\beta$  or APE1 (Fig. 1A). It is our hypothesis that MPG overexpression shifts the rate-limiting step in the BER pathway, significantly enhancing BER initiation that may result in the accumulation of BER intermediates such as 5'dRP. In support of this hypothesis, an increased sensitivity to TMZ is observed when MPG is overexpressed in these cells (Fig. 1B), in-line with that of Rinne et al. (2004). Because MPG performs the first step in the BER pathway and is essential to manifest the Pol  $\beta$  null phenotype observed in mouse cells (Sobol et al., 2003), we reasoned that MPG overexpression saturates endogenous Pol  $\beta$  and yields an apparent Pol  $\beta$  deficiency, because glycosylase overexpression could cause an overall BER imbalance (Coquerelle et al., 1995). To determine whether the endogenous level of Pol  $\beta$  is rate-limiting when MPG is overexpressed, the MPG overexpressed MDA-MB-231 human breast cancer cells were modified to also overexpress Pol  $\beta$ , increasing expression severalfold compared with endogenous levels. For this, the MPG overexpression cells were complemented with human epitope-tagged Pol  $\beta$  (Fig. 2A). There is no change in expression of endogenous Pol  $\beta$  protein or in expression of the other BER proteins, but these cells exhibit robust expression of the transgenic FLAG-Pol  $\beta$  (Fig. 2A). By immunoblot analysis, we estimate that these cells express 5 times greater transgenic Pol  $\beta$  than the endogenous level of Pol  $\beta$  in these cells. However, overexpression of wild-type Pol  $\beta$  in our MPG overexpression cells completely restores resistance to TMZ (Fig. 2B), whereas cells with vector control are just as sensitive to TMZ as the original cells, demonstrating that the hypersensitivity to TMZ of cells that overexpress MPG is due to unrepaired Pol  $\beta$  substrates such as the cytotoxic 5'dRP lesions that would accumulate after TMZ exposure if the Pol  $\beta$  step in BER was rate-limiting.

**Human DNA Pol  $\beta$  Protein Is Required for Resistance to the Cytotoxicity of the Alkylating Agent TMZ.** To facilitate the analysis of the BER pathway and Pol  $\beta$  in particular, in human tumor cells, we designed shRNA-expressing vectors, specific for different regions of Pol  $\beta$  mRNA. As shown in Fig. 3A, five shRNA expression plasmids specific to human Pol  $\beta$  were developed based on GenBank sequence NM\_002690. Of those, four sequences were specific for the human Pol  $\beta$  open reading frame (ORF), and one sequence was specific for a region outside the ORF, within the 3'UTR. It is noteworthy that the 3'UTR-targeted shRNA proved most effective. The sequence for each shRNA target is shown in Fig. 3B. Each vector (either plasmid or lentivirus) was used to develop single-cell clones, and each was analyzed for effectiveness of Pol  $\beta$  knockdown by probing Pol  $\beta$  expression via immunoblot analysis of nuclear proteins. The pSuper-Retro shRNA expression vectors were not effective in Pol  $\beta$  knockdown (data not shown). It is noteworthy that the ORF-specific shRNA lentiviral vectors were only partially or minimally effective (pFIV-H1-Puro-hpol $\beta$ .2 and pFIV-H1-Puro-hpol $\beta$ .3) in leading to a decrease in Pol  $\beta$  expression (Fig. 3C). Although some clones were identified that had lost most expression of Pol  $\beta$ , many presented with partial expression and some with as much as 50% expression compared with the parental cell line or an empty vector control (data not shown). However, all cell clones expressing the 3'UTR-specific Pol  $\beta$  shRNA (pFIV-H1-Puro-hpol $\beta$ .1) led to complete loss of Pol  $\beta$  protein expression (Fig. 3D). Note that the expression of

human Pol  $\beta$ -specific shRNA does not affect expression of other BER proteins (Fig. 3D). PCNA expression is shown as a loading control (Fig. 3D). This loss of Pol  $\beta$  expression has been observed for cells in culture as long as 4 months; we have not analyzed them after longer periods in culture. We next tested the cells for TMZ sensitivity, so as to evaluate the impact of loss of Pol  $\beta$ . Human tumor cells that have lost Pol



**Fig. 4.** Overexpression of human MPG in Pol  $\beta$  down-regulated MDA-MB-231 cells results in further increase in sensitivity to temozolomide. A, MPG overexpression as determined by immunoblot analysis of nuclear proteins isolated from the MDA-MB-231 or MPG overexpressing MDA-MB-231 cells transduced with a Pol  $\beta$  shRNA lentiviral vector. Proteins isolated from two separate MPG-overexpressing clones are shown [MDA-MB-231/Pol  $\beta$ -KD(10)/MPG(1) and MDA-MB-231/Pol  $\beta$ -KD(10)/MPG(2); lanes 3 and 4] compared with proteins isolated from control cells (MDA-MB-231; lane 1) or Pol  $\beta$  knockdown cells (MDA-MB-231/Pol  $\beta$ -KD(10); lane 2]. Pol  $\beta$  and APE1 expression was determined by immunoblot. PCNA expression is shown as a loading control (bottom). B, MDA-MB-231 cells (○) or MDA-MB-231 cells expressing Pol  $\beta$ -specific shRNA [MDA-MB-231/Pol  $\beta$ -KD(10), ◆] or MPG overexpression in Pol  $\beta$  down-regulated MDA-MB-231 cells [MDA-MB-231/Pol  $\beta$ -KD(10)/MPG(1), ▲; and MDA-MB-231/Pol  $\beta$ -KD(10)/MPG(2), ▼] were cultured in 96-well plates for 24 h before exposure to TMZ. Viable cells were determined using a modified MTT assay as described in Fig. 1.



**Fig. 5.** Reconstitution of human FLAG-Pol  $\beta$  in Pol  $\beta$  down-regulated MDA-MB-231 cells restores resistance to temozolomide. **A**, DNA Pol  $\beta$  expression as determined by immunoblot (IB) analysis of nuclear proteins isolated from the MDA-MB-231 cells or MDA-MB-231/Pol  $\beta$  KD cells transfected with the FLAG-Pol  $\beta$ . Proteins isolated from three separate FLAG-Pol  $\beta$ -expressing clones are shown [MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -WT(2), MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -WT(3), and MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -WT(4); lanes 4–6] compared with proteins isolated from control cells (MDA-MB-231; lane 1), MDA-MB-231/Pol  $\beta$  KD cells [MDA-MB-231/Pol  $\beta$ -KD(10); lane 2], or pIRES-Neo-transfected MDA-MB-231/Pol  $\beta$  KD cells [MDA-MB-231/Pol  $\beta$ -KD(10)/Neo(1); lane 3]. FLAG and APE1 expression was determined by immunoblot. PCNA expression is shown as a loading control (bottom). For IP, cell lysate from the above-mentioned cell lines was incubated overnight with anti-FLAG antibodies followed by 1-h incubation with protein G Dynabeads. Immunoprecipitates were eluted with 3× FLAG peptide, separated on SDS-PAGE, and transferred to nitrocellulose filters and probed with anti-Pol  $\beta$  antibody. **B**, MDA-MB-231 cells ( $\ominus$ ) or MDA-MB-231 cells expressing Pol

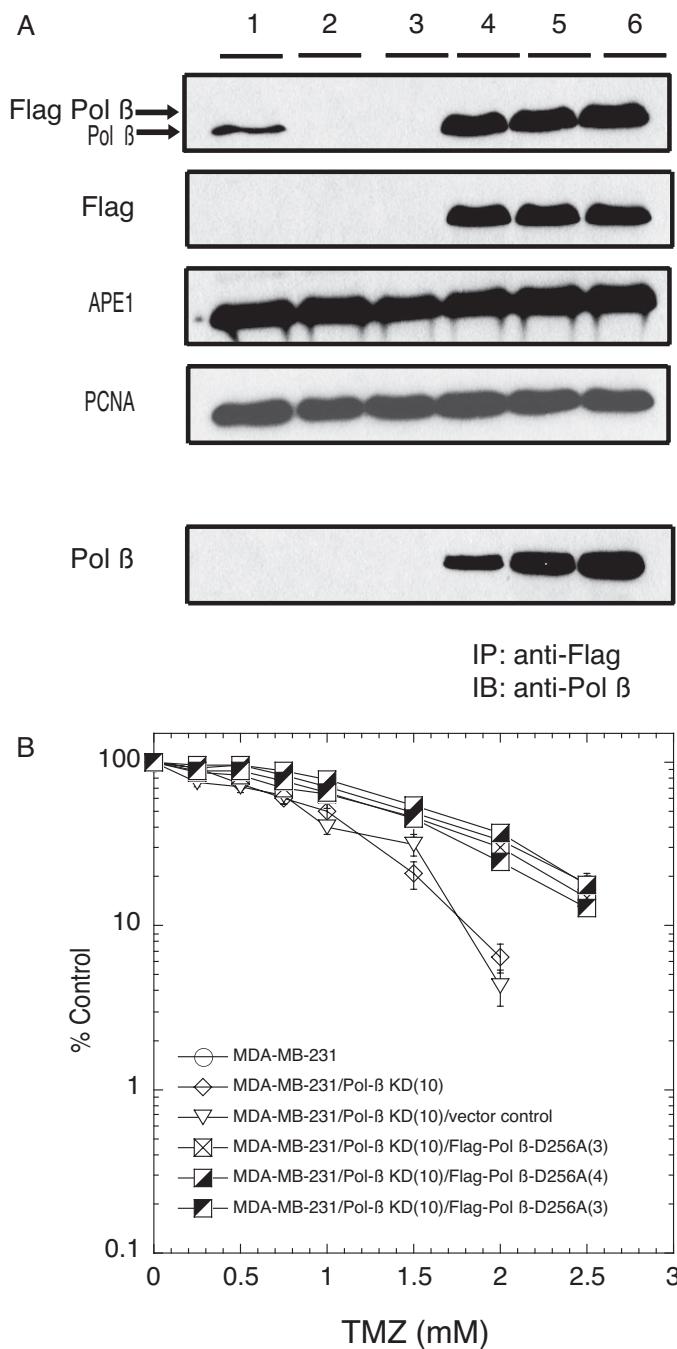
$\beta$  expression are significantly more sensitive to TMZ than the parental cells (Fig. 3E).

We next evaluated the role of Pol  $\beta$  in the breast cancer cells with elevated expression of MPG. For these analyses, we combined MPG overexpression and Pol  $\beta$  knockdown to demonstrate that the Pol  $\beta$ -deficient hypersensitivity to TMZ is mediated by MPG-mediated repair initiation. Immunoblots are shown demonstrating that these human tumor cells can harbor both MPG overexpression plus the loss of Pol  $\beta$  expression (Fig. 4A). We find that overexpression of MPG combined with Pol  $\beta$  knockdown drastically increases the sensitivity of human tumor cells to TMZ, a decrease in IC<sub>50</sub> value of almost 10-fold compared with the parental cells (Fig. 4B). Pol  $\beta$  therefore plays an important role in BER and protects from TMZ-induced cell death, supporting our hypothesis that an active BER pathway could be partially responsible for the TMZ-mediated resistance seen in some tumors. Most significantly, these results emphasize the intimate balance in repair protein expression that must be maintained to prevent accumulation of cytotoxic and genotoxic lesions such as repair intermediates (e.g., 5'dRP).

**Hypersensitivity of Human Pol  $\beta$  Knockdown Cells Is Due to Unrepaired 5'dRP Lesions.** We have shown that loss of Pol  $\beta$  expression, as mediated by shRNA expression, or saturation of the Pol  $\beta$  step in the pathway by MPG over-expression, leads to hypersensitivity to TMZ. To demonstrate that the observed TMZ hypersensitivity is specific to Pol  $\beta$  (e.g., not to an aberrant RNAi-mediated nonspecific effect), we complemented our Pol  $\beta$  knockdown cells with RNAi-resistant human epitope-tagged Pol  $\beta$ . This is feasible by taking advantage of our 3'UTR-specific Pol  $\beta$  shRNA system (Fig. 3A) and to compliment Pol  $\beta$  knockdown human tumor cells using expression vectors lacking the shRNA-targeted 3'UTR. As shown, there is no expression of endogenous Pol  $\beta$  but robust expression of the transgenic FLAG-Pol  $\beta$  (Fig. 5A). In addition, note that there is no change in the expression of APE1 (Fig. 5A). To further confirm that the observed expression of Pol  $\beta$  is not by re-expression of endogenous Pol  $\beta$ , we characterized the expression of the transgene by IP using the anti-FLAG antibody and immunoblot (IB) analysis of the precipitated material with a Pol  $\beta$  monoclonal antibody, confirming the presence of FLAG epitope-tagged Pol  $\beta$  only in the transfected cell lines (Fig. 5A, lanes 4–6). It is noteworthy that expression of wild-type FLAG-Pol  $\beta$  in our human tumor Pol  $\beta$  knockdown cells completely restores resistance to TMZ, whereas cells with a vector control are just as sensitive to TMZ as the original Pol  $\beta$  knockdown cells (Fig. 5B).

However, as we have shown previously in mouse cells, the Pol  $\beta$  substrate and BER intermediate 5'dRP is highly toxic (Sobol et al., 1996, 2000), suggesting that the observed hypersensitivity of human Pol  $\beta$  knockdown cells to TMZ may be due to loss of 5'dRP lyase activity of Pol  $\beta$ . To this end, we complemented our Pol  $\beta$  knockdown cells with human

$\beta$ -specific shRNA [MDA-MB-231/Pol  $\beta$ -KD(10),  $\diamond$ ] or pIRES-Neo-transfected Pol  $\beta$  KD/MDA-MB-231 cells [MDA-MB-231/Pol  $\beta$ -KD(10)/Neo(1),  $\ominus$ ] or Pol  $\beta$  KD/MDA-MB-231 cells expressing FLAG-Pol  $\beta$  [MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -WT(2), squares with diagonal lines; MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -WT(3),  $\blacksquare$ ; and MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -WT(4), dotted squares] were cultured in 96-well plates for 24 h before exposure to TMZ. Viable cells were determined using a modified MTT assay as described in Fig. 1.



**Fig. 6.** Reconstitution of polymerase-inactive mutant human FLAG-Pol  $\beta$  (D256A) in Pol  $\beta$  down-regulated MDA-MB-231 cells restores resistance to temozolomide. **A**, DNA Pol  $\beta$  expression as determined by immunoblot (IB) analysis of nuclear proteins isolated from the MDA-MB-231 or Pol  $\beta$  KD/MDA-MB-231 cells transfected with polymerase-inactive mutant FLAG-Pol  $\beta$  (D256A). Proteins isolated from three separate FLAG-Pol  $\beta$  (D256A)-expressing clones are shown [MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -D256A(3), MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -D256A(4), and MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -D256A(5); lanes 4–6] compared with proteins isolated from control cells (MDA-MB-231; lane 1), MDA-MB-231/Pol  $\beta$  KD cells [MDA-MB-231/Pol  $\beta$ -KD(10); lane 2], or pIREs-Neo-transfected MDA-MB-231/Pol  $\beta$  KD cells [MDA-MB-231/Pol  $\beta$ -KD(10)/Neo(1); lane 3]. FLAG and APE1 expression was determined by immunoblot. PCNA expression is shown as a loading control (bottom). For IP, cell lysate from the above-mentioned cell lines was incubated overnight with anti-FLAG antibodies followed by 1-h incubation with protein G Dynabeads. Immunoprecipitates were eluted with 3 $\times$  FLAG peptide, separated on SDS-PAGE, and transferred to nitrocellulose filters and probed with anti-Pol  $\beta$  antibody. **B**, MDA-MB-231 cells (○) or MDA-MB-231 cells expressing Pol  $\beta$ -specific shRNA [MDA-MB-231/Pol  $\beta$ -KD(10), ◊]. pIREs-Neo-transfected

epitope-tagged Pol  $\beta$  encoding a D256A mutation in the polymerase active site that retains complete 5'dRP lyase activity yet is devoid of polymerase activity (Fig. 6A). As with the cells complemented with Pol  $\beta$ , there is no expression of endogenous Pol  $\beta$  but robust expression of the transgenic FLAG-Pol  $\beta$  D256A mutant (Fig. 6A). In addition, there is no change in the expression of APE1 (Fig. 6A). As noted above, expression of the transgene was confirmed by a combined IP/IB (Fig. 6A, lanes 4–6). Most importantly, expression of the polymerase-defective/5'dRP lyase-active D256A Pol  $\beta$  mutant in our human tumor Pol  $\beta$  knockdown cells completely restores resistance to TMZ (Fig. 6B), whereas cells with vector control are just as sensitive to TMZ as the original Pol  $\beta$  knockdown cells, indicating that the hypersensitivity of cells to TMZ that have lost Pol  $\beta$  expression (due to Pol  $\beta$ -specific shRNA) is due to unrepaired cytotoxic 5'dRP lesions.

**Minimal or No Contribution of Apoptosis and Autophagy to 5'dRP Lesion-Induced Cell Death in MDA-MB-231 Cells after Temozolomide Treatment.** To investigate the involvement of apoptosis due to loss of Pol  $\beta$  or MPG overexpression in MDA-MB-231 cells after TMZ treatment, we measured caspase-3 activation. In addition, we also determined whether TMZ-induced cell death in these cells could be prevented by pretreatment with Z-VAD-FMK, a pan-caspase inhibitor (Fig. 7). ETO is a widely used DNA-damaging agent to induce apoptosis and was used as a positive control in this study (Bockbrader et al., 2005). The dose response of ETO is essentially the same for all the cell lines used herein (Fig. 7A) and pretreatment with 50  $\mu$ M Z-VAD-FMK prevented ETO-induced apoptosis in all these cells (Fig. 7B), indicating a functional apoptotic pathway. It is noteworthy that pretreatment with 50  $\mu$ M Z-VAD-FMK failed to prevent TMZ-induced cell death (Fig. 7C), suggesting that 5'dRP-induced cell death is not via apoptosis. This is supported by the results from the caspase-3 activation assay, in which we observed a 1- to 2-fold increase in caspase-3 activation at 6, 12, or 24 h and a 3-fold increase at 48 h after TMZ exposure in all the cell lines tested, suggesting a minor level of caspase-3 activation that is independent of 5'dRP lesion accumulation. As expected, we observed a 6- to 8-fold increase in caspase-3 activation within 24 h after ETO exposure (Fig. 7D). Recently Mhaidat et al. (2007) reported that TMZ does not induce apoptosis in melanoma cells, similar to our results in the MDA-MB-231 cell line presented herein. The results from the present study support the view that TMZ-induced 5'dRP lesion-induced cell death in MDA-MB-231 cells is independent of caspase-3 activation and not via the onset of apoptosis.

We next evaluated the impact of 3-MA, a specific inhibitor of autophagy, on 5'dRP lesion-mediated cell death in BER-defective cells (due to loss of Pol  $\beta$  or MPG overexpression) after TMZ treatment. 3-MA treatment alone has a similar effect on all the MDA-MB-231 cells used herein (Fig. 8A), and pretreatment with 5 mM 3-MA, as shown in Fig. 8B, failed to

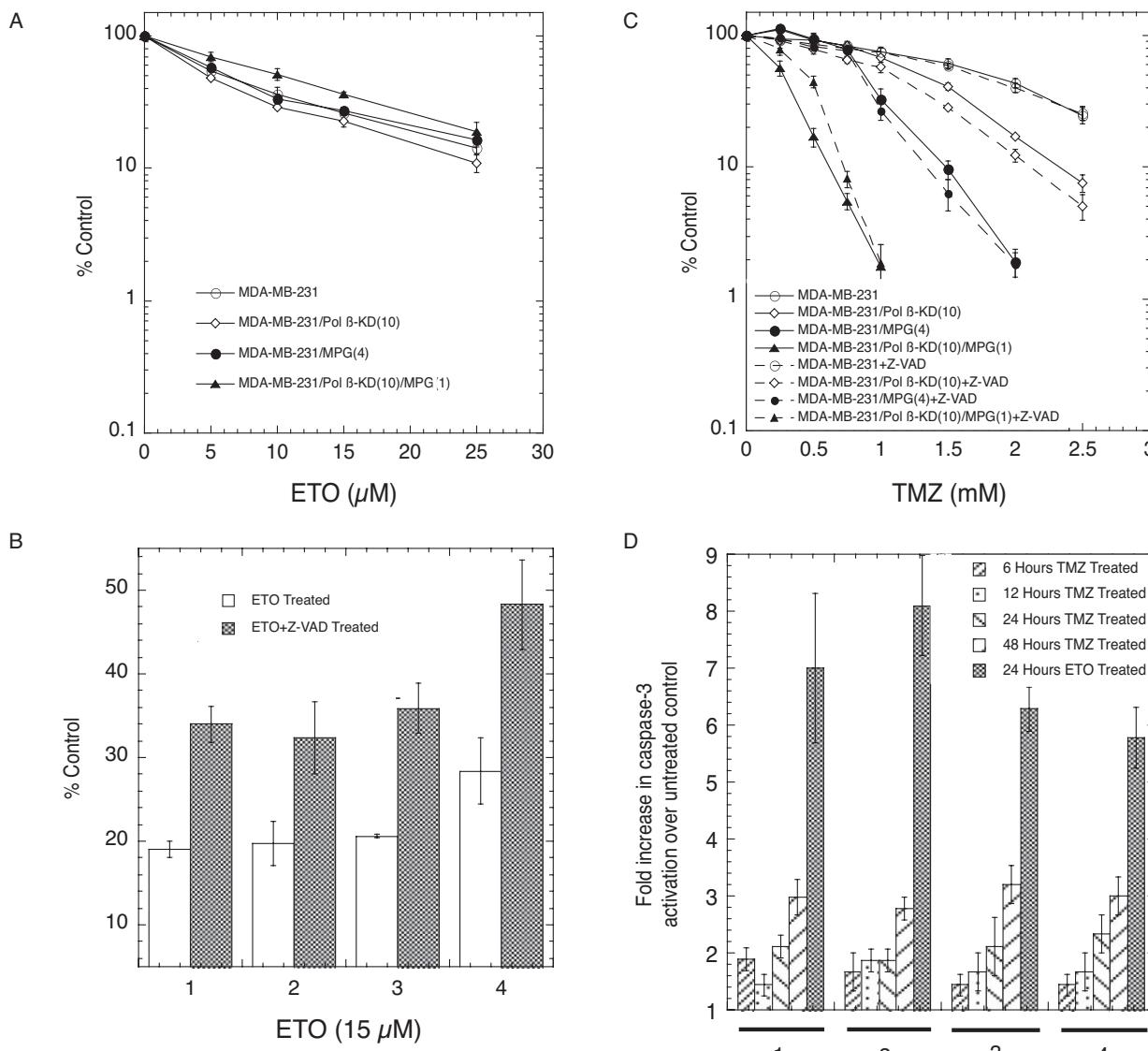
MDA-MB-231/Pol  $\beta$  KD cells [MDA-MB-231/Pol  $\beta$ -KD(10)/Neo(1), ▽] or MDA-MB-231/Pol  $\beta$  KD cells expressing FLAG-Pol  $\beta$  (D256A) [MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -D256A(3), □; MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -D256A(4), ▨; and MDA-MB-231/Pol  $\beta$ -KD(10)/FLAG-Pol  $\beta$ -D256A(5), ▩] were cultured in 96-well plates for 24 h before exposure to TMZ. Viable cells were determined using a modified MTT assay as described in Fig. 1.

prevent TMZ-induced cell death. Consistent with these results, we find that 5'dRP-mediated cell death does not induce the formation of autophagosomes, as measured by GFP-LC3 localization. Greater than 99% of all cells, regardless of BER status or TMZ exposure, presented a diffuse pattern of GFP-LC3 staining (Fig. 8C). Less than 1% of the cells were observed with a punctate expression pattern of GFP-LC3, indicative of spontaneous formation of autophagosomes (Fig. 8D). However, this pattern was not altered by TMZ exposure or BER protein expression status (data not shown), suggesting

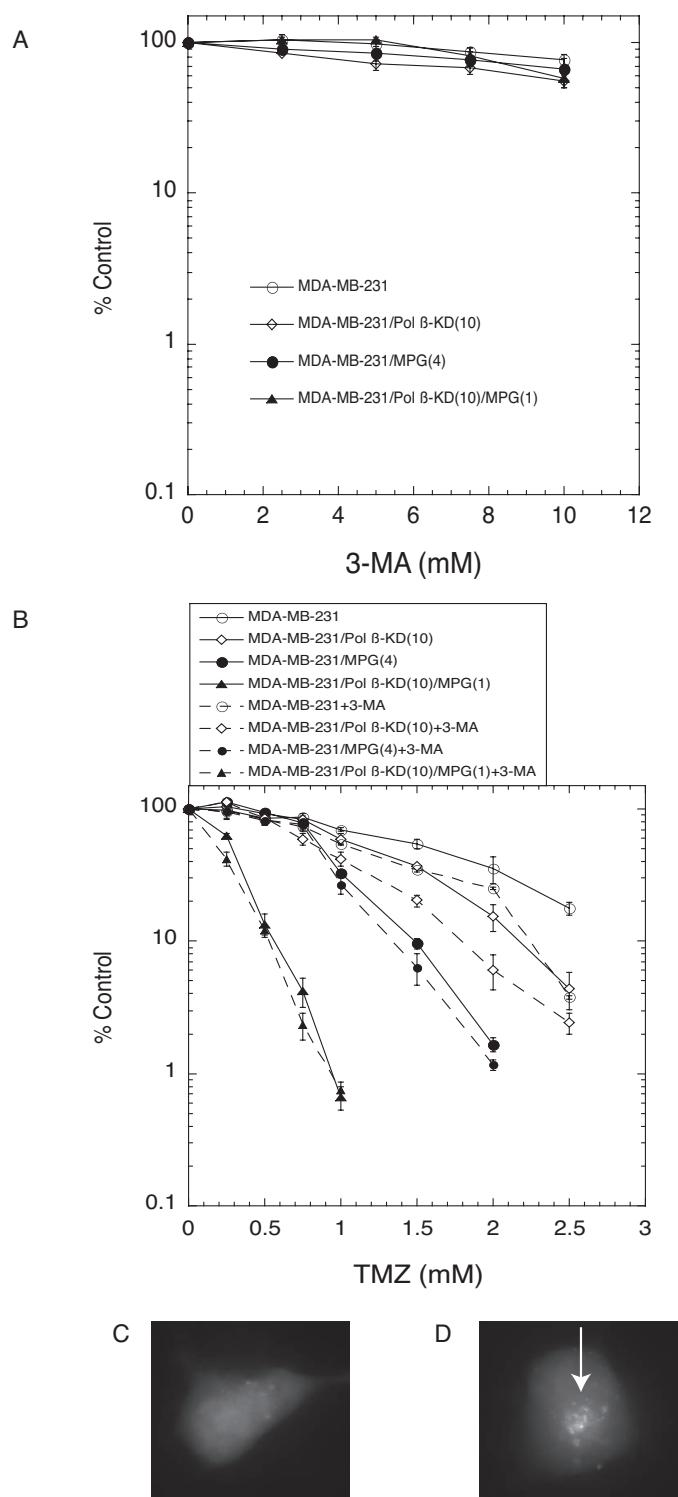
a low background level of autophagosome formation in all the cell lines used herein.

## Discussion

Overexpression of methyl-specific DNA glycosylases has been reported to have varying effects in mammalian cells, ranging from no effect to eliciting an increase in or a decrease in sensitivity to alkylating agents (Ibeau et al., 1992; Klungland et al., 1992; Habraken and Laval, 1993). We and



**Fig. 7.** Analyzing the contribution of apoptosis to 5'dRP lesion-induced cell death in MDA-MB-231 cells after temozolomide treatment. **A**, ETO-induced dose response was determined by culturing cells (as labeled in the figure) in 96-well plates for 24 h before exposure to ETO. Viable cells were determined using a modified MTT assay as described in Fig. 1. **B**, Cells [MDA-MB-231 cells, group 1; MDA-MB-231 cells expressing Pol  $\beta$ -specific shRNA (MDA-MB-231/Pol  $\beta$ -KD(10), group 2; MPG-overexpressing MDA-MB-231 cells (MDA-MB-231/MPG(4), group 3; and MPG overexpression in Pol  $\beta$  down-regulated MDA-MB-231 cells (MDA-MB-231/Pol  $\beta$ -KD(10)/MPG(1), group 4] were cultured in 96-well plates for 24 h before exposure with media or Z-VAD-FMK (50  $\mu\text{M}$ ) for 1 h. After Z-VAD-FMK pretreatment, cells were treated with ETO (15  $\mu\text{M}$ ), and viable cells were determined using a modified MTT assay as described in Fig. 1. **C**, cells (as labeled in the figure), were cultured in 96-well plates for 24 h before exposure to media or Z-VAD-FMK (50  $\mu\text{M}$ ) for 1 h. After Z-VAD-FMK pretreatment, cells were treated with TMZ (0.25–2.5 mM), and viable cells were determined using a modified MTT assay as described in Fig. 1. **D**, caspase-3 activation was determined as follows. MDA-MB-231 cells (group 1), MDA-MB-231 cells expressing Pol  $\beta$ -specific shRNA [MDA-MB-231/Pol  $\beta$ -KD(10), group 2], MPG-overexpressing MDA-MB-231 cells [MDA-MB-231/MPG(4), group 3], and MPG overexpression in Pol  $\beta$  down-regulated MDA-MB-231 cells [MDA-MB-231/Pol  $\beta$ -KD(10)/MPG(1), group 4] were treated with 2.0, 0.8, 0.6, or 0.3 mM TMZ (the corresponding TMZ IC<sub>50</sub> value for each cell line; Table 1) or with media for 6, 12, 24, and 48 h. These cells were also treated with 15  $\mu\text{M}$  ETO or media for 24 h. After drug treatment, the cell pellet was resuspended in 1× cell lysis buffer, and cytosolic extract was used for the assay of caspase-3 activation.



**Fig. 8.** Measuring the contribution of autophagy to 5'dRP lesion-induced cell death in MDA-MB-231 cells after temozolomide treatment. **A**, 3-MA-induced dose response was determined by culturing cells (as labeled in the figure) in 96-well plates for 24 h before exposure to 3-MA. Viable cells were determined using a modified MTT assay as described in Fig. 1. **B**, TMZ-induced cytotoxicity after 3-MA pretreatment was evaluated by culturing cells (as labeled in the figure) in 96-well plates for 24 h before exposure with media or 3-MA (5 mM) for 30 min. After 3-MA pretreatment, cells were then treated with TMZ (0.25–2.5 mM) for 48 h, and viable cells were determined using a modified MTT assay as described in Fig. 1. Representative image showing diffuse pattern of pEGFP-LC3 expression observed in >99% of the cells (**C**) and a punctate pattern of pEGFP-LC3 expression (see arrow, **D**) and localization observed in less than 1% of the cell population. This expression pattern was independent of BER status, TMZ treatment, or 3-MA treatment.

others have found that MPG overexpression induces a DNA-damage sensitivity phenotype in mouse fibroblasts (Trivedi et al., 2005) as well as human breast and ovarian tumor cells (Rinne et al., 2004; Fishel et al., 2007). In this study, we demonstrate that both MPG overexpression and Pol  $\beta$  depletion (via shRNA-mediated knockdown) similarly affect the sensitivity of human breast cancer (MDA-MB-231) cells to alkylating damage. However, it is the collective expression of both of these DNA repair proteins that defines the sensitivity phenotype. Herein, we demonstrate that the Pol  $\beta$  substrate 5'dRP (a BER intermediate) is the cytotoxic lesion in both MPG-overexpressed and Pol  $\beta$  KD human tumor cells. This TMZ-induced cell death (due to 5'dRP lesion accumulation) is not likely via caspase-mediated apoptosis, because we do not observe caspase-3 activation after TMZ exposure. Although autophagy has been reported to play a role in TMZ-induced cell death in some glioma cell lines when observed 72 h after exposure (Kanzawa et al., 2004), this study suggests that 5'dRP-mediated cytotoxicity does not induce the formation of autophagosomes, as measured by GFP-LC3 localization, suggesting a possible role of a necrotic mechanism of cell death due to failed repair of the cytotoxic 5'dRP BER intermediate. It also seems that p53 is not involved in the genotoxin-induced cell death observed, because MDA-MB-231 cells express a mutant form of p53 (R280K; <http://www.sanger.ac.uk/genetics/CGP/CellLines/>), albeit at elevated levels (Hui et al., 2006), in-line with our earlier observations in p53-null mouse cells (Sobol et al., 2003). MPG is the BER-initiating enzyme, and DNA Pol  $\beta$  is generally considered the rate-limiting enzyme in BER. In this study, we tested the hypothesis that MPG overexpression induces a BER imbalance that saturates the Pol  $\beta$  5'dRP lyase activity in the BER pathway, leading to 5'dRP-mediated cell death in cells that overexpress MPG. If left unrepaired, 5'dRP moieties could give rise to an increase in genomic instability (Sobol et al., 2003), eventually leading to cell death (Sobol et al., 2000).

As the first enzyme in the BER process, MPG expression is required for the initiation of BER to repair-alkylated bases (Wood et al., 2001). There is a wealth of information regarding the enzymology and structural biology of MPG (Wyatt and Pittman, 2006). However, little is known about the mechanism of MPG-initiated BER in vivo. It has been reported that MPG expression levels vary considerably (Cerda et al., 1998; Kim et al., 2003). In addition, MPG is also reported to have multiple post-translational modifications and to form protein-protein interactions with many DNA repair proteins, including XRCC1 and hR23A, among others (Almeida and Sobol, 2007). But the role of these post-translational modifications and protein-protein interactions are unknown. Because of the many binding partners of MPG, it might be possible that overexpression of MPG leads to cellular sensitivity by negatively affecting the function of these binding proteins. The studies described here suggest that MPG overexpression increases repair initiation of alkylated bases in vivo. Increased cellular sensitivity is only observed when cells are exposed to TMZ, and the TMZ-induced cell sensitivity is altered by the expression level of Pol  $\beta$ . The increased repair initiation then would lead to an imbalance in BER, in this case by saturating the Pol  $\beta$  step in the repair pathway.

DNA Pol  $\beta$  has long been considered a DNA repair polymerase (Sobol, 2007). Pol  $\beta$  is a 335-amino acid, bifunctional enzyme, facilitating both the DNA synthesis step in BER as

well as the gap-tailoring step to remove the 5'dRP group formed during BER after APE1 hydrolysis of the abasic site (Almeida and Sobol, 2007). Extensive enzymology, protein biochemistry, and structural biology of Pol  $\beta$  have provided intimate details of the structure of the protein (Beard and Wilson, 2006) and the mechanisms of DNA synthesis and 5'dRP lyase activity conducted by Pol  $\beta$  using both purified recombinant enzyme and extracts from mouse and human cells (Prasad et al., 2005; Beard and Wilson, 2006). Pol  $\beta$  facilitates repair via the formation of protein complexes at the lesion site with several BER proteins, including APE1, DNA ligase I, XRCC1, PCNA, and poly(ADP-ribose) polymerase-1 and -2, among others (Almeida and Sobol, 2007). By genetic complementation of Pol  $\beta$  null mouse fibroblasts, it was determined that the increased sensitivity of Pol  $\beta$  null mouse cells to alkylation damage was the result of a failure to repair the 5'dRP BER intermediate (Sobol et al., 1996, 2000). However, little is known about the significance of the 5'dRP lyase activity of Pol  $\beta$  in human cells and the impact of Pol  $\beta$  expression and function on the human cellular response to DNA damage. Clearly, these studies demonstrate that variations in Pol  $\beta$  expression affect cellular sensitivity to TMZ in an MPG-dependent manner and due to a failure to repair the 5'dRP repair intermediate.

Expression of Pol  $\beta$  is highly variable in human cells (Srivastava et al., 1999). Pol  $\beta$  is up-regulated in adenocarcinoma (Srivastava et al., 1999), ovarian tumor (Bergoglio et al., 2001), and glioma cells (Gomi et al., 1996) and is found to be overexpressed in more than 35% of human tumors (Starcevic et al., 2004). Furthermore, Pol  $\beta$  function may be altered by post-translational modification, including methylation, acetylation, and ubiquitylation (Sobol, 2008a), suggesting a cellular requirement to regulate Pol  $\beta$  function. In addition, many Pol  $\beta$  variants with altered function have been isolated from human tumors (Tan et al., 2005; Lang et al., 2007).

DNA Pol  $\beta$  seems to be the major BER polymerase in MDA-MB-231 human breast cancer cells. RNAi-mediated expression loss of Pol  $\beta$  does not affect cell viability yet Pol  $\beta$  KD cells are hypersensitive to the cytotoxic effects of the clinical alkylating agent TMZ, and this is further enhanced when MPG expressed in increased. This increase in cellular sensitivity to TMZ is consistent with earlier studies using mouse knockout fibroblasts (Horton et al., 2005; Trivedi et al., 2005). By using this knockdown/knockin strategy, we show that the hypersensitive phenotype is a direct result of the loss of Pol  $\beta$  expression, eliminating concerns that the knockdown phenotype may be the result of off-target effects of RNA interference-mediated gene silencing.

This study addresses the problem of cellular resistance of alkylation damage induced by the chemotherapeutic agent TMZ, yet it may be considered a model for many types of base lesions that are repaired by BER. We focused this study on biochemical and molecular mechanisms that mediate the repair of alkylation-induced DNA damage in human tumor cells. Overall, we show that MPG, Pol  $\beta$ , and base excision repair contribute significantly to the repair of alkylation-induced DNA damage in human cells. Therefore, modulating the BER pathway by MPG overexpression or by Pol  $\beta$  inhibition/loss of expression could enhance the chemotherapeutic index of agents that damage DNA and initiate BER. Furthermore, cellular conditions that affect Pol  $\beta$  expression and/or BER balance can predispose a given human cell population to

5'dRP-mediated genome instability and cytotoxicity. However, the overall BER capacity must be considered when evaluating cellular response to TMZ and similar DNA-damaging agents. The experiments presented herein suggest that it will be important to investigate whether an active BER pathway could be partially responsible for the TMZ-mediated resistance seen in some tumors and that overall BER capacity may help predict sensitivity to TMZ. Furthermore, it will be important to evaluate whether a BER imbalance will predispose human primary cells to cellular and environmental genotoxins, inducing an increase in genome instability, cellular senescence, or in the case of postmitotic cells (neurons), neurodegeneration as a result of failed 5'dRP lesion repair. It is also interesting to speculate that the increase in genome instability due to a BER imbalance combined with chronic inflammation (ulcerative colitis) may be brought about by a deficiency in the repair of the BER intermediate 5'dRP (Hofseth et al., 2003). Finally, these findings may have clinical applicability toward the design of specific Pol  $\beta$  and BER modulators as adjuvant treatments for reversing a TMZ-resistant phenotype and suggest that alkylating agents such as TMZ combined with BER modulators may be explored as possible therapeutics in the treatment of breast cancer.

#### Acknowledgments

We thank Drs. R. D. Wood (University of Pittsburgh, Pittsburgh, PA), K. A. Eckert (The Pennsylvania State University College of Medicine, Hershey, PA), and K. H. Almeida (Rhode Island College, Providence, RI) for providing helpful discussions.

#### References

- Almeida KH and Sobol RW (2007) A unified view of base excision repair: lesion-dependent protein complexes regulated by post-translational modification. *DNA Repair* **6**:695–711.
- Beard WA and Wilson SH (2006) Structure and mechanism of DNA polymerase beta. *Chem Rev* **106**:361–382.
- Bergoglio V, Canitrot Y, Hogarth L, Minto L, Howell SB, Cazaux C, and Hoffmann JS (2001) Enhanced expression and activity of DNA polymerase  $\beta$  in human ovarian tumor cells: impact on sensitivity towards antitumor agents. *Oncogene* **20**:6181–6187.
- Bockbrader KM, Tan M, and Sun Y (2005) A small molecule Smac-mimic compound induces apoptosis and sensitizes TRAIL- and etoposide-induced apoptosis in breast cancer cells. *Oncogene* **24**:7381–7388.
- Cerda SR, Turl PW, Thor AD, and Weitzman SA (1998) Altered expression of the DNA repair protein, N-methylpurine-DNA glycosylase (MPG) in breast cancer. *FEBS Lett* **431**:12–18.
- Coquerelle T, Dosch J, and Kaina B (1995) Overexpression of N-methylpurine-DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents—a case of imbalanced DNA repair. *Mutat Res* **336**:9–17.
- Elder RH, Jansen JG, Weeks RJ, Willington MA, Deans B, Watson AJ, Mynett KJ, Bailey JA, Cooper DP, Rafferty JA, et al. (1998) Alkylpurine-DNA-N-glycosylase knockout mice show increased susceptibility to induction of mutations by methyl methanesulfonate. *Mol Cell Biol* **18**:5828–5837.
- Engelward BP, Dreslin A, Christensen J, Huszar D, Kurahara C, and Samson L (1996) Repair-deficient 3-methyladenine DNA glycosylase homozygous mutant mouse cells have increased sensitivity to alkylation-induced chromosome damage and cell killing. *EMBO J* **15**:945–952.
- Fishel ML, He Y, Smith ML, and Kelley MR (2007) Manipulation of base excision repair to sensitize ovarian cancer cells to alkylating agent temozolomide. *Clin Cancer Res* **13**:260–267.
- Gomi A, Shinoda S, Sakai R, Hirai H, Ozawa K, and Masuzawa T (1996) Elevated expression of DNA polymerase beta gene in glioma cell lines with acquired resistance to 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3-(2-chloroethyl)-3-nitrosourea. *Biochem Biophys Res Commun* **227**:558–563.
- Habraken Y and Laval F (1993) Increased resistance of the Chinese hamster mutant irs1 cells to monofunctional alkylating agents by transfection of the E. coli or mammalian N3-methyladenine-DNA-glycosylase genes. *Mutat Res* **293**:187–195.
- Hofseth LJ, Khan MA, Ambrose M, Nikolayeva O, Xu-Welliver M, Kartalou M, Hussain SP, Roth RB, Zhou X, Mechanic LE, et al. (2003) The adaptive imbalance in base excision-repair enzymes generates microsatellite instability in chronic inflammation. *J Clin Invest* **112**:1887–1894.
- Horton JK, Stefanick DF, Naron JM, Kedar PS, and Wilson SH (2005) Poly(ADP-ribose) polymerase activity prevents signaling pathways for cell cycle arrest following DNA methylating agent exposure. *J Biol Chem* **280**:15773–15785.

- Horton JK, Watson M, Stefanick DF, Shaughnessy DT, Taylor JA, and Wilson SH (2008) XRCC1 and DNA polymerase beta in cellular protection against cytotoxic DNA single-strand breaks. *Cell Res* **18**:48–63.
- Hui L, Zheng Y, Yan Y, Bargiometti J, and Foster DA (2006) Mutant p53 in MDA-MB-231 breast cancer cells is stabilized by elevated phospholipase D activity and contributes to survival signals generated by phospholipase D. *Oncogene* **25**:7305–7310.
- Ibeau G, Hartenstein B, Dunn WC, Chang LY, Hofmann E, Coquerelle T, Mitra S, and Kaina B (1992) Overexpression of human DNA repair protein N-methylpurine-DNA glycosylase results in the increased removal of N-methylpurines in DNA without a concomitant increase in resistance to alkylating agents in Chinese hamster ovary cells. *Carcinogenesis* **13**:1989–1995.
- Kanzawa T, Germano IM, Komata T, Ito H, Kondo Y, and Kondo S (2004) Role of autophagy in temozolomide-induced cytotoxicity for malignant glioma cells. *Cell Death Differ* **11**:448–457.
- Kim NK, Ahn JY, Song J, Kim JK, Han JH, An HJ, Chung HM, Joo JY, Choi JU, Lee KS, et al. (2003) Expression of the DNA repair enzyme, N-methylpurine-DNA glycosylase (MPG) in astrocytic tumors. *Anticancer Res* **23**:1417–1423.
- Klungland A, Fairbairn L, Watson AJ, Margison GP, and Seeberg E (1992) Expression of the *E. coli* 3-methyladenine DNA glycosylase I gene in mammalian cells reduces the toxic and mutagenic effects of methylation agents. *EMBO J* **11**:4439–4444.
- Lang T, Dalal S, Chikova A, Dimaio D, and Sweasy JB (2007) The E295K DNA polymerase beta gastric cancer-associated variant interferes with base excision repair and induces cellular transformation. *Mol Cell Biol* **27**:5587–5596.
- Lindahl T and Wood RD (1999) Quality control by DNA repair. *Science* **286**:1897–1905.
- Mhaidat NM, Zhang XD, Allen J, Avery-Kiejda KA, Scott RJ, and Hersey P (2007) Temozolomide induces senescence but not apoptosis in human melanoma cells. *Br J Cancer* **97**:1225–1233.
- Ono Y, Furuta T, Ohmoto T, Akiyama K, and Seki S (1994) Stable expression in rat glioma cells of sense and antisense nucleic acids to a human multifunctional DNA repair enzyme, APEX nuclease. *Mutat Res* **315**:55–63.
- Pail J, Duncan T, Lindahl T, and Sedgwick B (2005) Sensitization of human carcinoma cells to alkylating agents by small interfering RNA suppression of 3-alkyladenine-DNA glycosylase. *Cancer Res* **65**:10472–10477.
- Poeschla EM, Wong-Staal F, and Looney DJ (1998) Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat Med* **4**:354–357.
- Prasad R, Batra VK, Yang XP, Krahn JM, Pedersen LC, Beard WA, and Wilson SH (2005) Structural insight into the DNA polymerase beta deoxyribose phosphate lyase mechanism. *DNA Repair* **4**:1347–1357.
- Rinne M, Caldwell D, and Kelley MR (2004) Transient adenoviral N-methylpurine DNA glycosylase overexpression imparts chemotherapeutic sensitivity to human breast cancer cells. *Mol Cancer Ther* **3**:955–967.
- Sobol RW (2007) DNA polymerase  $\beta$  null mouse embryonic fibroblasts harbor a homozygous null mutation in DNA polymerase iota. *DNA Repair* **6**:3–7.
- Sobol RW (2008a) CHIPping away at base excision repair. *Mol Cell* **29**:413–415.
- Sobol RW (2008b) Temozolomide, in *Encyclopedia of Cancer*, 2nd ed (Schwab M ed), Springer, Berlin, Germany.
- Sobol RW, Horton JK, Kuhn R, Gu H, Singhal RK, Prasad R, Rajewsky K, and Wilson SH (1996) Requirement of mammalian DNA polymerase- $\beta$  in base-excision repair. *Nature* **379**:183–186.
- Sobol RW, Kartalou M, Almeida KH, Joyce DF, Engelward BP, Horton JK, Prasad R, Samson LD, and Wilson SH (2003) Base excision repair intermediates induce p53-independent cytotoxic and genotoxic responses. *J Biol Chem* **278**:39951–39959.
- Sobol RW, Prasad R, Evenski A, Baker A, Yang XP, Horton JK, and Wilson SH (2000) The lyase activity of the DNA repair protein  $\beta$ -polymerase protects from DNA-damage-induced cytotoxicity. *Nature* **405**:807–810.
- Srivastava DK, Husain I, Arteaga CL, and Wilson SH (1999) DNA polymerase  $\beta$  expression differences in selected human tumors and cell lines. *Carcinogenesis* **20**:1049–1054.
- Starcevic D, Dalal S, and Sweasy JB (2004) Is there a link between DNA polymerase beta and cancer? *Cell Cycle* **3**:998–1001.
- Tan XH, Zhao M, Pan KF, Dong Y, Feng GJ, Jia G, and Lu YY (2005) Frequent mutation related with overexpression of DNA polymerase beta in primary tumors and precancerous lesions of human stomach. *Cancer Lett* **220**:101–114.
- Trivedi RN, Almeida KH, Fornasaglio JL, Schamus S, and Sobol RW (2005) The role of base excision repair in the sensitivity and resistance to temozolomide mediated cell death. *Cancer Res* **65**:6394–6400.
- Walker LJ, Craig RB, Harris AL, and Hickson ID (1994) A role for the human DNA repair enzyme HAP1 in cellular protection against DNA damaging agents and hypoxic stress. *Nucleic Acids Res* **22**:4884–4889.
- Wood RD, Mitchell M, Sguoros J, and Lindahl T (2001) Human DNA repair genes. *Science* **291**:1284–1289.
- Wyatt MD and Pittman DL (2006) Methylating agents and DNA repair responses: methylated bases and sources of strand breaks. *Chem Res Toxicol* **19**:1580–1594.

**Address correspondence to:** Dr. Robert W. Sobol, Hillman Cancer Center, University of Pittsburgh Cancer Institute, Research Pavilion, Suite 2.6, 5117 Centre Ave., Pittsburgh, PA 15213-1863. E-mail: rws9@pitt.edu