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Parg deficiency confers radio-sensitization through enhanced cell death in mouse ES cells exposed to various forms of ionizing radiation

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ABSTRACT

Poly(ADP-ribose) glycohydrolase (Parg) is the main enzyme involved in poly(ADP-ribose) degradation. Here, the effects of *Parg* deficiency on sensitivity to low and high linear-energy-transfer (LET) radiation were investigated in mouse embryonic stem (ES) cells.

Mouse *Parg*$^{-/-}$ and poly(ADP-ribose) polymerase-1 deficient (*Parp-1*$^{-/-}$) ES cells were used and responses to low and high LET radiation were assessed by clonogenic survival and biochemical and biological analysis methods.

Parg$^{-/-}$ cells were more sensitive to γ-irradiation than *Parp-1*$^{-/-}$ cells. Transient accumulation of poly(ADP-ribose) was enhanced in *Parg*$^{-/-}$ cells. Augmented levels of phosphorylated H2AX (γ-H2AX) from early phase were observed in *Parg*$^{-/-}$ ES cells. The induction level of p53 phophorylation at ser18 was similar in wild-type and *Parp-1*$^{-/-}$ cells and apoptotic cell death process was mainly observed in the both genotypes. These results suggested that the enhanced sensitivity of *Parg*$^{-/-}$ ES cells to γ-irradiation involved defective repair of DNA double strand breaks. The effects of *Parg* and *Parp-1* deficiency on the ES cell response to carbon-ion irradiation (LET13 and 70 keV/μm) and Fe-ion irradiation (200 keV/μm) were also examined. *Parg*$^{-/-}$ cells were more sensitive to LET 70 keV/μm carbon-ion irradiation than *Parp-1*$^{-/-}$ cells. Enhanced apoptotic cell death also accompanied augmented levels of γ-H2AX in a biphasic manner peaked at 1 and 24 h. The induction level of p53 phophorylation at ser18 was not different between wild-type and *Parg*$^{-/-}$ cells. The augmented level of poly(ADP-ribose) accumulation was noted after carbon-ion irradiation compared to γ-irradiation even in the wild-type cells. An enhanced poly(ADP-ribose) accumulation was further observed in *Parg*$^{-/-}$ cells. Both *Parg*$^{-/-}$ cells and *Parp-1*$^{-/-}$ cells did not show sensitization to Fe-ion irradiation.

Parg deficiency sensitizes mouse ES cells to a wide therapeutic range of LET radiation through the effects on DNA double strand break repair responses and enhanced cell death.

1. Introduction

Radiation therapy is an important component of cancer treatment and various sources of low-to-high linear-energy-transfer (LET) radiation are used in cancer therapy [1]. Chemotherapeutic agents that target the DNA damage response (DDR) are strong candidates for radio-sensitizers. For example, a Chk1 inhibitor is currently in clinical trials [2]. An hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin [3], which targets homologous recombination repair, is also in preclinical and clinical testing. PolyADP-ribosylation catalyzed by poly(ADP-ribose) polymerase (Parp) is a critical step in the repair of DNA single and double strand breaks (SSBs and DSBs) [4]. Parp inhibitors have been shown to enhance the cell lethality by γ-irradiation [5].

Poly(ADP-ribose) (PAR) degradation is catalyzed mainly by poly(ADP-ribose) glycohydrolase (Parg) [6–8]. Parg has been implicated in DNA break repair through an interaction with Parp-1 [9].

Abbreviations: Parg, poly(ADP-ribose) glycohydrolase; LET, linear-energy-transfer; Parp-1, poly(ADP-ribose) polymerase 1; PAR, poly(ADP-ribose); γ-H2AX, phosphorylated H2AX; DDR, DNA damage response; SSB, single strand break; DSB, double strand break; PAR, poly(ADP-ribose); AIF, apoptosis-inducing factor; ES, embryonic stem; NAD, nicotinamide adenine dinucleotide; FACS, fluorescence activated cell sorting; p-H3, phosphorylated form of H3.

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Recruitment of Parg to DSB foci has been shown to be mediated by an interaction with proliferating cell nuclear antigen [10]. *Parg* deficiency is reported to induce delays in SSB and DSB repair [11] and enhances the mitotic catastrophe after γ-irradiation in cancer cells [12]. *Parg* deficient (*Parg*$^{-/-}$) mice, in which nuclear expression of full-length *Parg* is lost due to the disruption of exons 2 and 3, show enhanced sensitivity to γ-irradiation and alkylating agents [7]. *Parg* deficiency enhances cell death through PAR accumulation and induction of apoptosis inducing factor (AIF)-dependent cell death after oxidative stress [13].

To elucidate the role of Parg in the DDR after exposure to low and high LET radiation and to determine the significance of Parg functional inhibition in radiation therapy, we used mouse embryonic stem (ES) cells. Mouse ES cells are able to form tumors when grafted into immune-compromised mice. This tumorigenic potential of ES cell has been proposed to be due to the upregulation of the *E-Ras* gene [14]. ES cells have a high degree of genomic stability, which is one of the properties that enable ES cells to eventually develop into the various tissues of the body. In contrast, many human tumor cell lines possess a variety of mutations, and their responses to DNA damaging agents differ. ES cells can thus be considered to have both normal and tumor cell properties, harboring low levels of genomic alterations, allowing one to extrapolate the sensitivity profiles of normal and tumor cells in part from ES cell data. The *Parg*$^{-/-}$ ES cells, which carry a disruption of exon 1 of *Parg*, exhibit reduced PAR degradation activity to approximately 10% of wild-type levels, and are hypersensitive to the alkylating agents, cisplatin and γ-irradiation [8].

In the current study, the sensitivity of *Parg* deficient ES cells to low and high LET radiation was investigated. *Parg* deficiency resulted in the increased sensitization to both low and high LET radiations, suggesting that Parg inhibition could be a useful approach to sensitizing at least certain tumor cells possessing stem cell properties to low and high LET radiation.

2. Materials and methods

2.1. ES cell culture and clonogenic survival assay

Parg$^{-/-}$[8] and *Parp-1*$^{-/-}$[15] ES cells previously established were cultured, as described previously [8]. ES cells were irradiated and inoculated onto gelatin-coated dishes in triplicate and allowed to grow for 8 days. Colonies were counted after fixation with a 1% formalin solution and staining with a 0.02% crystal violet solution, as previously described.

2.2. γ-Irradiation and particle-ion irradiation

Cells were exposed to γ-irradiation using a ^{60}Co γ-irradiator at 0.29 Gy/s at the National Cancer Center Research Institute in Japan. Cells were irradiated with carbon-ion and Fe-ion beams generated by the HIMAC accelerator at the National Institute of Radiological Sciences in Japan. The initial energy of the carbon-ion and Fe-ion beams was 290 MeV/n and 500 MeV/n, respectively. The energy at the irradiation site was obtained by comparing the calculated and measured depth-dose distribution. Two different LET values were achieved using Lucite absorbers of varying thicknesses to change the energy of the beams. At the sample position, the LET values (dose averaged LET) for all experiments were estimated to be 13 and 70 keV/μm. The dose rate of both LET beams was approximately 1.2 Gy/min. The LET value for the Fe-ion beams was 200 keV/μm.

2.3. Measurement of nicotinamide adenine dinucleotide (NAD) and PAR levels

ES cells were extracted with 0.5 N perchloric acid and then neutralized with 3 M potassium peroxide-0.7 M glycine-glycine (pH 7.4). NAD levels in the supernatant were analyzed by HPLC on a Develosil C30-UG-5 column (ϕ46 × 250 mm, Nomura Chemicals), as described elsewhere. The precipitate containing PAR was subjected to digestion using a recombinant glutathione-*s*-transferase-fusion protein of rat Parg, and then the ADP-ribose produced from PAR was analyzed by HPLC, as described above.

2.4. Immunoblot analysis

ES cells were extracted with Laemmli's buffer and then sonicated, as previously described. Proteins were subjected to electrophoresis on an SDS–polyacrylamide gel followed by transfer to a Sequi-Blot™ PVDF membrane (Bio-Rad). Immunoblot analysis was carried out using the following primary antibodies: anti-γ-H2AX (1:1000; Millipore), anti-phospho (Ser15) p53 (1:1000, Cell Signaling), anti-p53 (pan, 1:1,000; Cell Signaling), anti-phospho-RPA32 (1:1000, Bethyl), anti-phospho histone H3 (1:1000, Abcam), anti-PARP-1 (1:1000; Upstate Biotechnology), and anti-β-actin (1:10,000, Sigma–Aldrich). The secondary antibodies were horseradish peroxidase-linked immunoglobulin. Immune complexes were detected using an enhanced chemiluminescence reaction kit (Millipore). When the samples were electrophoresed on the different gels, they were included in the same bag during incubation with antibodies, washed in a container and exposed to the same autography films.

2.5. Flow cytometry

ES cells were trypsinized and then fixed with 70% ethanol. Following treatment with RNase A, cells were stained with propidium iodide and then analyzed by fluorescence activated cell sorting (FACS) using a FACS Calibur system (Beckton and Dickinson).

2.6. DNA ladder formation assay for apoptosis

After trypsinization, cells and culture supernatants were collected; suspended in lysis buffer containing 10 mM Tris–Cl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100; and then placed on ice for 10 min. The suspension was subjected to centrifugation at 17,000 *g* for 20 min and then the supernatant was collected and incubated with RNase A/proteinase K. DNA was isolated by ethanol precipitation and analyzed by 2% agarose gel electrophoresis. The fragmented DNA obtained from the same numbers of inoculated cells was subjected to electrophoresis. We used a procedure to recover only fragmented DNA by ethanol precipitation. The fragmented DNA obtained from the same number of inoculated cells was subjected to electrophoresis. Therefore, the DNA at the origin corresponds to degraded DNA of the large size (approximately more than 100 kb) but not undegraded DNA.

2.7. Statistical analysis

Differences were assessed using the Kruskal–Wallis test (Macintosh version, JMP).

3. Results

Both *Parp-1*and *Parg* deficiency have been shown in separate experiments to result in increased lethality following γ-irradiation

in ES cells [8,16]. Here, enhanced sensitivity of *Parp-1*$^{-/-}$ and *Parg*$^{-/-}$ ES cells to γ-irradiation was directly compared. Compared to parental J1 ES cells, *Parg*$^{-/-}$ ES cells were more sensitive to γ-irradiation (Fig. 1) than *Parp-1*$^{-/-}$ ES cells. In *Parp-1*$^{-/-}$ ES cells, Parp activity is decreased to about 6% of wild-type levels [15], while in *Parg*$^{-/-}$ ES cells, PAR degradation activity is reduced to 10% of wild-type cells [8].

The mechanism of enhanced cell death by *Parg* deficiency was further investigated. In *Parg*$^{-/-}$ ES cells, there was a 2-fold increase in total PAR levels 1 h after 5 Gy irradiation (Fig. 2A). PAR levels were subsequently reduced 5 h after irradiation but remained higher than in wild-type cells. Accumulation of poly(ADP-ribosylated) protein was peaked at 1 h post-irradiation and again increased 24 h post-irradiation in *Parg*$^{-/-}$ cells (Fig. 2B). It is thus suggested that accumulation of PAR could be associated with the sensitization by *Parg* deficiency. It is also noted that in both wild-type and *Parg*$^{-/-}$ cells, there was a transient decrease in NAD 1 h after irradiation (Fig. 2A).

Because Parg is suggested to be involved in DNA repair [9], the level of phosphorylated H2AX (γ-H2AX), which is a marker for DSBs [17], was examined by immunoblot. As shown in Fig. 2B, γ-H2AX levels were higher 5 h after irradiation in *Parg*$^{-/-}$ cells as observed in *Parp-1*$^{-/-}$ cells, which suggested higher levels of residual DSBs and/or delayed repair of DSBs. Time course analysis of γ-H2AX foci formation was also carried out (Fig. 2C). *Parg*$^{-/-}$ ES cells showed higher numbers of γH2AX foci 1 h after 5 Gy irradiation. We noted that a low correlation between the levels of γ-H2AX foci number and the level of γ-H2AX detected by western blot in the time course analysis. The persistent presence of high levels γ-H2AX protein at 24 h was detected only by western blot in wild-type, *Parg*$^{-/-}$ and *Parp-1*$^{-/-}$ cells. In this study ES cells were cultured in the presence of leukemia inhibitory factor, thus the subpopulation, including differentiated cells, was only infrequently observed and we saw γH2AX distribution evenly, as reported [18].

In wild-type cells, the increase of polyADP-ribosylated proteins was not detected after irradiation, although in the *Parg*$^{-/-}$ cells we observed increase of polyADP-ribosylated proteins of larger than 250 kDa (Fig. 2B). On the other hand, as shown in Fig. 2A, we detected total PAR, includingfree and protein-attached PAR, in the wild-type cells by HPLC analysis. By the Parg activity, PAR could be easily cleaved from poly(ADP-ribosylated) proteins in wild-type ES cells and they could not have been detected by western blot using PAR antibody 10H. 10H can only detect PAR of more than 10 residues of PAR [19]. In Fig. 2A, all PAR molecules including protein-attached and free PAR molecules were quantified. This could be the reason why we were able to detect total PAR levels in Fig. 2A in wild-type cells. The prime poly(ADP-ribosylation) target after radiation may be mainly Parp-1, but may include other molecules.

Both *Parg*$^{-/-}$ and wild-type ES cells were arrested in G2/M by 10 h post-irradiation and there was a subsequent increase in the

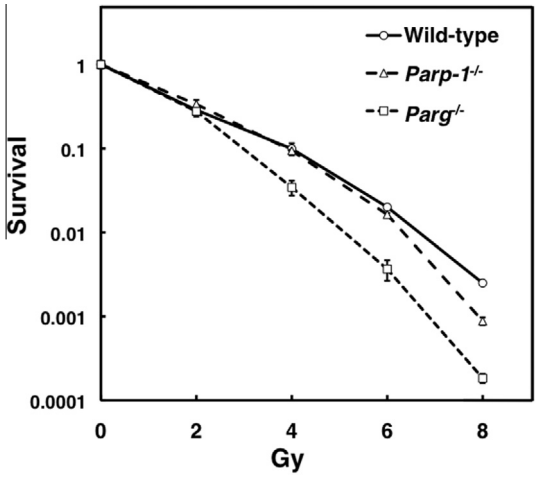

Fig. 1. Enhanced sensitivity of *Parg*$^{-/-}$ ES cells to γ-irradiation. A clonogenic survival assay was performed in triplicate as described in Section 2. The results from representative triplicate irradiation experiments are shown.

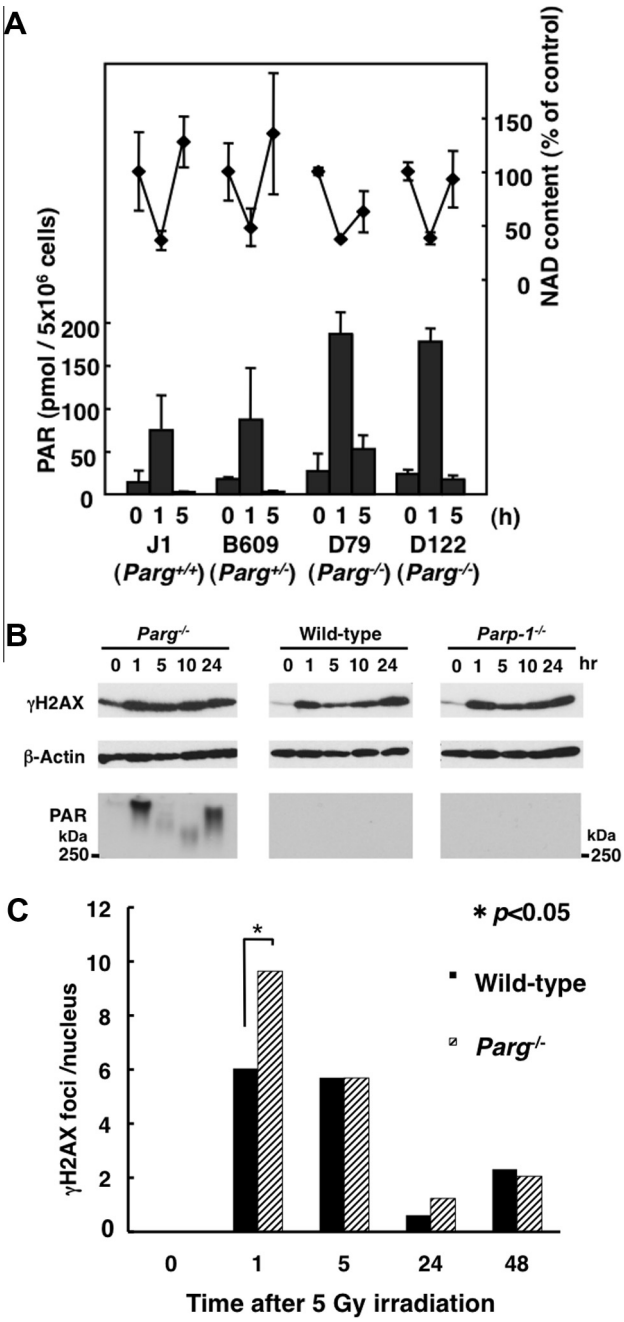

Fig. 2. DNA damage response of *Parg*$^{-/-}$ and wild-type ES cells. (A) PAR and NAD levels following exposure to 5 Gy irradiation are shown. Total PAR was extracted, digested to ADP-ribose, and then analyzed by HPLC. NAD levels in acid-soluble fractions were also analyzed by HPLC. (B) Levels of γ-H2AX, a marker of DNA DSBs, and PAR were analyzed by western blot following exposure to 5 Gy irradiation. PolyADP-ribosylated proteins of more than 250 kDa were analyzed because of the presence of non-specific bands at the lower regions. (C) Time course analysis of γ-H2AX foci formation after 5 Gy irradiation. Foci numbers per nucleus were counted. Fifty cells were counted for each sample.

apoptotic sub-G1 fraction 24 h post-γ-irradiation (Fig. 3A). Nucleosomal DNA fragmentation was similarly observed between the genotypes, which could be associated with apoptosis (Fig. 3B). The band intensities of the intact and cleaved bands of Parp-1 both became weaker at 24 h post-irradiation in *Parg*$^{-/-}$ cells compared to wild-type (Fig. 3C). This could be due to further degradation of Parp-1 caused by accelerated apoptosis under *Parg* deficiency. These results suggested the possibility of delayed enhancement of cell death in *Parg*$^{-/-}$ ES cells. In contrast, similar levels of activation of p53, as estimated by phosphorylation at Ser18 following 5 Gy irradiation, were observed in both *Parg*$^{-/-}$ and wild-type cells.

The effects of *Parg* and *Parp-1* deficiency on sensitivity to high LET radiation were then examined. Figs. 4 A-C show clonogenic survivals following carbon-ion and Fe-ion (LET 200 keV/μm) irradiation. Increased lethality after 5 Gy but not 2 Gy or lower doses of LET 70 carbon-ion irradiation was observed in *Parg*$^{-/-}$ ES cells, whereas *Parp-1*$^{-/-}$ cells did not exhibit a clear increase in sensitivity (Fig. 4A). After LET13 (LET 13 keV/μm) irradiation, the weaker sensitization effect was observed in *Parg*$^{-/-}$ cells only at 5 Gy (Fig. 4A). There was no augmentation of lethality by Fe-ion irradiation in either *Parg*$^{-/-}$ or *Parp-1*$^{-/-}$ cells (Fig. 4C).

To understand the mechanism of sensitization under *Parg* deficiency, the DDR was evaluated with immunoblot analysis (Fig. 4D). The γH2AX level was about 2-fold higher 1 and 5 h after LET13 irradiation in *Parg*$^{-/-}$ ES cells and at 24 h, 4-fold higher level was observed. With LET70 irradiation, the level of γH2AX was not different 1 h after irradiation but at 24 h, the level increased to 3-fold in *Parg*$^{-/-}$ cells. The p53 phosphorylation at ser18 was similar in *Parg*$^{-/-}$ and wild-type ES cells after LET 13 and LET 70 irradiation. On the other hand, the level of phosphorylated RPA32 was lower in *Parg*$^{-/-}$ cells after LET 13 and LET 70 irradiation. Therefore, the difference in processing of DSBs between *Parg*$^{-/-}$ and wild-type cells was also noted after exposure to carbon-ion irradiation. The decrease of phosphorylated form of H3 (p-H3), an M-phase marker, occurred in both *Parg*$^{-/-}$ and wild-type cells after LET 13 irradiation and the increase of the level was observed already at 10 h with wild-type cells but not in *Parg*$^{-/-}$ cells, although the level in wild-type and *Parg*$^{-/-}$ cells reached the same level at 24 h. With the LET70 irradiation the time profile of the decrease and increase of p-H3, which suggests M-phase arrest, was similar in both genotypes.

The augmented level of PAR accumulation was noted after carbon-ion irradiation compared to γ-irradiation even in the wild-type cells. PAR levels were further augmented in *Parg*$^{-/-}$ cells and remained higher up to 24 h after LET 13 and LET 70 irradiation (Fig. 4D). With the PAR size after carbon-ion irradiation, the smearing was distributed to larger sizes in *Parg*$^{-/-}$ than in wild-type cells, possibly due to longer residues and/or higher numbers of PAR chains for the acceptor molecules.

The effects of *Parg* deficiency on acute apoptosis after carbon-ion irradiation were also examined. Although cleavage of Parp-1

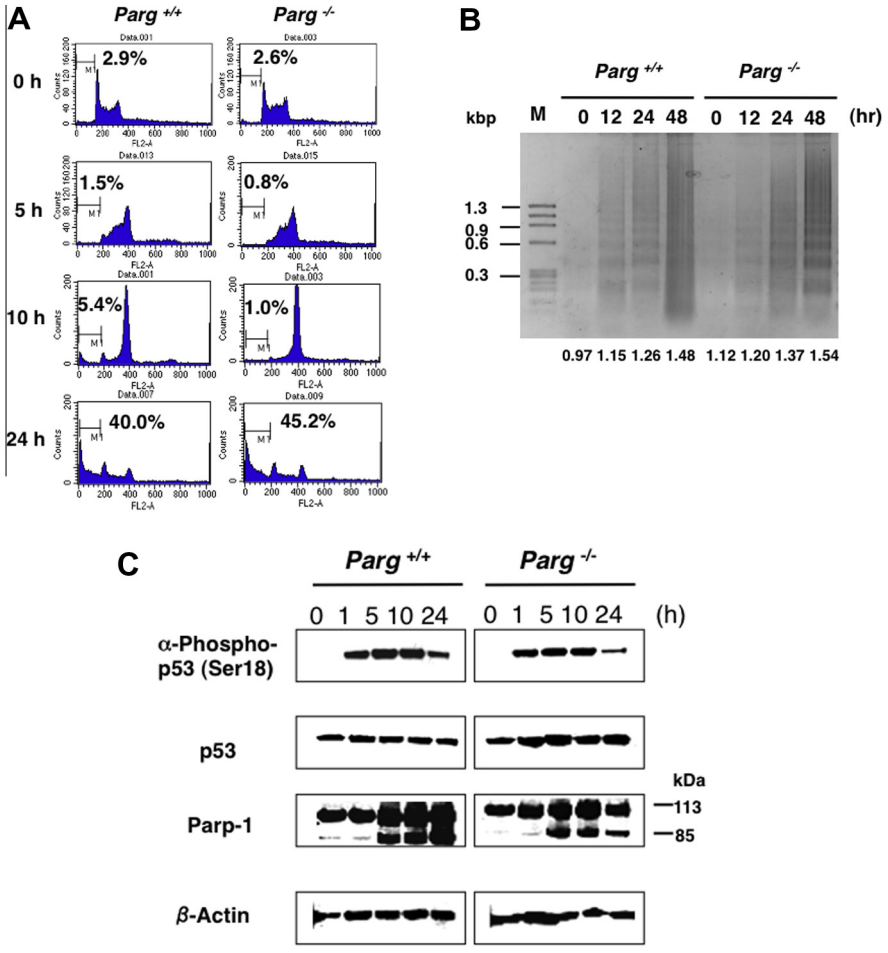

Fig. 3. Time course of cell death in *Parg*$^{-/-}$ ES cells after exposure to 5 Gy irradiation. (A) Flow cytometry was performed following propidium iodide staining and fixation. (B) Nucleosomal DNA ladder formation was assessed by agarose gel electrophoresis. The fragmented DNA obtained from the same number of inoculated cells was subjected to electrophoresis. Therefore the DNA at the origin corresponds to degraded DNA of the large size (approximately more than 100 kb) but undegraded DNA is not detected by this procedure. The values under the lanes show the quantification results of the apoptosis-derived bands larger than 100 bp. (C) Immunoblot analysis of p53 activation, using an anti-phosphorylated (ser18) p53 antibody, and Parp-1 cleavage.

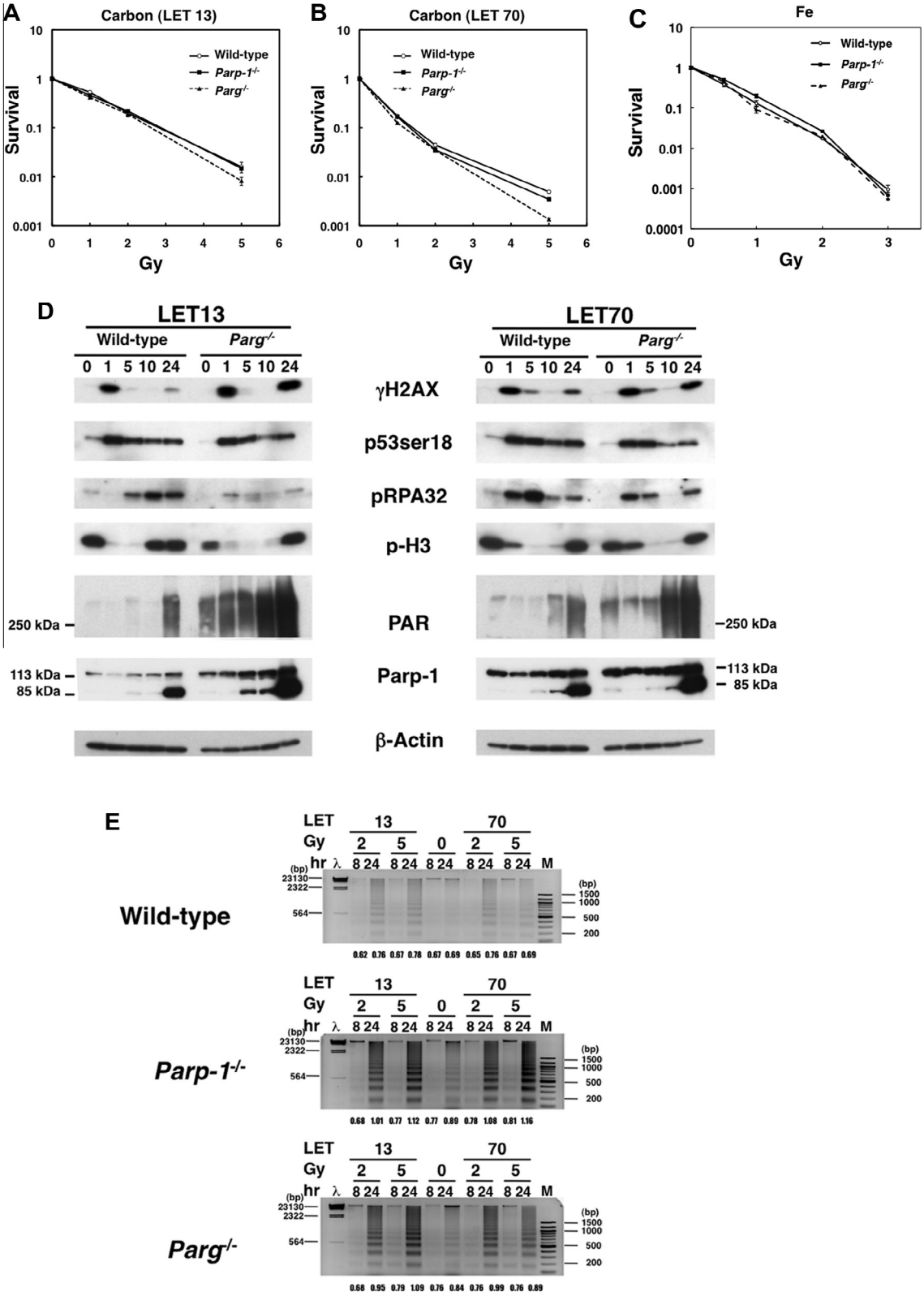

Fig. 4. Enhanced sensitivity of *Parg*$^{-/-}$ ES cells to carbon-ion irradiation. A and B. Clonogenic survival assay following exposure to LET 13 (A) and LET 70 (B) carbon-ion irradiation. (C) Clonogenic survival assay after Fe-ion irradiation. Clonogenic survival assays were carried out repeatedly and representative data are shown. (D) Levels of γ-H2AX, p53 phosphorylation at ser18, RPA32 phosphorylation, histone H3 phosphorylation, PAR, Parp-1 cleavage, and β-actin after carbon-ion irradiation at 5 Gy were analyzed by immunoblot. PolyADP-ribosylated proteins of more than 250 kDa were analyzed because of the presence of non-specific bands at the lower regions. (E) DNA fragmentation in *Parp-1*$^{-/-}$ and *Parg*$^{-/-}$ ES cells after carbon-ion irradiation was analyzed by agarose gel electrophoresis. Lane λ: λ phage DNA digested with *Hin*d III; Lane M: 100 base ladders. The values under the gels show the quantified densities of the bands larger than 100 bp after normalization to the markers of 100 base ladders (M) on each gel.

after LET 13 and LET 70 irradiation was the same level in wild-type and *Parg*$^{-/-}$ cells (Fig. 4D), *Parg*$^{-/-}$ cells exhibited an enhanced apoptotic DNA ladder formation compared to wild-type cells 24 h after LET 13 and LET 70 carbon-ion irradiation (Fig. 4E). However, there was almost no difference in the augmented level of apoptotic DNA ladder formation between *Parg*$^{-/-}$ and *Parp-1*$^{-/-}$ cells both after LET 13 and LET 70 radiation. Considering that *Parg*$^{-/-}$ but not *Parp-1*$^{-/-}$ cells showed sensitization by clonogenic survival assay after carbon-ion irradiation, this suggests that acute apoptosis might not contribute to sensitization by *Parg* deficiency during clonogenic survival. The increase of M phase marker, phosphorylated histone H3, 24 h after LET 13 and 70 carbon-ion irradiation in wild-type and *Parg*$^{-/-}$ cells suggests a possibility that mitotic catastrophe may also be involved. The above results suggest that *Parg* deficiency led to enhanced cell death pathways accompanying defective DSB processing and PAR accumulation after low and high LET radiations.

4. Discussion

Parg deficiency resulted in the increased sensitivity of mouse ES cells to γ-irradiation as well as high LET carbon-ion irradiation. *Parg* deficiency caused stronger sensitization effects compared to *Parp-1* deficiency for both γ-irradiation and carbon-ion irradiation. Under *Parp-1* deficiency, the sensitization effect was decreased with increasing LET radiation. This is understandable given that DNA repair processes are less efficient in dealing with the increase in irrepairable clustered DNA strand breaks induced at higher levels of LET radiation [20]. *Parg* deficiency might sensitize ES cells via additional mechanisms to impairment of DNA repair. *Parg* deficiency enhanced cell death substantially accompanying defective DSB processing and accumulation of PAR after carbon-ion irradiation. The basic sensitization mechanism by *Parg* deficiency seems to be common between low and high LET radiation involving the enhanced γ-H2AX response. Furthermore, we noted the accumulation of PAR transiently after γ-irradiation and over a longer period after carbon-ion irradiation. Because PAR accumulation is reported to cause apoptosis-inducing factor (AIF)-dependent cell death, there may be a possibility that AIF-induced cell death [13] is accompanied after carbon-ion irradiation. Because phosphorylated histone H3 showed a higher level in wild-type and *Parg*$^{-/-}$ ES cells after carbon-ion irradiation, there may be also a possibility of the involvement of mitotic catastrophe. The detailed mechanism of enhanced cell death after low and high LET irradiation remains the subject of further study.

In the radiation therapy with carbon-ion, only tumor tissues are exposed to high LET radiation. As we showed that a higher sensitization by *Parg* deficiency was observed at LET 70 than at LET 13 radiation, tumor tissues could be rather selectively sensitized. Inaddition, *Parg* deficiency was not associated with increased sensitization at lower doses (1–2 Gy) of LET 13 and 70 radiation. Thus, while Parg inhibition might have minimal sensitizing effects on normal cells at low doses, it may have greater sensitizing effects at doses higher than 2 Gy in target tumor tissues. Screening of tumor cells for effective sensitization by Parg inhibition is an important subsequent step in the development of appropriate strategies for therapeutic applications.

At present, our knowledge of factors that can induce sensitization to high LET radiation is limited. For example, inactivating mutations of both Bcl-2 and p53 render cells resistant to low LET radiation but have no effect on sensitivity to carbon-ion irradiation [1]. The results of the current study suggest that Parg functional inhibition may be a useful approach to study the sensitization of certain cancer cells to both low and high LET radiation.

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