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Parp1 activation in mouse embryonic fibroblasts promotes Pol β -dependent cellular hypersensitivity to alkylation damage

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Abstract

Alkylating agents induce cell death in wild-type (WT) mouse embryonic fibroblasts (MEFs) by multiple mechanisms, including apoptosis, autophagy and necrosis. DNA polymerase β (Pol β) knockout (KO) MEFs are hypersensitive to the cytotoxic effect of alkylating agents, as compared to WT MEFs. To test the hypothesis that Parp1 is preferentially activated by methyl methanesulfonate (MMS) exposure of Pol β KO MEFs, we have examined the relationship between Pol β expression, Parp1 activation and cell survival following MMS exposure in a series of WT and Pol β deficient MEF cell lines. Consistent with our hypothesis, we observed elevated Parp1 activation in Pol β KO MEFs as compared to matched WT MEFs. Both the MMS-induced activation of Parp1 and the MMS-induced cytotoxicity of Pol β KO MEFs are attenuated by pre-treatment with the Parp1/Parp2 inhibitor PJ34. Further, elevated Parp1 activation is observed following knockdown (KD) of endogenous Pol β , as compared to WT cells. Pol β KD MEFs are hypersensitive to MMS and both the MMS-induced hypersensitivity and Parp1 activation is prevented by pre-treatment with PJ34. In addition, the MMS-induced cellular sensitivity of Pol β KO MEFs is reversed when *Parp1* is also deleted (Pol β /*Parp1* double KO MEFs) and we observe no MMS sensitivity differential between Pol β /*Parp1* double KO MEFs and those that express recombinant mouse Pol β . These studies suggest that Parp1 may function as a sensor of BER to initiate cell death when BER is aborted or fails. Parp1 may therefore function in BER as a tumor suppressor by initiating cell death and preventing the accumulation of cells with chromosomal damage due to a BER defect.

Keywords

Base excision repair; alkylating agents; Parp1; RNAi; DNA glycosylase; DNA polymerase beta

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1. Introduction

DNA polymerase β (Pol β) plays a critical role in the repair of genomic base damage and is considered the predominant DNA polymerase in the base excision repair (BER) pathway [1]. The development of mouse embryonic fibroblasts (MEFs) deficient in Pol β clearly defined a requirement for Pol β in the repair of base damage mediated by the BER pathway [2]. Pol β is a bi-functional, two-domain, single-polypeptide 39kDa enzyme [3]. The polymerase activity responsible for gap-filling DNA synthesis in BER resides in the C-terminal 31kDa domain whereas the N-terminal 8kDa domain encodes a 5'deoxyribose phosphate (5'dRP) lyase activity that removes the sugar-phosphate lesion (5'dRP) [3]. The 5'dRP lesion is the product of AP endonuclease 1 (Ape1)-mediated hydrolysis of the abasic site generated following DNA glycosylase action [3]. The polymerase function of Pol β is responsible for gap-filling DNA synthesis in both the short-patch and long-patch sub-pathways of BER [4] yet alternate DNA polymerases have been demonstrated to complement the long-patch BER related DNA synthesis activity of Pol β in its absence when evaluated in cell extracts *in vitro* [5]. It is possible that the polymerase activity of Pol β is also complemented *in vivo*, as it is the 5'dRP lyase function of Pol β [6] that is essential and sufficient for alkylating-agent resistance in MEFs [7] and in human breast cancer cells [8]. In the absence of Pol β , cells are unable to efficiently repair the highly toxic 5'dRP moiety and therefore are hypersensitive to the cytotoxic effect of different alkylating agents such as methylmethane sulfonate (MMS), *N*-methyl-*N*-nitrosourea and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) [2], the thymidine analog 5-hydroxymethyl-2'-deoxyuridine [9] as well as the therapeutic alkylating agent temozolomide [8-10].

The 5'dRP lyase enzymatic activity, an essential BER gap-tailoring function, was first identified in mammalian cells as an enzymatic activity due to a 47kDa enzyme [10] but was later shown to be encoded by Pol β [6]. It is somewhat surprising however that the 5'dRP lyase function of Pol β is not complemented *in vivo* since there are several mammalian proteins that have 5'dRP lyase enzymatic activity. In addition to Pol β , DNA polymerase λ (Pol λ) and DNA polymerase ι (Pol ι), as well as the mitochondrial polymerase Pol γ have 5'dRP lyase activity and can function (*in vitro*) in the removal of the 5'dRP BER intermediate [11-16]. Further, the endonuclease VIII-like DNA glycosylases NEIL1 and NEIL2 both excise 5'dRP lesions with similar efficiency to Pol β [17]. However, the severe 5'dRP-mediated cell death observed in Pol β knockout (KO) MEFs [7] and Pol β deficient human cells [8] indicates that potential complementing activities provide little, if any, repair of the 5'dRP lesion generated during BER. This would suggest that the un-repaired 5'dRP lesion is not freely and readily available by additional cellular proteins outside of the BER complex that forms at the lesion site [1].

The molecular mechanism responsible for 5'dRP-mediated cell death is unknown. However, left un-repaired, 5'dRP lesions trigger mouse fibroblast and human breast cancer cell death via a p53-independent mechanism that does not involve apoptosis and does not induce the formation of autophagosomes [8,18]. Initial reports suggested a possible apoptotic mechanism of cell death [19] concomitant with an elevated level of chromosome aberrations following alkylating-agent exposure [19]. It was subsequently suggested that the observed apoptosis was the result of DNA double-strand breaks (DSBs) that form secondary to failed BER (in the absence of Pol β) following replication [20]. The formation of replication-dependent DSBs in Pol β KO MEFs following MMS-exposure is consistent with the increase in sister chromatid exchange (SCE) events observed in Pol β KO MEFs following MMS treatment [18]. However, the significantly long time-frame for the appearance of DSBs is not consistent with the relatively rapid (2 hrs) alterations in the cell cycle observed in Pol β KO MEFs following MMS exposure [18]. We have therefore hypothesized that the un-repaired 5'dRP lesion functions as a signal for cell death, possibly via protein binding and activation.

The DNA binding and signaling molecules Parp1 and Parp2 have each been implicated in BER [1], although the precise enzymatic role of either Parp isoform in BER is un-defined. Robust BER can be recapitulated *in vitro* in the absence of Parp1 [21] and neither bacteria (*E. coli*) nor yeast (*S. cerevisiae* or *S. pombe*) expresses a Parp homolog yet each is proficient in BER [22]. Interestingly, BER has been reported as both impaired [23] and proficient [24] in the absence of Parp1 [23,24]. Current models suggest that Parp1 and possibly Parp2 function as scaffold proteins in BER, facilitating the formation of BER complexes at the site of DNA damage via multiple protein-protein interactions, including interactions with Pol β , Xrcc1 and DNA LigIII α [1,25]. Parp1 was observed to bind to the lesion site prior to Xrcc1 or Pol β [26,27]. Further, Parp1 was found in a complex of BER proteins cross-linked to a BER intermediate [28] and more recently it was observed that Parp1 and Ape1 from MEF extracts compete for the same 5'dRP BER intermediate [29]. It has been postulated that local, strand-break induced activation of Parp1 and the resultant synthesis of poly(ADP-ribose) (PAR) facilitates recruitment of the BER proteins Xrcc1 and Pol β to stimulate DNA repair [30]. In short, Parp1 (and Parp2) are involved in BER complex formation [1], Parp1 can recognize and bind to BER intermediates [29], Parp1 activation is observed upon BER initiation [30] and in un-related studies, it has been shown that Parp1 hyperactivation is recognized as an inducer of necrotic cell death [31].

We have hypothesized that Parp1 is the signaling protein that triggers cell death in MEFs when 5'dRP lesions are not repaired. In this study, we used genetically defined *Pol β* KO and knockdown (KD) mouse cell lines to define a role for Parp1 in the cellular response to alkylation damage in MEFs when BER fails or is aborted. These studies suggest that Parp1 (and likely Parp2) may function not only in BER protein recruitment but may also act as a sensor of completed BER and therefore Parp1 may be considered a BER sensor protein. As a BER sensor or checkpoint protein, Parp1 may therefore function as a tumor suppressor by preventing chromosomal damage that could accumulate due to a BER defect.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media and supplies were from InVitrogen-Gibco (Carlsbad, CA). Fetal bovine serum was from Atlanta Biologicals (Lawrenceville, GA). Methyl methanesulfonate (MMS), DTT, Phosphocreatine di(tris) salt, Creatine Phosphokinase and NAD⁺ were purchased from Sigma-Aldrich (St. Louis, MO). ATP and Parp Inhibitor VIII (PJ34) were obtained from GE Healthcare (Piscataway, NJ) and Calbiochem (San Diego, CA), respectively. The hairpin DNA substrate with a 3'-biotinylated end was synthesized by Integrated DNA Technologies (IDT; Coralville, IA). We used the following primary antibodies: DNA polymerase beta Ab-2; clone 61 (NeoMarker, Fremont, CA); Ape1 Ab (EMD Biosciences, Inc, San Diego, CA); Xrcc1 Ab (Bethyl Labs, Montgomery, TX); Parp1 mAb (Novus Biologicals, Littleton, CO); PCNA mAb (Santa Cruz Biotechnology, Santa Cruz, CA); PAR Ab (BD Biosciences, San Jose, CA); anti-human MPG (Mab; clone 506-3D)[8]. Secondary antibodies GAM-HRP and GAR-HRP conjugates and signal generation substrates were from Bio-Rad (Hercules, CA) and Pierce (Rockford, IL), respectively. All electrophoresis reagents were from Bio-Rad (Hercules, CA).

2.2. Cell lines and culture conditions

Transformed WT MEF cells (16tsA, clone 1B5; 36.3; 53TAg and 92TAg), *Pol β* KO MEF cells (38 Δ 4; 50TAg and 88TAg), *Pol β /Pol ι* double KO MEF cells (19tsA, clone 2B2) and *Pol β /Parp1* double KO MEF cells have been described previously [2]. Transformed WT MEFs HCC384 were derived from c57BI/6 day 14 embryos, as described previously [2]. The cell lines 16tsA, clone 1B5; 19tsA, clone 2B2; 88TAg and 92TAg are available from the ATCC. 92TAg cells engineered to express MPG have been reported previously [32]. The *Pol β /*

Parp1 double KO MEF cells were kindly provided by Dr. J. Menissier-de Murcia (CNRS). MEFs were cultured at 37°C in a humidified incubator with 10% CO₂ in DMEM supplemented with 10% fetal bovine serum, penicillin (50 units/mL), streptomycin (50 µg/mL) and Glutamax (4 mmol/L). The *Pol β/Parp1* double KO MEF cells were then immortalized with SV40 T antigen, as we have previously described [32].

2.3. Cloning and expression of mouse *Pol β*

Total RNA was isolated from 5×10^6 92Tag cells using Qiagen RNeasy reagents. Total cDNA was prepared using a first-strand cDNA synthesis kit (InVitrogen) as described by the manufacturer, using oligo-dT as the primer. The mouse *Pol β* cDNA was then amplified by PCR using primers mBetaF (caccatgagcaaacgcaaggcgccg) and mBetaR (tcattcattctatccttggg). EGFP cDNA was also amplified by PCR from pIRES2-EGFP (Clontech) using primers FEGFP (caccatggtgagcaaggcgagga) and REGFP (ttactgtacagctcgtccatgccgagag). Each cDNA was then cloned via a Topoisomerase cloning procedure into the pENTR-D cloning plasmid (InVitrogen), as per the manufacturers protocol. The cloned mouse *Pol β* cDNA and EGFP cDNA were both sequence verified to be identical to that reported earlier (NM_011130.1 and www.addgene.com; respectively). The mouse *Pol β* open reading frame (ORF) and the EGFP ORF were each transferred from pENTR-mPolBeta or pENTR-EGFP; respectively, to a Gateway modified pIRES-Puro plasmid via LR recombination, as per the manufacturer (InVitrogen).

Pol β KO (88Tag) and *Pol β/Parp1* double KO MEF cells were modified to express transgenic mouse *Pol β* or EGFP as follows: briefly, 1.5×10^5 cells were seeded into 60mm dishes and incubated for 24-30 hours at 10% CO₂ at 37°C. The mouse *Pol β* expression plasmid (pIRES-Puro-mBeta) or the EGFP expression plasmid (pIRES-Puro-EGFP) was transfected using FuGene 6 Transfection Reagent (Roche Diagnostic Corp, Indianapolis, IN) according to the manufacturer's instructions. Stable cell lines were selected in puromycin (7 µg/ml) for 2 weeks, individual clones (stably expressing mouse *Pol β* or EGFP) were amplified and 30 µg of nuclear extract was analyzed by immunoblotting for the expression of mouse *Pol β* protein as described below.

2.4. Lentivirus expressing mouse *Pol β* specific shRNA

We have previously described a highly effective retroviral shRNA vector specific for mouse *Pol β* [32]. Using the same target sequence (gatcagtactactgtggtg), we cloned the corresponding hairpin sequence into the lentiviral vector pLL3.7 [33]. Lentiviral particles were generated by transfection of four plasmids (the control plasmid pLL3.7 or the mouse *Pol β*-specific shRNA plasmid pLLOxmPol β, plus pMD2.g(VSVG), pRSV-REV and pMDLg/pRRE) into 293-FT cells [33,34] using FuGene 6 transfection reagent (Roche Diagnostic Corp, Indianapolis, IN). Culture media from transfected cells was collected 48 hours after transfection to isolate the viral particles, passed through 0.45 µm filters, used immediately or stored at -80°C in single-use aliquots. Transduction of 92Tag and HCC384 cells with control lentivirus (GFP expression only) and mouse *Pol β* specific shRNA lentivirus was completed as follows: Briefly, 6.0×10^4 cells were seeded into 6-well plates and incubated for 24-30 hours at 10% CO₂ at 37°C. Cells were transduced for 18 hours with virus at 32°C and cultured for 72 hours at 37°C and then seeded in 96-well plates at limiting dilution (0.3 cells/well) for the isolation of single-cell clones. Individual clones (control and stable KD of mouse *Pol β* protein) were amplified and 30 µg of nuclear extract was analyzed by immunoblotting for the expression of endogenous mouse *Pol β* protein as described below.

2.5. Cell extract preparation and immunoblot assays

Preparation of whole cell extract from 92Tag cells expressing MPG was as described [35]. Briefly, cells were washed three times with ice cold PBS and the pellet was suspended in buffer

containing 20 mM Hepes-KCl, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 10% glycerol and 1 mM DTT. Cells were disrupted by sonication for 10 seconds (four times) with 30-second incubations on ice in between each burst. The cell suspension was centrifuged for 20 minutes at 16400 RPM (4°C) to obtain whole cell extract and dialyzed overnight against re-suspension buffer (25 mM Hepes pH 7.9, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA, 5% Glycerol, 2 mM DTT).

Nuclear extracts were prepared using the NucBuster nuclear protein extraction reagent (Novagen). Protein concentration was determined using Bio-Rad protein assay reagents according to the manufacturer's instructions. Nuclear protein (30 µg) was separated by electrophoresis in a 4-12% SDS-polyacrylamide gel and electrotransferred to a 0.45 µm nitrocellulose membrane (Trans-Blot, Bio-Rad). Antigens were detected using standard protocols. Primary antibodies: anti-Pol β, (NeoMarkers, #MS-1402-P0), 1,000×; anti-Ape1, (Novus, #NB-100-116), 3,000×; anti-Xrcc1, (Bethyl Labs, #A300-065A) 1,000×; anti-Parp, (Novus, #NB-100-112) 3,000×; anti-PCNA, (Santa Cruz, #sc-56) 1,000×; anti-MPG, [8] 1000× and the horseradish peroxidase (HRP)-conjugated secondary antibody (GAM-HRP or GAR-HRP; Bio-Rad) were diluted 10,000× in TBST/5% milk. Each membrane was stripped and reprobed with anti-PCNA antibodies to correct for differences in protein loading.

2.6. PAR assay

Cells (1.5×10^6) were seeded in 100mm dishes 24 hours before treatment. After 24 hours, media was removed and replaced with fresh media or media supplemented with Parp Inhibitor VIII (PJ34; Calbiochem, #528150) at a final concentration of 2 µM. After 30 minutes, cells were lysed immediately (0 time point) or media was replaced with MMS at a final concentration of 1.25 mM (diluted in media) and incubated for 15 or 30 minutes, as indicated in the figure legends. Extracts were prepared by washing the cells with PBS and preparing cell extract with 400 µl of 2× Laemmli Buffer. 20 µl of the cell extract was analyzed by immunoblot with a 1,000-fold dilution of an anti-PAR primary antibody (BD Pharmingen, #551813) followed by a 5,000-fold dilution of the horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit Ab (Pierce; cat#1858415).

2.7. Cell cytotoxicity assays

MMS induced cytotoxicity was determined by growth inhibition assays, essentially as we have described previously [32]. Briefly, cells were seeded in 96-well dishes at 1,200 cells per well in two sets. After 24 hours one set was pre-treated with the Parp inhibitor PJ34 at a final concentration of 2 µM. After 30 minutes of media or PJ34 pre-treatment, cells were treated with a range of concentrations of MMS for one hour (+/- PJ34, 2 µM). Drug-containing medium was replaced with fresh media or media containing PJ34 (2 µM) and the plates were incubated at 37°C for 48 hours at which point the total cell number was determined by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (MTS; Promega, Madison, WI) [36]. Metabolically active cells were quantified by the bio-reduction of the MTS tetrazolium compound by recording absorbance at 490nm using a microplate reader. Results were calculated from the average of four separate experiments and are reported as the % of treated cells relative to the cells in control wells (% Control).

3. Results

3.1. Increased Parp1 activation following MMS exposure in the absence of Pol β

These studies were designed to address our hypothesis that Parp1 is the signaling protein that triggers cell death in MEFs when cells are deficient in Pol β and the BER intermediate 5'dRP is not repaired. We utilized a series of SV40 T-Antigen transformed WT and Pol β KO MEF cell lines. As depicted in Fig. 1, each of the cell lines have similar expression levels of the BER

proteins Parp1, Xrcc1 and Ape1. Our human MPG antibody [8] does not cross-react with mouse Mpg/Aag and repeated attempts to validate antibodies specific for mouse Mpg were unsuccessful. However, as we have reported [32], Mpg glycosylase activity of both MEF WT and MEF *Pol β* knockout (KO) cell lines is low but uniform and suggests that MEFs generally have a low, yet sufficient level of expression of Mpg to initiate repair of the MMS-induced lesions, in-line with a previous report from one of us [18]. Further, these studies confirm that the WT cells all express similar levels of *Pol β* protein (lanes 1,3,5 & 7) and as expected, no *Pol β* protein is detected in the *Pol β* KO cell lines (lanes 2,4,6 & 8). All of the cell lines express *Pol ι* except for the *Pol β/Pol ι* double KO cell line 19tsA, as we and others have reported [37,38].

Parp1 is part of the BER machinery [37] and can be found in a complex with other BER proteins, as was shown previously by two different experimental methodologies [26,28]. Using a similar approach, we confirmed that Parp1, as well as the BER proteins Ape1, *Pol β*, MPG and Xrcc1 can bind to a BER substrate by incubating a hairpin oligonucleotide containing a single MPG substrate (Etheno-2'-deoxyAdenosine) with whole cell extracts prepared from MEFs expressing human MPG [32]. Proteins bound to the substrate were cross-linked by incubation with formaldehyde (see Supplementary data and methods, Appendix A) and purified from unbound proteins by affinity purification via the biotin moiety linked to the 3' end of the DNA hairpin oligonucleotide, essentially as described [26]. Purified proteins were released from the DNA by heating to reverse the formaldehyde-induced crosslinks, thereby separating the protein complexes. The purified BER proteins were then analyzed by immunoblot, as shown in Fig. S1. This is similar to that reported by Dianov and colleagues [26] and importantly, demonstrates that Parp1 binds to a BER substrate, as do the other BER proteins MPG, Ape1, Xrcc1 and *Pol β*.

Parp1 binding to a BER substrate supports the current BER model in which Parp1 is recruited to the lesion site to facilitate BER complex formation, including the recruitment of Xrcc1/DNA ligase III and *Pol β* to complete repair [1,26,40]. Once bound to the lesion site, Parp1 undergoes rapid, low-level auto-modification (ADP-ribosylation). This self-imposed post-translational modification helps facilitate recruitment of Xrcc1 to complete repair [38]. We therefore reasoned that inability to complete repair due to the absence of *Pol β* would perpetuate the DNA strand-break induced activation of Parp1, preferentially in the *Pol β* KO cells. Activation of Parp1 in cells can be monitored by the presence of PAR in whole cell lysates, probing for PAR synthesis and accumulation by immunoblot analysis. In support of this hypothesis, DNA repair proficient cells (WT) show little or no evidence of Parp1 activation following challenge to 1.25 mM MMS (Fig. 2A-D, left panel). Note however that over-exposure of these immunoblots reveals a very low level of Parp1 activation in WT cells (Fig. 4C and data not shown). However, cells deficient in *Pol β* expression show a rapid and robust activation of Parp1 within 15 minutes after exposure of the cells to the same low dose of MMS (1.25 mM). Further, an even greater level of Parp1 activation is observed 30 minutes after MMS treatment (Fig. 2A-D, left panel). In 3 of the 4 *Pol β* KO cell lines, all of the observed Parp1 activation can be blocked by a 30 minute pre-treatment with the Parp1/Parp2 inhibitor PJ34 (2 μM) (Fig. 2A-C, right panel). In the fourth *Pol β* KO cell line (19tsA), a majority of the Parp1 activation can be blocked by pre-incubation with PJ34 (Fig. 2D, right panel). These data show very clearly that failed BER due to loss of *Pol β* leads to rapid and robust Parp1 activation following DNA damage induction mediated by MMS exposure.

3.2. Inhibition of Parp1 in *Pol β* null MEFs provides partial resistance to MMS

The hallmark phenotype of *Pol β* KO MEFs is an increase in cytotoxicity following exposure to alkylating agents such as MMS, MNNG and temozolomide [2]. We therefore evaluated the four matched WT and *Pol β* KO cell pairs for sensitivity to the alkylating agent MMS, as this

has been the most commonly used alkylating agent for the characterization of *Pol β* KO cells [2]. Based on our observation that MMS induces an elevated level of Parp1 activation in *Pol β* KO MEFs (as compared to WT MEFs) and that Parp1 inhibition can protect MEFs, cortical neurons, PC12, SH-SY5Y and HeLa cells from DNA damage-induced cell death [31,39-42], we reasoned that a potent Parp inhibitor may provide some level of phenotypic rescue, leading to an increase in the survival of *Pol β* KO cells exposed to MMS. Parp1 and Parp2 were inhibited by either pre-treating cells (30 min) with PJ34 before addition of MMS or treating cells simultaneously with PJ34 and MMS. PJ34 was used at a concentration (2 μM) sufficient to prevent MMS-induced Parp-activation in the *Pol β* KO cells (Fig. 2). After an hour of MMS exposure, cells were re-fed normal growth media (+/- PJ34, 2 μM), grown for 48 hours and surviving cells were measured by an MTS assay (Fig. 3). In line with reports that Parp inhibition can potentiate the cell killing effect of many DNA damaging agents, including alkylating agents [38,43-45], blocking Parp activation in the WT MEFs increased the cell killing effect of MMS at most doses (Fig. 3). This potentiation effect was observed for both the pre-treatment and simultaneous treatment regimen. However, the MMS-induced cytotoxicity of *Pol β* KO cells was not increased by Parp inhibition. Conversely the MMS-induced cytotoxicity of *Pol β* KO cells was reduced when Parp was inhibited. Two of the *Pol β* KO MEF cell lines (88TAG; Fig. 3A,E & 50TAG; Fig. 3B,F) showed a marked improvement in cell survival when Parp was inhibited and the cells were challenged with MMS, as evidenced by as much as a 10-fold increase in survival for the +PJ34 treated *Pol β* KO cells at the highest doses of MMS. In some cases, the survival curve of the *Pol β* KO cells + PJ34 was similar to the WT cells treated with PJ34 (Fig. 3A & F). One *Pol β* KO cell line (38Δ4) showed an intermediate or variable response to the Parp inhibitor. Whereas simultaneous treatment of the 38Δ4 cells with PJ34 and MMS restored survival equal to that of the WT (36.3) cells (MMS+PJ34, Fig. 3G), pre-treatment with PJ34 provided rescue at only the highest doses of MMS (Fig. 3C). Finally, the *Pol β/Pol ι* double KO cell line was only mildly rescued by Parp inhibition. This was not entirely unexpected, as PJ34 treatment was found to only prevent some (a majority) of the MMS-induced Parp activation (Fig. 2D, right panel). The involvement of Parp1 in the hypersensitive phenotype of *Pol β* KO MEFs is consistent with earlier reports that un-repaired 5'dRP lesions trigger MEF [7,32] and human breast cancer [8] cell death via a mechanism that does not involve apoptosis and does not induce the formation of autophagosomes [8], suggesting a necrotic form of cell death [31].

3.3. *Pol β* down-regulation in WT MEFs leads to elevated Parp activation and Parp-dependent cell death following MMS exposure

The general trend observed in *Pol β* KO cells is that MMS treatment leads to hyper-activation of Parp1 that contributes to the elevated level of cell death in *Pol β* KO cells as compared to WT cells (Fig. 2 & 3). As with many studies using MEF cells isolated from gene-targeted KO mice, direct comparison is made between a *Pol β* KO cell line and a WT cell line, each derived from embryos of the same litter to minimize genetic variation (i.e., an isogenic cell line pair). However, this may not guarantee a true isogenic system. For example, the WT cell line 16tsA is WT for *Pol ι* while the littermate-derived *Pol β* KO cell line 19tsA is null for *Pol ι* [37,38]. To avoid any suggestion of genetic variation that may complicate interpretation of the role of Parp1 in the hypersensitive phenotype of *Pol β* KO MEFs, we developed a lentiviral system for expression of mouse *Pol β* specific shRNA, based on the target sequence for our retroviral shRNA system reported earlier [32]. We transduced two different WT MEF cells: 92TAG and HCC384. Stable cell lines expressing the mouse *Pol β* specific shRNA are devoid of detectable *Pol β* expression, as determined by immunoblot analysis (Fig. 4A, B). Expression of the BER proteins Ape1, Xrcc1 and Parp1 are similar when comparing the 92TAG and HCC384 cells and the corresponding lentiviral-transduced cells that show a *Pol β* KD phenotype (Fig. 4A, B). Similar to the results discussed above for the *Pol β* KO cells, the *Pol β* KD cells have a highly elevated level of Parp1 activation following MMS exposure (Fig. 4C, D) whereas the

92TAg and HCC384 WT cells show little or no Parp1 activation following MMS treatment. The Pol β KD cells are functionally similar to the gene targeted KO cells. Both the 92TAg/Pol β KD and HCC384/Pol β KD cells are hypersensitive to MMS as compared to the corresponding WT cell line (Fig. 4E, F). This is consistent with our previous results and others that strong knockdown of Pol β in MEFs leads to hypersensitivity to alkylating agents [32, 46]. Most importantly, this isogenic system clearly demonstrates the role of Parp1 activation in the hypersensitivity of Pol β deficient MEFs. For both the 92TAg/Pol β KD and HCC384/Pol β KD cells, Parp1 inhibition by pre-treatment with the Parp1/Parp2 inhibitor PJ34 significantly enhances survival following MMS exposure (Fig. 4E, F). Interestingly, there is some slight variation in response even when comparing the 92TAg cells (Fig. 4E) and the HCC384 cells (Fig. 4F), supporting the significance of developing a truly isogenic system using shRNA-mediated gene expression knockdown. For example, the 92TAg cells were slightly sensitized to MMS when Parp1 was inhibited whereas the viability of the HCC384 cells was not altered by Parp1 inhibition (Fig. 4E, F). In both cases however, Parp1 inhibition provides a significant survival advantage for the corresponding Pol β KD cells (92TAg/Pol β KD and HCC384/Pol β KD), supporting our hypothesis that Parp1 activation is involved in the hypersensitivity of Pol β deficient MEFs to alkylating agents.

3.4. Complementation of Pol β KO MEFs and Pol β /Parp1 double KO MEFs with mouse Pol β – impact of Parp1 expression on MMS sensitivity in the absence of Pol β

The use of small molecule Parp inhibitors such as PJ34 to inhibit Parp1 and Parp2 activation provides significant support for our hypothesis that Parp1 activation promotes the hypersensitivity of Pol β deficient MEFs to alkylating agents. However, there are 18 Parp family members that are involved in many biological processes [47]. Although only Parp1 and Parp2 have been implicated in BER and in response to DNA damage [37], potential off-target effects of inhibitors such as PJ34 (and others) is always a distinct possibility. Therefore, we reasoned that if Parp1 was the primary Parp-family member that regulates the cellular response to failed repair of the BER intermediate 5'dRP, then we should take advantage of the previously developed Pol β /Parp1 double KO MEF cells [51]. To directly compare the cellular response of the Pol β KO and Pol β /Parp1 double KO MEFs to those that express Pol β , we cloned the mouse Pol β cDNA and developed stable cell lines that express either GFP (as a control) or mouse Pol β . An immunoblot analysis of the Pol β KO and Pol β /Parp1 double KO cells as well as the corresponding GFP or mouse Pol β expressing stable cell lines are shown (Fig. 5A, B). Pol β is only expressed in the transfected, stable cell lines (shown in lanes 4&5) and Parp1 is only expressed in the Pol β KO cells, with no Parp1 expression observed in the Pol β /Parp1 double KO cells (Fig. 5A, B). Comparable to the results shown above, the Pol β KO and Pol β KO/GFP cells show an MMS-induced hyper-activation of Parp1, as evidenced by the elevated level of PAR formation 15 minutes following MMS treatment of the Pol β KO and Pol β KO/GFP cells (Fig. 5C, group 1 & 2). Further, analogous to that shown above when comparing WT and Pol β KO cell lines (Fig. 2), ectopic expression of Pol β in the Pol β KO cell line suppresses MMS-induced Parp1 activation (Fig. 5C, group 3). Analysis of the Pol β /Parp1 double KO cells and the derived GFP and mouse pol β expressing clones show no PAR synthesis following MMS exposure (Fig. 5D), suggesting that most if not all of the PAR synthesized following MMS exposure of Pol β KO cells (Fig. 5C) is mediated by Parp1.

We next extended our functional comparison of the Pol β KO and Pol β /Parp1 double KO cells, as well as, the corresponding GFP or mouse Pol β expressing stable cell lines by exposing each to MMS (1.5 mM) and measuring survival, as described in the Methods section. Both the 88TAg cells and the derived GFP expressing cells (88TAg/GFP) were highly sensitive to MMS whereas the 88TAg cells expressing recombinant mouse Pol β (88TAg/mPol β) showed marked resistance, similar to other genetically WT cells such as 92TAg (Fig. 5E). Similar to the WT cells described above (Fig. 4E), the 88TAg/mPol β cells were slightly sensitized to MMS by

Parp1 inhibition (Fig. 5E). However, Parp1 inhibition provided a significant survival advantage for the 88TA_g and GFP-expressing cells (Fig. 5E). On the contrary, there is little or no difference in survival following MMS exposure when comparing GFP-expressing and mouse Pol β expressing *Pol β /Parp1* double KO cells (*Pol β /Parp1* KO/GFP vs. *Pol β /Parp1* KO/mPol β) (Fig. 5F). In support of the overall hypothesis put forth herein, the MMS-induced hypersensitive phenotype of *Pol β* KO cells (as compared to Pol β expressing cells) requires or is exacerbated by Parp1 activation (Fig. 5).

4. Discussion

Alkylators such as MMS induce a spectrum of DNA base lesions [48] as well as single-strand and double-strand DNA breaks. Failure to repair these lesions or DNA breaks triggers cell death via apoptosis, autophagy and necrosis. The role of Pol β in repair of MMS-induced lesions is restricted to base lesions removed by the methylation-specific DNA glycosylase MPG (AAG) [18]. Once repair is initiated, Pol β is essential for repair gap tailoring to remove the 5' dRP BER intermediate. Although it is clear that the un-repaired 5' dRP BER intermediate is the cytotoxic lesion that renders *Pol β* KO MEFs hypersensitive to alkylating agents [7], the specific cell death mechanism that is triggered by failure to repair the 5' dRP BER intermediate (BER failure) following alkylation damage has not been adequately resolved.

This study tested the hypothesis that Parp1 is a sensor of "BER failure" and that a major function of Parp1 within the context of the BER pathway is to monitor the status of BER and initiate cell death if the Pol β -dependent repair process fails (i.e., BER failure). To test this, we utilized a series of WT and *Pol β* KO MEF cell lines (Fig. 1). Each littermate cell line pair contains a Pol β WT and Pol β KO cell line and each expresses Parp1. Treatment of WT and *Pol β* KO MEFs with MMS induces a rapid and robust activation of Parp1 in Pol β KO cells, with little or no measurable activation in WT cells (Fig. 2). As an alternative, we developed a lentiviral system to down-regulate or KD Pol β in MEFs. This lentiviral-based mouse Pol β specific shRNA expression system leads to a complete loss of Pol β expression in MEFs (Fig. 4) and in preliminary studies, it appears that KD in mice is sufficient to induce post-natal lethality (not shown), similar to that observed in gene-targeted KO mice [49]. As with all four *Pol β* KO cell lines evaluated, exposure of Pol β KD cells to MMS initiates rapid Parp1 activation, with little or no MMS-induced Parp1 activation in the parental WT cells (Fig. 4). Data presented from these studies of *Pol β* KO and KD cells therefore supports our hypothesis that Parp1 provides a cellular signal of failed BER via PAR synthesis.

Alkylation damage can induce cell death via a mismatch repair dependent process [50,51], activation of caspases [52] or calpains [53], induction of autophagy [54] or the generation of DNA double strand breaks and H2AX recruitment [55]. In a multitude of studies of alkylation-induced cell death in MEFs, Parp1 activation and the onset of necrosis is the primary means of cell death [45,47,52,56-58]. Importantly, the specific molecular activator of Parp1 due to alkylation damage has not been clearly defined. Parp1 is activated by breaks to ssDNA and dsDNA [47,58], by Erk1/2-mediated phosphorylation [59] or by a direct interaction with ERK2, the latter independent of DNA damage [60]. In DNA repair proficient cells, the expectation is that alkylation-induced Parp1 activation is via DNA breaks [47,58], but it is not clear if activation is mediated by failed BER or via replication-induced specific ssDNA breaks, dsDNA breaks or a spectrum of both. It is also possible that cell death arises from mitotic catastrophe [61]. To address the connection between Pol β status, Parp1 activation and cell death, we utilized Parp1/Parp2 inhibitors [31,39,62] to prevent Parp1 activation resulting from BER failure. Since we found that a deficiency in Pol β caused DNA damage-induced Parp1 activation, we hypothesized that blocking Parp1 activation may provide some level of phenotypic rescue in the *Pol β* KO and KD cells. In support of this hypothesis, we find that the alkylation-sensitive phenotype of all four *Pol β* KO cells used in this study was rescued, either

completely or partially, by Parp1 inhibition (Fig. 3). An even stronger phenotypic rescue is observed in two independently derived Pol β KD MEF cell lines (Fig. 4). The phenotypic (cell death) rescue observed herein is similar to that observed in neurons [63,64], cardiac myocytes [65-69] and ischemia-reperfusion stroke models [63,64] whereby cell death is Parp1 dependent and inhibition of Parp1 activation prevents stress-induced cell death. Additional studies will be required to determine if this resistant phenotype is long-lived or if the resulting un-repaired BER intermediates lead to the formation of DNA double-strand breaks and the onset of apoptosis after several rounds of replication [70].

Complicating the interpretation of the DNA-damage induced survival curves of the Pol β KO and Pol β KD cell lines (Fig. 3, 4) is the well-documented potentiation of DNA damaging agents by Parp1 inhibition [43]. It is hypothesized that blocking Parp1 activation prevents Parp1-regulated DNA repair processes, increasing the cell killing and anti-tumor effect of many DNA damaging agents, including alkylating agents, cisplatin, radiation and topoisomerase-inhibitors [71,72]. Many of these earlier studies utilized DNA repair proficient MEFs and exposure to extremely high doses of DNA damaging agent. It is likely that Parp1 inhibition shifts the cell death mechanism from necrosis to apoptosis [45]. In the context of BER-mediated repair of alkylated bases, blocking Parp1 should therefore prevent BER complex recruitment [73,74], leading to accumulation of cytotoxic replication-induced DSBs [75]. In-line with the potentiating effect of Parp1-inhibition, we do observe an increase in MMS-induced cell killing in WT cells when Parp1 is inhibited (Fig. 3,4). However, Parp1 inhibition provides a survival advantage to Pol β KO cells, equal to that of the Parp1 inhibited WT cells for most doses of MMS (Fig. 3,4). This led us to speculate that the alkylation-damage mediated hypersensitive phenotype of Pol β KO (and KD) cells requires Parp1 activation. Although we observe a phenotypic rescue by Parp1 inhibition, we recognize that the ‘damage’ from a combination of alkylation treatment and BER failure will undoubtedly lead to an accumulation of DSBs and enhanced apoptotic cell death [43,45].

To directly test the role of Parp1 in the alkylation-damage mediated hypersensitive phenotype of Pol β deficient cells, we compared the alkylation response of Pol β KO cells and Pol β /Parp1 double KO cells. In support of our hypothesis that Parp1 activation is the result of BER failure (i.e., Pol β deficiency), ectopic expression of mouse Pol β in Pol β KO MEFs restored MMS resistance and attenuated MMS-induced Parp1 activation (Fig. 5). The Pol β /Parp1 double KO cells did not show any MMS-induced PAR synthesis, implicating Parp1 as the major DNA damage response Parp-family member, as expected [47]. In support of our hypothesis that Parp1 is required for the Pol β hypersensitive phenotype, there was no MMS-induced cytotoxic difference when comparing Pol β /Parp1 double KO cells expressing GFP and those that express mouse Pol β (Fig. 5).

In summary, we propose that Pol β KO MEFs are hypersensitive to alkylating agents due to hyper-activation of Parp1. Once repair is initiated and the DNA backbone is hydrolyzed, Parp1 is engaged to recruit the necessary proteins required for the completion of repair [1], including Xrcc1 and Pol β [73,74]. However, we show that BER failure (due to Pol β deficiency) leads to a rapid, elevated level of Parp1 activation, implicating Parp1-mediated necrosis as the cause of the Pol β hypersensitive phenotype [8,10]. This work shows one way by which defective BER can lead to cell death (via Parp1 activation), but there may be other ways as well. These studies are in-line with our recent report that Pol β deficiency in human cells gives rise to cellular sensitivity to the chemotherapeutic alkylating agent temozolomide by a mechanism that does not involve apoptosis and does not lead to the formation of autophagosomes [8]. Together with the Parp1 activation described here, it is our expectation that cell death due to failed BER is mediated by Parp1 activation. Continuing studies will explore how Parp1 and related PAR- and NAD⁺-metabolic enzymes impact BER and in turn, affect DNA damage or

chemotherapeutic response in BER-proficient as compared to BER-deficient or BER-inhibited cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A

Supplementary Data

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Fig. 1. WT and Pol β KO MEFs used in this study

Expression of the DNA repair proteins Pol β , Ape1, Xrcc1, Parp1 and PCNA in MEFs as determined by immunoblot analysis of nuclear proteins isolated from the following MEF cells: 92TAG (lane 1), 88TAG (lane 2), 53TAG (lane 3), 50TAG (lane 4), 36.3 (lane 5), 38 Δ 4 (lane 6), 16tsa (lane 7) and 19tsa (lane 8).

Fig. 2. Preferential Parp1 activation in *Pol β* KO cells following MMS exposure

Parp1 activation was determined in MEF cell extract by immunoblot analysis, measuring the synthesis of PAR in the absence (left panel) or in the presence of PJ34 (2 μM, right panel), after exposure with MMS (1.25 mM) for 0, 15 or 30 minutes in [92TAg (WT) and 88TAg (*Pol β* KO); **Panel-A**], [53TAg (WT) and 50TAg (*Pol β* KO); **Panel-B**], [36.3 (WT) and 38Δ4 (*Pol β* KO); **Panel-C**] and [16tsa (WT) and 19tsA (*Pol β/Pol ι* double KO); **Panel-D**] MEFs. Parp1 and PCNA expression was determined by immunoblot. PCNA expression is shown as a loading control.

Fig. 3. Chemical inhibition of Parp1 sensitizes WT MEFs yet provides partial resistance to MMS in *Pol β* KO MEFs

Wild type (92TAg, 53TAg, 36.3 and 16tsa; open squares), *Pol β* KO (88TAg, 50TAg and 38Δ4; open circles) and *Pol β/Pol ι* double KO (19tsa; open circles) MEFs were seeded in 96-well plates in two sets. After 24 hours, cells were pre-treated with media (open squares or open circles) or PJ34 (filled squares or filled circles) at a final concentration of 2 μM. After 30 minutes of media or PJ34 pre-treatment, cells were treated with a range of concentrations of MMS for one hour (A, B, C and D). A second set was treated with media (open squares or open circles) or media supplemented with PJ34 (2 μM) and MMS (0.25-2.0 mM) simultaneously (filled squares or filled circles) for one hour (E, F, G and H). Drug-containing

medium was replaced with fresh media or media containing PJ34 and the plates were incubated at 37°C for 48 hours and viable cells were determined using a modified MTT assay. Plots show the % viable cells as compared to untreated (control) cells. Means are calculated from quadruplicate values in each experiment. Results indicate the mean $\pm$ S.E. of four independent experiments.

Fig. 4. Mouse Pol β knockdown causes robust Parp1 activation and Parp1 dependent cell death following MMS exposure in MEFs

(A & B) BER protein expression as determined by immunoblot analysis of nuclear proteins isolated from the 92TA cells or 92TA cells transduced with a mouse Pol β shRNA lentiviral vector (A) or the HCC384 cells or HCC384 cells transduced with a mouse Pol β shRNA lentiviral vector (B). (A) Proteins isolated from the shRNA expressing clone (92TA/Polβ-KD (3); lane 2) as compared to proteins isolated from control cells (92TA; lane 1) are shown in (A) and from the shRNA expressing clone (HCC384/Polβ-KD(1); lane 2) as compared to proteins isolated from control cells (HCC384; lane 1) are shown in (B). Pol β, Ape1, Xrcc1 and Parp1 expression was determined by immunoblot. PCNA expression is shown as a loading

control. **(C & D)** Parp1 activation was determined in cell extracts by immunoblot analysis, measuring the synthesis of PAR after exposure with MMS (1.25 mM) for 0, 15 or 30 minutes in (C) 92TAg cells [WT] or 92TAg cells transduced with a mouse Pol β shRNA lentiviral vector, clone 3 [Pol β KD] or (D) HCC384 cells [WT] or HCC384 cells transduced with a mouse Pol β shRNA lentiviral vector, clone 1 [Pol β KD]. PCNA expression is shown as a loading control. **(E & F)** MMS-induced cytotoxicity following PJ34 pre-treatment was evaluated by culturing (E) 92TAg cells (open squares) or 92TAg cells transduced with a mouse Pol β shRNA lentiviral vector (92TAg/Pol β -KD(3); open circles) or (F) HCC384 cells (open squares) or HCC384 cells transduced with a mouse Pol β shRNA lentiviral vector (HCC384/Pol β -KD(1); open circles), in 96-well plates for 24 hours prior to exposure with media (open squares or open circles) or PJ34 (filled squares or filled circles; 2 μ M) for 30 minutes. After PJ34 pre-treatment, cells were then treated with MMS and viable cells were determined using a modified MTT assay as described in Fig. 3.

Fig. 5. Impact of Parp1 expression on MMS sensitivity in the absence of Pol β

(A & B) BER protein expression as determined by immunoblot analysis of nuclear proteins isolated from the (A) 88Tag cells or 88Tag cells complemented with mouse Pol β or (B) Pol β /PARP-1 double KO cells or Pol β /PARP-1 double KO cells complemented with mouse Pol β . Proteins isolated from two separate mouse Pol β (pIRES-Puro-mBeta) expressing clones are shown: (A) [88Tag/mPol β (27) and 88Tag/mPol β (28); lanes 4 and 5] as compared to proteins isolated from control cell (88Tag; lane 1) or pIRES-Puro-EGFP transfected in 88Tag cell [88Tag/EGFP (1) and 88Tag/EGFP (2); lanes 2 and 3]. (B): [Pol β /PARP-1 double KO/mPol β (6) and Pol β /PARP-1 double KO/mPol β (7); lanes 4 and 5] as compared to proteins isolated from control cells (Pol β /PARP-1 double KO cell; lane 1) or pIRES-Puro-EGFP transfected in

Pol β /*Parp1* double KO cells [Pol β /*Parp1* double KO/EGFP (1) and Pol β /*Parp1* double KO/EGFP (2); lanes 2 and 3]. Pol β , Ape1, Xrcc1 and Parp1 expression was determined by immunoblot. PCNA expression is shown as a loading control. **(C & D)** Parp1 activation was determined in cell extract by immunoblot analysis, measuring the synthesis of PAR after exposure with MMS (1.25 mM) for 0 or 15 minutes in (C) 88Tag cells (group 1) or 88Tag cell transfected with pIRES-Puro-EGFP (group 2) or 88Tag cells transfected with pIRES-Puro-mBeta (group 3) or (D) Pol β /*Parp1* double KO cells (group 1) or Pol β /*Parp1* double KO cells transfected with pIRES-Puro-EGFP (group 2) or Pol β /*Parp1* double KO cells transfected with pIRES-Puro-mBeta (group 3). PCNA expression is shown as a loading control. **(E)** MMS-induced cytotoxicity following PJ34 pre-treatment was evaluated by culturing 88Tag cells [88Tag], 88Tag cells transfected with pIRES-Puro-EGFP [88Tag/GFP] or 88Tag cells complemented with mouse Pol β [88Tag/mPol β], in 96-well plates for 24 hours prior to exposure with media [open bars] or PJ34 (2 μ M) [filled bars] for 30 minutes. After PJ34 pre-treatment, cells were then treated with MMS (1.5 mM) and viable cells were determined using a modified MTT assay as described in Fig. 3. **(F)** MMS-induced cytotoxicity following PJ34 pre-treatment was evaluated by culturing Pol β /*Parp1* double KO cells transfected with pIRES-Puro-EGFP or Pol β /*Parp1* double KO cell complemented with mouse Pol β , in 96-well plates for 24 hours prior to exposure with media [open bars] or PJ34 (2 μ M) [filled bars] for 30 minutes. After PJ34 pre-treatment, cells were then treated with MMS (1.5 mM) and viable cells were determined using a modified MTT assay as described in Fig. 3.