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Lack of DNA Polymerase θ (POLQ) Radiosensitizes Bone Marrow Stromal Cells *In Vitro* and Increases Reticulocyte Micronuclei after Total-Body Irradiation

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Abstract

Mammalian POLQ (pol θ) is a specialized DNA polymerase with an unknown function *in vivo*. Roles have been proposed in chromosome stability, as a backup enzyme in DNA base excision repair, and in somatic hypermutation of immunoglobulin genes. The purified enzyme can bypass AP sites and thymine glycol. Mice defective in POLQ are viable and have been reported to have elevated spontaneous and radiation-induced frequencies of micronuclei in circulating red blood cells. To examine the potential roles of POLQ in hematopoiesis and in responses to oxidative stress responses, including ionizing radiation, bone marrow cultures and marrow stromal cell lines were established from $Polq^{+/+}$ and $Polq^{-/-}$ mice. Aging of bone marrow cultures was not altered, but $Polq^{-/-}$ cells were more sensitive to γ radiation than were $Polq^{+/+}$ cells. The D_0 was 1.38 ± 0.06 Gy for $Polq^{+/+}$ cells compared to 1.27 ± 0.16 and 0.98 ± 0.10 Gy ($P = 0.032$) for two $Polq^{-/-}$ clones. $Polq^{-/-}$ cells were moderately more sensitive to bleomycin than $Polq^{+/+}$ cells and were not hypersensitive to paraquat or hydrogen peroxide. ATM kinase activation appeared to be normal in γ-irradiated $Polq^{-/-}$ cells. Inhibition of ATM kinase activity increased the radiosensitivity of $Polq^{+/+}$ cells slightly but did not affect $Polq^{-/-}$ cells. $Polq^{-/-}$ mice had more spontaneous and radiation-induced micronucleated reticulocytes than *Polq*+/+ and $^{+/-}$ mice. The sensitivity of POLQ-defective bone marrow stromal cells to ionizing radiation and bleomycin and the increase in micronuclei in red blood cells support a role for this DNA polymerase in cellular tolerance of DNA damage that can lead to double-DNA breaks.

Intrdoduction

Lesions in DNA caused by endogenous sources or external agents such as ionizing radiation create lesions in DNA templates that can cause DNA replication forks to stall and collapse. Specialized enzymes termed “translesion” or “bypass” DNA polymerases can insert nucleotides opposite the lesions and provide an important strategy for cells to survive DNA

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damage. Studies in human cells currently aim to understand which specialized DNA polymerases are involved in bypass of particular lesions in specific cell types (1,2). For example, human DNA POLH (pol η inserts bases across from UV-radiation-induced cyclobutane pyrimidine dimers in DNA, and a deficiency in this enzyme causes the hereditary skin cancer-prone disorder xeroderma pigmentosum variant (*XP-V*) (3). The related enzyme DNA polymerase ι also inserts bases opposite UV-radiation-induced lesions (4,5).

DNA polymerase θ (POLQ) was first identified as an enzyme encoded in the human genome with similarity to "A-family" DNA polymerases such as *E. coli* pol I (6,7). Its sequence and domain arrangement are related to the *Drosophila melanogaster mus308* gene product. Mutant alleles of *mus308* increase the sensitivity of *Drosophila* to DNA interstrand crosslinking agents (8). However, accumulating evidence indicates that vertebrate POLQ is not a strict ortholog of POLQ and is involved in DNA damage tolerance pathways other than crosslink repair. Recombinant human DNA polymerase theta can catalyze efficient DNA synthesis opposite an AP site or a thymine glycol on synthetic templates (9). Human POLQ has low fidelity (9), with a pronounced tendency to form +1 frameshift mutations (10). These properties may be given to vertebrate POLQ by amino acid insertions in the catalytic domain of the DNA polymerase that are not present in other A-family DNA polymerases such as Mus308 (9). There is limited information on the relative sensitivity of *Polq*-deficient cells. POLQ-deficient chicken DT40 cells have some increased relative sensitivity to hydrogen peroxide but are not sensitive to cisplatin or mitomycin C (agents that can form interstrand DNA crosslinks) (11). It is possible that POLQ in DT40 cells has some role in the tolerance of reactive oxygen species damage to DNA, either in the bypass of lesions during DNA replication or in a backup role in DNA base excision repair (11). Mouse POLQ has also been suggested to participate in somatic hypermutation of immunoglobulin genes (12–15), although this role appears to be minor one if it does exist (16).

Mice with defective POLQ were first isolated in a screen for mutants exhibiting an increased frequency of micronuclei in circulating red blood cells (17). The chaos1 mutant is ascribed to a Ser to Pro amino acid change at residue 1932 of mouse POLQ. $Polq^{-/-}$ mice develop normally, but they have elevated frequencies of spontaneous and radiation-induced micronuclei, indicating some role for the enzyme in maintaining genomic integrity during the development of reticulocytes (17,18). The spontaneous viability of *Polq*-defective mice is severely compromised by an additional mutation in *Atm*, suggesting distinct roles for POLQ and ATM in mammalian embryogenesis (18). POLQ is expressed in cells, including cancer cells, and is particularly well expressed in hematopoietic tissues (19). Further study of the function of POLQ in hematopoietic cells and its relationship to radiation sensitivity is therefore of particular interest. Thus we examined how deletion of the *Polq* gene affects the ionizing radiation sensitivity of mouse bone marrow cell lines *in vitro* and the effects on hematopoiesis in mice and in long-term bone marrow cultures.

Materials and Methods

Long-Term Bone Marrow Cultures

Long-term bone marrow cultures were established from C57BL/6J ($Polq^{+/+}$) and $Polq^{-/-}$ mice as described previously (20,21). $Polq^{-/-}$ mice (17,18) were obtained from N. Shima (University of Minnesota). Mice were genotyped by PCR as $Polq^{+/+}$, $^{+/-}$ and $^{-/-}$ from DNA extracted from tail skin samples. The contents of a femur and tibia from $Polq^{-/-}$ and control C57BL/6J mice were flushed into McCoy's 5A medium (Gibco, Gaithersburg, MD) supplemented with 25% horse serum (Cambrex, Rockland ME), and 10^{-5} M hydrocortisone sodium hemisuccinate. Cultures were incubated at 33°C in 93% air/7% CO_2. For maintenance of continuous hematopoiesis, half medium changes were performed weekly.

After 4 weeks, the horse serum was replaced with 25% FBS (Gibco). The cultures were observed weekly for hematopoietic cell production and cobblestone island formation. Cobblestone islands of greater than or equal to 50 cells were scored weekly in each flask (20,21). Statistical analysis was done using a two-sided two-sample t test comparing the number of cobblestone islands from the $Polq^{+/+}$ and $^{-/-}$ cultures each week. *P* values less than 0.05 were regarded as significant.

Hematopoietic Cell Colony-Forming Assays

Each week the nonadherent cells from each of eight $Polq^{-/-}$ and eight $Polq^{+/+}$ control long-term bone marrow culture flasks were combined into two pools. A total of 1.65×10^5 nonadherent cells from the $Polq^{-/-}$ and control cultures were removed and 5×10^4 cells/dish were plated in triplicate in semi-solid medium consisting of methylcellulose in Iscove's MDM, fetal bovine serum (FBS), 10% bovine serum albumin (BSA), WEHI conditioned medium (as a source of IL-3), L-glutamine, 3 U/ml erythropoietin, and 2-mercaptoethanol. Colony-forming unit granulocyte-macrophage (CFU-GM) colonies of 50 cells or greater were counted on days 7 and 14 after plating. Statistical analysis was done using a two-sided two-sample *t* test comparing the number of colonies from the $Polq^{+/+}$ and $^{-/-}$ cultures each week. *P* values < 0.05 were regarded as significant.

Clonal Bone Marrow Stromal Cell Lines

Thirty-four-week-old $Polq^{+/+}$ and $^{-/-}$ long-term marrow culture adherent layers were expanded by passage into Dulbecco's modified Eagle's medium (DMEM) with 10% FBS to establish bone marrow stromal cell cultures. Clonal cell lines were established by expanding single cells in the same medium.

Radiation Survival Curves: Bone Marrow Stromal Cell Lines

$Polq^{+/+}$ and $^{-/-}$ cells were irradiated using a ^{137}Cs γ-ray source with doses ranging from 0 to 8 Gy and plated in Nunc four-well tissue culture dishes (Fisher Scientific, Pittsburgh, PA) at concentrations of 500, 1000 or 5000 cells/well. The small molecule KU55933 (KuDOS Pharmaceuticals), which was used to inhibit ATM kinase activity in cells, was reconstituted in DMSO and used at a concentration of 10 μ*M* (22). After irradiation, the $Polq^{+/+}$ and $^{-/-}$ cells were incubated with KU55933 for 4 h. The medium was then replaced with fresh medium. Seven days later, the cells were stained with crystal violet, and colonies of 50 cells or greater were counted. Data were analyzed using linear-quadratic and single-hit multitarget models (23).

Quantification of Paraquat, Hydrogen Peroxide and Bleomycin Toxicity

Two clonal $Polq^{+/+}$ cell lines and two $Polq^{-/-}$ cell lines were exposed to various concentrations of paraquat (0–100 m*M*), hydrogen peroxide (0–120 μ*M*) or bleomycin (0–7 μg/ml) for 1 h prior to plating in Nunc four-well tissue culture dishes at concentrations ranging from 500 to 8000 cells/well. After 7 days, the cells were stained with crystal violet, and colonies of 50 or more cells were counted. Statistical significance was determined using a standard analysis of covariance (ANCOVA) model with the surviving fraction as the dependent variable and genotype, clone, dose and dose × dose as the independent variables.

The data for the number of colonies were divided by the number of cells plated and normalized using the number of colonies and number of cells at zero dose for the same clonal line, compound and experimental group. The normalized numbers of colonies were then log transformed and the ANCOVA model was fitted to them for each compound to determine the significance of the difference between cell lines. The clonal line effect and the quadratic term of dose were included as well as their interaction terms. The model was fitted

using SAS PROC GLM, and the difference between cell lines was examined with an *F* test. For each group, the 50% killing dose was also estimated based on the fitted ANCOVA model.

Micronucleus Assay

In two separate experiments, groups of adult male and female *Polq*$^{+/+}$, $^{+/-}$ and $^{-/-}$ mice were irradiated with 75 cGy or 7 Gy in a ^{137}Cs irradiator at a dose rate of 80 cGy/min. Unirradiated control mice of each genotype were also used. Blood was collected from the tail vein from mice receiving 75 cGy 40 h after the irradiation. Blood was collected on days 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, 34 and 88 from mice receiving 7 Gy. The mice receiving 7 Gy were divided into three groups with five mice of each genotype in each group. Each group had blood drawn no more than every 10 days. Approximately 100 μl of blood was collected from each mouse into tubes containing 350 μl of heparin solution. Blood samples were then fixed in ultra-cold methanol according to the protocol in the Mouse MicroFlowBasic Kit (Litron Laboratories, Rochester, NY). The fixed samples were stored at −85°C until the flow cytometry analysis was performed. Methanol-fixed blood samples were washed and labeled with anti-CD71-FITC, anti-CD61-PE and PI for highspeed flow cytometry using CellQuest software, v5.2 (Becton Dickinson, San Jose, CA). For each sample, 2×10^4 CD71-positive reticulocytes were analyzed for the presence of micronucleated reticulocytes. Flow cytometers were calibrated by staining *Plasmodium berghei*-infected rodent blood (malaria biostandards) in parallel with test samples on each day of analysis (24–26). Statistical analysis was performed using the Student's *t* test or one-way ANOVA followed by Tukey's test.

Differential Blood Counts

An aliquot of the blood, drawn for the analysis for micronucleated reticulocytes from the *Polq*$^{+/+}$, $^{+/-}$ and $^{-/-}$ mice at each of the times, was used for differential blood cell counts. Complete blood counts were performed using an automated veterinary hematology analyzer set with a mouse software card (VetABC, Scil Veterinary Diagnostics). For red blood cells, the parameters were number per mm^3, mean corpuscular volume, hematocrit, hemoglobin, mean corpuscular hemoglobin concentration, mean hemoglobin concentration and red blood cell distribution width. Total white blood cell numbers per mm^3 and a three-part differential (lymphocytes, monocytes, granulocytes) were determined by the analyzer. Platelet counts and mean platelet volume were also obtained. Data were summarized as means ± SE and compared using the two-sided two-sample *t* test.

ATM Immunoprecipitation and Immunoblotting

Whole cell extracts were prepared 1 h after irradiation in TGN lysis buffer (50 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 50 m*M* NaF, 1% Tween-20 and 0.5% NP40) with protease inhibitors. Cleared extracts were standardized for total protein. Phosphorylated ATM was immunoprecipitated using 5 μl ATM phospho-specific (pS1981) rabbit monoclonal antibody clone EP1890Y (no. 2152-1, Epitomics, Burlingame, CA). Immunocomplexes were washed two times in TGN lysis buffer and resolved by SDS-PAGE. Total ATM levels in the immunocomplexes were determined by immunoblotting with total ATM mouse monoclonal antibody clone MAT3-4G10/8 (A1106, Sigma, St Louis, MO). Total ATM levels were also determined in whole cell extracts. While ATM phospho-specific (pS1981) rabbit monoclonal antibody clone EP1890Y selectively recognizes murine ATM phosphorylated on the equivalent serine (S1987) in solution in cell extract, this antibody does not selectively recognize denatured murine ATM phosphorylated on serine 1987 during immunoblotting.

Animal Welfare

The Institutional Animal Care and Use Committee of the University of Pittsburgh approved all protocols. Veterinary care was provided by the Department of Laboratory Animal Research of the University of Pittsburgh in strict accordance with the guidelines of the University of Pittsburgh Institutional Animal Care and Use Committee.

Results

Homozygous Disruption of Polq Elevates the Frequency of Micronucleated Reticulocytes Induced by Low- and High-Dose Radiation in Mice

In a screen for genetic determinants of spontaneous micronucleus formation in mouse red blood cells, Shima and colleagues isolated a mouse harboring a homozygous mutation termed *chaos1*. That was discovered to be a point mutation in mouse *Polq* (17). These data suggested that DNA POLQ functions in maintaining genomic integrity in hematopoietic cells, perhaps by helping cells tolerate normal stresses from reactive oxygen species. In the present study, we asked whether deletion of *Polq* affects hematopoiesis in mice or in long-term bone marrow cultures derived from them and the effect on ionizing radiation sensitivity of cells of mouse bone marrow cell lines.

We confirmed the occurrence of an increased frequency of micronucleated reticulocytes in *Polq* mice. Three-color flow cytometry was used to score micronucleated reticulocytes and total reticulocytes in peripheral blood (24). $Polq^{+/+}$, $^{+/-}$ and $^{-/-}$ mice were unirradiated or were exposed to a total-body dose of 75 cGy (to test low dose irradiation), and blood samples were collected 40 h later. Flow cytometry analysis (Fig. 1A) showed that, as anticipated, the spontaneous micronucleated reticulocyte frequencies in unirradiated $Polq^{-/-}$ mice were higher than in $Polq^{+/+}$ and $^{+/-}$ mice ($P < 0.0001$). These data show that radiation-induced micronuclei are elevated in mice carrying a complete disruption of *Polq* to levels very similar to those reported in mice with a *chaos1* point mutation in *Polq* (17). Thus the micronuclei are not caused by a dominant negative effect of mutant POLQ protein. Further, spontaneous and radiation-induced micronucleus frequencies are similar in $Polq^{+/-}$ and $Polq^{+/+}$ mice, indicating that there is no obvious effect of haploinsufficiency of this gene on genomic instability.

In a separate experiment, groups of $Polq^{+/+}$, $^{+/-}$ and $^{-/-}$ mice received 7 Gy, a sublethal total-body dose with significant toxicity to bone marrow. The proportion of reticulocytes was suppressed from 3 to 7 days after irradiation and then increased in a regenerative phase 14 to 21 days after irradiation (Fig. 1B). There were no significant differences in the percentages of reticulocytes in $Polq^{+/+}$, $9^{+/-}$ and $^{-/-}$ mice at any time (*F* test). $Polq^{-/-}$ mice had a significantly higher percentage of micronucleated reticulocytes than either the $Polq^{+/+}$ or $Polq^{+/-}$ mice (Fig. 1C) throughout the experiment, persisting for 88 days after irradiation (data not shown). There was no significant difference between the $Polq^{+/+}$ group and the $Polq^{+/-}$ heterozygote group.

Irradiated mice were also monitored for white blood cell, red blood cell, and platelet counts. Forty hours after 75 cGy irradiation, total white blood cell and lymphocyte counts were reduced to about half in $Polq^{+/+}$, $^{+/-}$ and $^{-/-}$ mice with no differences between genotypes. There were no significant differences in red blood cell or platelet counts (data not shown). After 7 Gy, blood cell counts were suppressed and then largely recovered by 34 days, but no differences in the response were detected between *Polq* genotypes (Fig. 2A–D).

An Intact and Stable Hematopoietic Microenvironment in Long-Term Bone Marrow Cultures from Polq$^{-/-}$ Mice

To examine potential effects of deletion of *Polq* on the duration of hematopoiesis, long-term bone marrow cultures were established that consisted of an adherent stromal supportive layer containing primitive hematopoietic stem cell islands termed “cobblestone islands” and a nonadherent cell compartment containing differentiated hematopoietic cells that are derived from the cobblestone islands. Establishment of an adherent layer was similar for *Polq*$^{+/+}$ and *Polq*$^{-/-}$ cultures. Both cultures showed a proliferation of adherent cells that resulted in >80% confluence by week 8 (Fig. 3A). The layers remained stable, with >98% confluence for the 34 weeks of culture. The adherent layer is important for the initiation and the persistence of hematopoiesis, and the persistence of cobblestone islands in this layer correlates with the longevity of production of nonadherent colony-forming progenitor cells (21). long-term bone marrow cultures from both *Polq*$^{+/+}$ and *Polq*$^{-/-}$ mice showed continuous maintenance of cobblestone islands in the adherent layer throughout the 34 weeks in culture (Fig. 3B), with the *Polq*$^{-/-}$ culture producing even more cobblestone islands between weeks 10 and 15. Production of nonadherent hematopoietic progenitor cells by the long-term bone marrow cultures was also maintained in both *Polq*$^{+/+}$ and $^{-/-}$ cultures for 34 weeks (Fig. 3C). These nonadherent cells produced similar numbers of colonies in methylcellulose (Fig. 3D and E), which is correlated with the release of progenitor cells into the nonadherent layer (21). The data suggest that *Polq*-null cells differ from *Polq*-proficient cells in the movement of hematopoietic cells from the adherent to the nonadherent compartment.

Polq$^{-/-}$ Clonal Bone Marrow Stromal Cell Lines are Radiosensitive

The above data suggested that the oxidative stress resulting from culture in atmospheric oxygen conditions (27) was not associated with a detectable increase in toxicity in *Polq*$^{-/-}$ long-term bone marrow cultures. To determine whether cells from *Polq*$^{+/+}$ and *Polq*$^{-/-}$ mice had a detectable difference in the response to a more acute oxidative stress, sensitivity to γ radiation was tested. Clonal bone marrow stromal cell lines were established from the adherent cells in long-term bone marrow cultures at 34 weeks. Although the plating efficiencies of two *Polq*$^{+/+}$ clones and two *Polq*$^{-/-}$ clones were similar, other differences in the cell lines were apparent. The *Polq*$^{-/-}$ bone marrow stromal cells were larger and had a saturation density that was about twofold lower than those of the *Polq*$^{+/+}$ cells. *Polq*$^{+/+}$ cells had a doubling time of 18 h compared to 24–30 h for *Polq*$^{-/-}$ clones. The *Polq*$^{+/+}$ and Polq$^{-/-}$ clonal cells were irradiated with doses from 0 to 8 Gy and plated at several densities. *Polq*$^{-/-}$ clonal bone marrow stromal cells were significantly more radiosensitive than the *Polq*$^{+/+}$ cells (Fig. 4). The D_0 values for the *Polq*$^{+/+}$ cells were 1.24 and 1.31 for clones 1 and 2, respectively, and 0.98 and 0.89 *Polq*$^{-/-}$ for clones 1 and 3, respectively (difference between the genotypes, $P < 0.05$).

Cells Lacking POLQ have Similar Sensitivities to Generators of Reactive Oxygen Species

To explore the role of POLQ in the ability of cells to tolerate DNA damage after exposure to other agents that induce oxidative stress, *Polq*$^{+/+}$ and *Polq*$^{-/-}$ cells were exposed to increasing concentrations of paraquat (Fig. 5A), hydrogen peroxide (H_2O_2) (Fig. 5B) or bleomycin (Fig. 5C) for 1 h prior to plating. When we compared two clonal cell lines of each genotype, no significant differences were apparent in the toxicity of paraquat and H_2O_2. For bleomycin, toxicity at low doses was greater for the *Polq*$^{-/-}$ clones (50% killing doses of 0.34, 0.23, <0.0001 and 0.0062 μg/ml for $^{+/+}$ clone 1,$^{+/+}$ clone 2, $^{-/-}$ clone 1 and $^{-/-}$ clone 3, respectively).

ATM Kinase Activation and Radiosensitivity of Polq$^{-/-}$ Cells

ATM deficiency is known to cause radiosensitivity of mammalian cells, and the present findings show that POLQ deficiency also causes radiosensitivity in mouse bone marrow stromal cells. The inability of mouse *Atm Polq* double mutants to thrive suggests that pathways involving *Atm* and *Polq* are complementary in helping to mediate tolerance to oxidative stresses encountered during normal cell growth. If one function of POLQ is to bypass sites of DNA damage caused by reactive oxygen species of endogenous origin, aberrant DNA structures might be present in *Polq*$^{-/-}$ cells that could cause constitutive activation of ATM. Further, in *Polq*$^{-/-}$ cells, the ability to activate ATM kinase might be particularly critical. To determine whether ATM kinase activation was altered in *Polq*$^{+/+}$ and *Polq*$^{-/-}$ bone marrow stromal cells, cultures were γ-irradiated (or sham-irradiated), and the levels of phosphorylated ATM protein and total ATM protein were visualized in whole cell extracts. The level of ATM phosphorylated on S1987, a surrogate marker of ATM kinase activity (28), did not appear to be constitutively increased in *Polq*$^{-/-}$ cells compared to *Polq*$^{+/+}$ cells. After irradiation, phosphorylated ATM was increased in both *Polq*$^{+/+}$ and *Polq*$^{-/-}$ bone marrow stromal cells to equivalent levels (Fig. 6), taking into account the amount of total ATM in the extracts (Fig. 6, lower panel).

We tested whether ATM kinase activation is particularly important to help mediate survival of irradiated POLQ-defective cells. The selective small molecule ATM kinase inhibitor KU55933 was used to inhibit ATM kinase activity *Polq*$^{+/+}$ and *Polq*$^{-/-}$ cells for 4 h (from +15 min to +4 h 15 min) after irradiation (29). Inhibition of ATM kinase decreased survival in *Polq*$^{+/+}$ cells (Fig. 7). However, although *Polq*$^{-/-}$ cells were more sensitive, there was no additional increase in radiosensitivity in the presence of the ATM inhibitor (Fig. 7).

Discussion

Radiosensitivity of Bone Marrow Stromal Cells from Polq-Defective Mice

DNA POLQ is recognized as a specialized DNA polymerase with many unique properties, but the physiological function of the enzyme is not well understood. POLQ and the related Mus308 enzyme are found only in metazoans and not in fungi or other unicellular organisms. Purified recombinant human DNA polymerase theta is a 2590 amino acid protein with low fidelity (9,10) and with the ability to efficiently bypass several types of DNA damage, including AP sites in DNA and thymine glycol, a major product of reactive oxygen species damage to DNA (9). There is limited information on the relative sensitivity of *Polq*-deficient cells to DNA-damaging agents. A targeted deletion of the POLQ polymerase core domain in mouse B-lymphoma cells resulted in cells with a slightly reduced growth rate (doubling time 19 h in parental cells and 29 h in POLQ-deleted cells, similar to the difference seen here with bone marrow stromal cells), and a slightly elevated sensitivity to all agents tested: mitomycin C, cisplatin, etoposide, γ radiation and UV radiation (30). On the other hand, POLQ-deficient chicken DT40 cells were not detectably sensitive to cisplatin, mitomycin C, UV radiation, γ radiation or MMS, but they had measurably increased sensitivity to hydrogen peroxide compared to POLQ-proficient DT40 cells (11). Primary fibroblasts from mouse *Polq*$^{-/-}$ cells were not hypersensitive to either ionizing radiation or mitomycin C, although extensive analysis of survival was not done (18).

Several observations suggest that a function of POLQ in defending cells against DNA damage may be cell-type specific. POLQ is expressed widely in different tissues but is particularly high in cells of the hematopoietic lineage as well as in human tumor cells (19). Mice with disruptions of POLQ function are viable and develop apparently normally. The increased spontaneous frequency of micronuclei suggests that the enzyme may be especially important in maintaining genetic stability in the reticulocyte/erythrocyte lineage. Therefore,

we specifically investigated aspects of blood cell function and radiosensitivity in cells derived from the bone marrow of POLQ-defective mice. Bone marrow stromal cell lines from POLQ-defective cells were hypersensitive to ionizing radiation. There was no strong evidence for hypersensitivity to the reactive oxygen species-generating agents hydrogen peroxide and paraquat, however, suggesting that POLQ may handle some of the adducts specifically caused by ionizing radiation, for example DNA strand breaks. The sensitivity of POLQ-defective cells to low doses of the strand-breaking agent bleomycin supports this suggestion.

Increased Ionizing Radiation-Induced Micronuclei in POLQ-Defective Cells

The micronucleus assay was used to assess *in vivo* chromosomal damage (31). Micronuclei represent chromosomal fragments left behind when the reticulocyte ejects its nucleus. They arise from unresolved chromosomal breakage events. They could have origins in an increased frequency of chromosomal breakage that overwhelms normal DNA repair systems or could arise from a difficulty with mitotic chromosome segregation. In mice, the *Polq* mutant *chaos1* was isolated by virtue of its increased spontaneous micronucleus formation. A mutant with a similar phenotype, *chaos3*, is a partially defective allele of *Mcm4*, a component of a helicase functioning in fork elongation during semiconservative DNA replication (32). Problems in maintaining efficient DNA replication fork progression may thus give rise to micronuclei.

One new finding in the present study regarding micronuclei is that mice with complete deletions in *Polq* also have increased ionizing radiation-induced micronucleus formation. Indeed, the frequencies found are nearly the same as with the *chaos1* point mutation (17). We also found no increase in spontaneous or radiation-induced micronuclei in $Polq^{+/-}$ heterozygous mice, indicating that there is no haploinsufficiency for this function. Finally, we found that micronucleated reticulocytes persist in irradiated *Polq*-defective mice for many weeks after irradiation, indicating that there are sustained difficulties giving rise to chromosome fragmentation. This may suggest that *Polq* is important in repairing a class of ionizing radiation-induced DNA lesions that otherwise leads to strand breaks and cannot be handled by other DNA polymerases.

Interplay of POLQ and ATM

Polq knockout mice are only marginally viable when they harbor an additional *Atm* deficiency (18). ATM has important roles in cellular response to DNA DSBs, which can be generated directly when ionizing radiation damages DNA or can be generated when stalled DNA replication forks collapse at adducts in DNA. This suggests that *Polq* either is involved in a process to repair DSBs or helps to prevent the generation of DSBs at stalled DNA replication forks. Because the drug bleomycin generates DSBs and the $Polq^{-/-}$ cells are more sensitive to bleomycin, it is possible that POLQ is involved in DSB repair; this subject bears further investigation. Another possibility is that POLQ is involved in translesion synthesis opposite some lesion generated by γ rays that is not a common product formed by hydrogen peroxide or paraquat.

ATM is implicated in DNA DSB repair, cell cycle checkpoint activation, and apoptosis in irradiated cells. These functions appear to be distinct and may be restricted to different tissues. For example, inactivation of *Lig4* results in massive neuronal lethality in the developing murine nervous system, indicating the occurrence of endogenously formed DNA DSBs. ATM deficiency rescues this apoptosis in all areas of the developing nervous system in *Lig4*-null mice, but ATM deficiency fails to rescue defects in immune differentiation (33).

The synthetic lethality observed in $Polq^{-/-}Atm^{-/-}$ mice is consistent with distinct or only partially overlapping functions for POLQ and ATM in the maintenance of genomic integrity, perhaps in response to different DNA lesions. Consistent with this hypothesis, in the surviving double mutants (approximately 10% of animals), the onset of thymic lymphoma is delayed and life span is significantly increased, suggesting that POLQ is essential for the proliferation and/or immortalization of $Atm^{-/-}$ thymocytes (18). We reasoned that since $Polq^{-/-}$ bone marrow stromal cells are radiosensitive, ATM kinase activity may be particularly important for $Polq^{-/-}$ bone marrow stromal cell survival after irradiation. The bone marrow stem cells were not further radiosensitized when ATM kinase was inhibited, suggesting that POLQ and ATM may respond to the same set of DNA lesions in bone marrow stromal cells.

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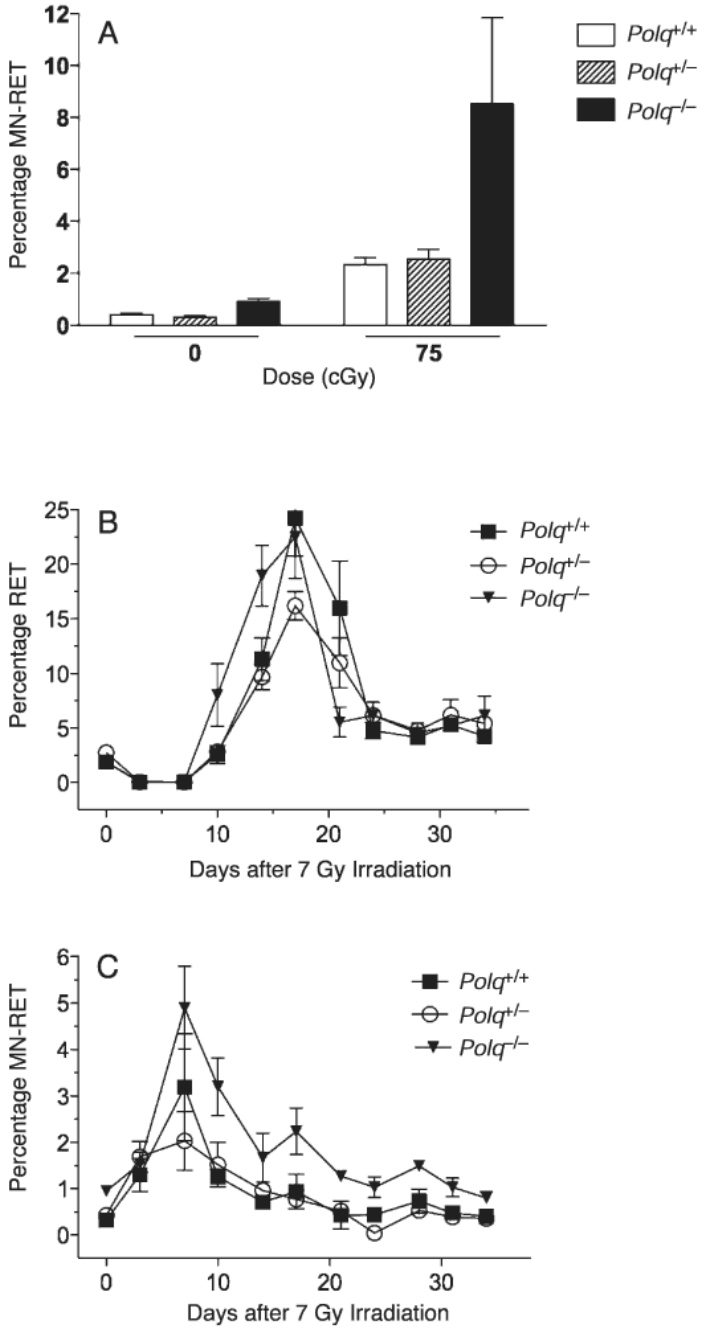

FIG. 1.

Panel A: Mean percentages of peripheral blood micronucleated reticulocytes (MN-RET) from $Polq^{+/+}$, $^{+/-}$ and $^{-/-}$ mice prior to irradiation and 40 h after 75 cGy total-body irradiation. $Polq^{-/-}$ mice had a significantly increased frequency of spontaneous ($P < 0.0001$) and radiation-induced micronucleated reticulocytes ($P = 0.0039$). Panels B and C: $Polq^{+/+}$, $^{+/-}$ and $^{-/-}$ mice were exposed to 7 Gy total-body irradiation. Blood samples were collected before and after irradiation. Panel B shows the mean percentages of peripheral blood reticulocytes (RET) and panel C the mean percentages of peripheral blood micronucleated reticulocytes. The difference in the percentages of MN-RET between the

Polq$^{-/-}$ genotype compared to the *Polq*$^{+/+}$ and $^{+/-}$ genotypes was significant ($P < 0.05$) except at days 3, 7 and 14. Data are expressed as means ± SE.

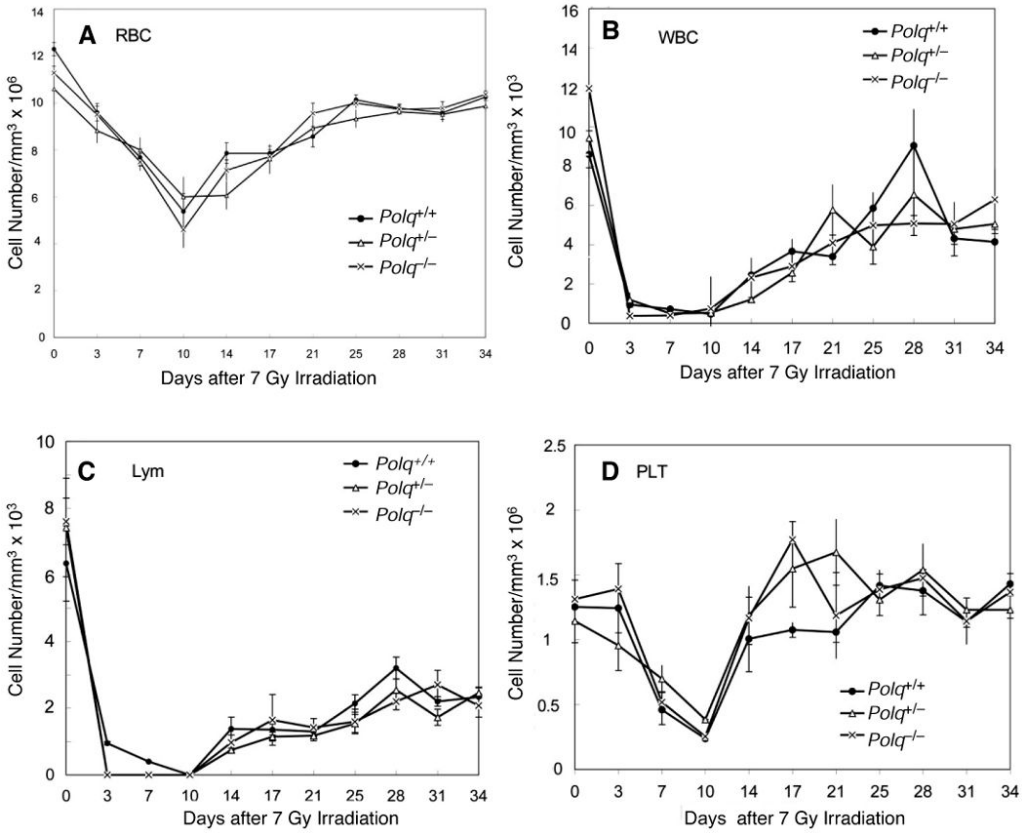

FIG. 2.

Differential blood cell counts from *Polq*$^{+/+}$, $^{+/-}$ and $^{-/-}$ mice after 7 Gy total-body irradiation. Blood samples were analyzed using an automated veterinary hematology analyzer, yielding counts for (panel A) red blood cells, (panel B) total white blood cells, (panel C) lymphocytes and (panel D) platelets. Data are expressed as means ± SE.

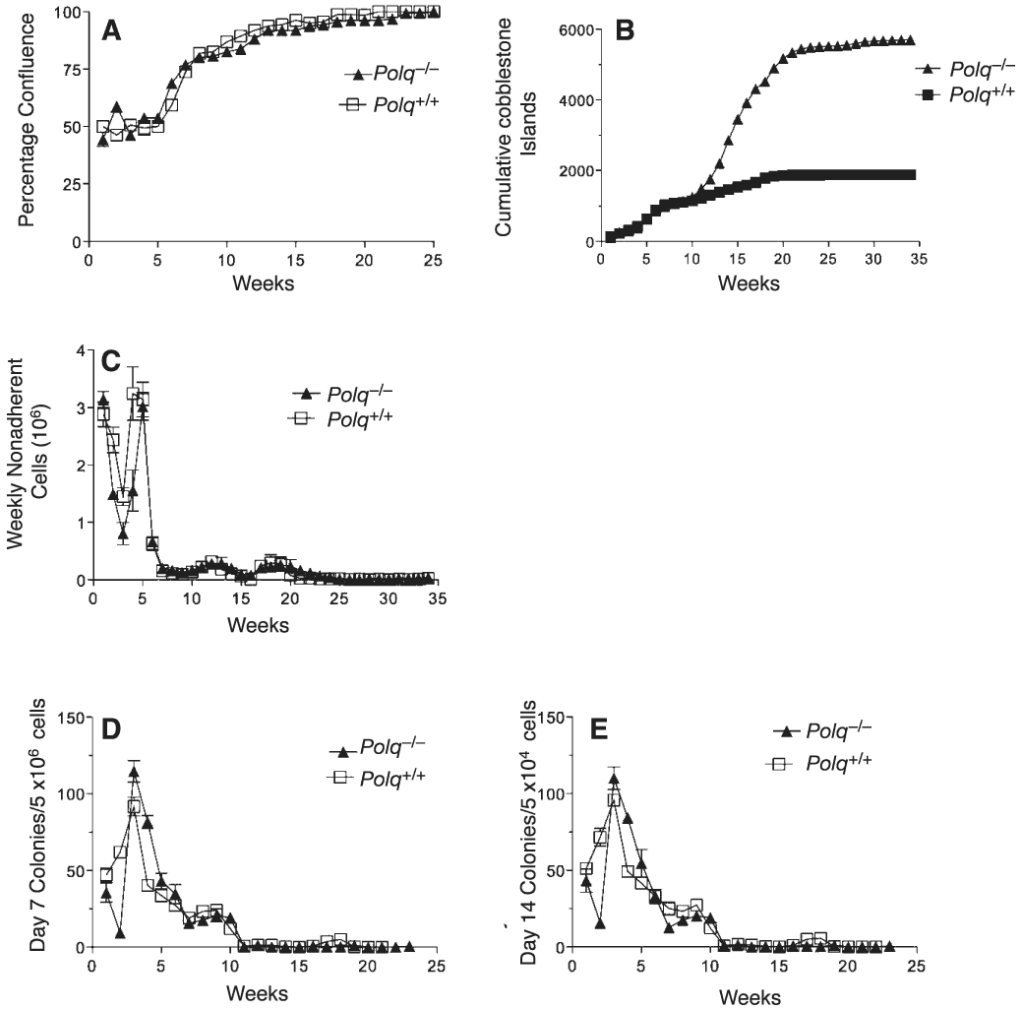

FIG. 3.

Long-term bone marrow cultures were established from *Polq*$^{+/+}$ and *Polq*$^{-/-}$ mice. Panel A: Adherent layer confluence (percentage of the flask surface area covered by the adherent layer) was scored weekly for each flask in each group. Results are presented as means ± SE of eight flasks per group per time. Panel B: Cumulative adherent cobblestone islands (50 cells or more) were counted weekly in each flask for each group. Points are means of eight flasks per group at each time. The increasing number of cobblestone islands reflects a capacity of the cultures to retain hematopoietic cells in the adherent compartment. Panel C: Weekly production of nonadherent cells. The results are the means ± SE of eight flasks per group. Panels D and E: Production of CFU-GM from nonadherent colony-forming cells harvested from *Polq*$^{+/+}$ and $^{-/-}$ long-term bone marrow cultures. Nonadherent cells harvested weekly from *Polq*$^{+/+}$ and $^{-/-}$ long-term bone marrow cultures were transferred to methycellulose. CFU-GM colonies of greater than 50 cells were scored on day 7 (panel D) and day 14 (panel E). Colony production in both *Polq*$^{+/+}$ and *Polq*$^{-/-}$ cultures began to decline in week 19. Points are means ± SE from eight flasks per group at each time over 23 weeks.

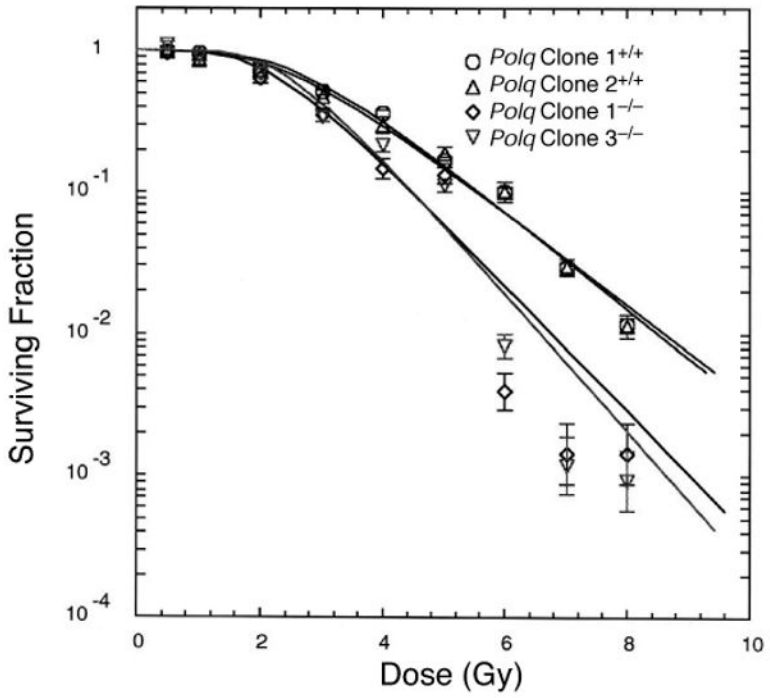

FIG. 4.

Radiation survival curves of clonal bone marrow stromal cell lines. Points are means ± SE for two clonal cell lines each for *Polq*$^{+/+}$ and *Polq*$^{-/-}$ cells.

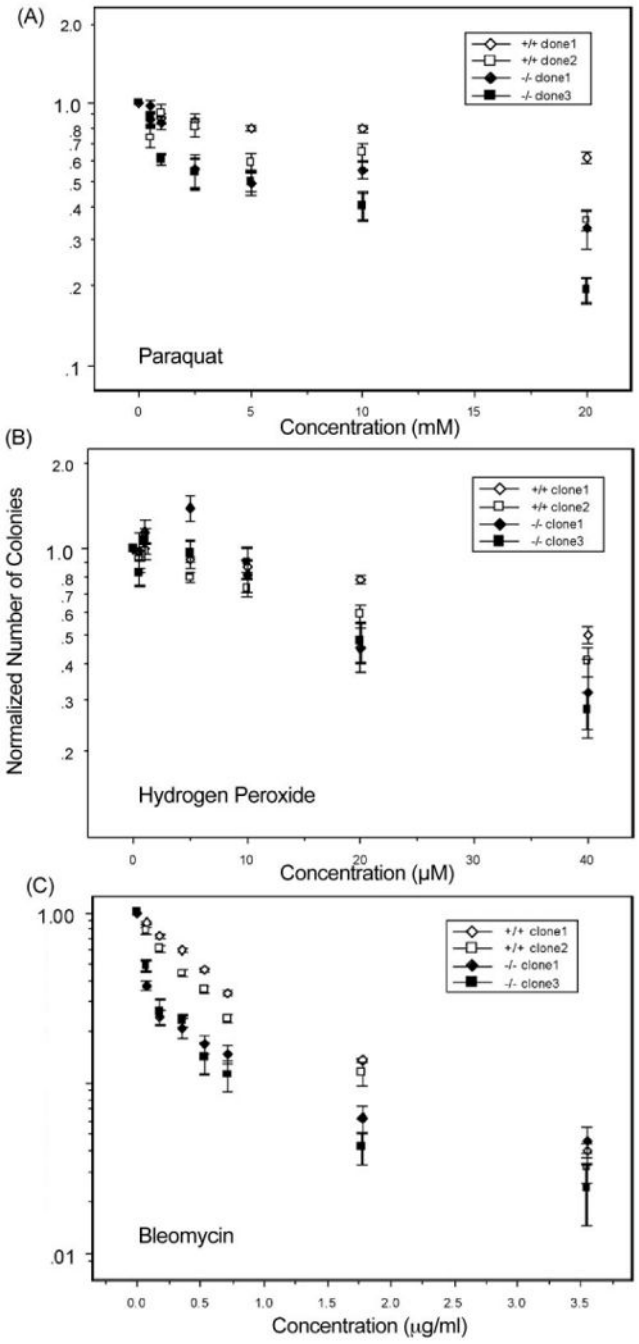

FIG. 5.

Colony-forming ability of *Polq*$^{+/+}$ and $^{-/-}$ clonal cell lines after 1 h exposure to (panel A) paraquat, (panel B) hydrogen peroxide or (panel C) bleomycin. Data are means ± SE from three experiments.

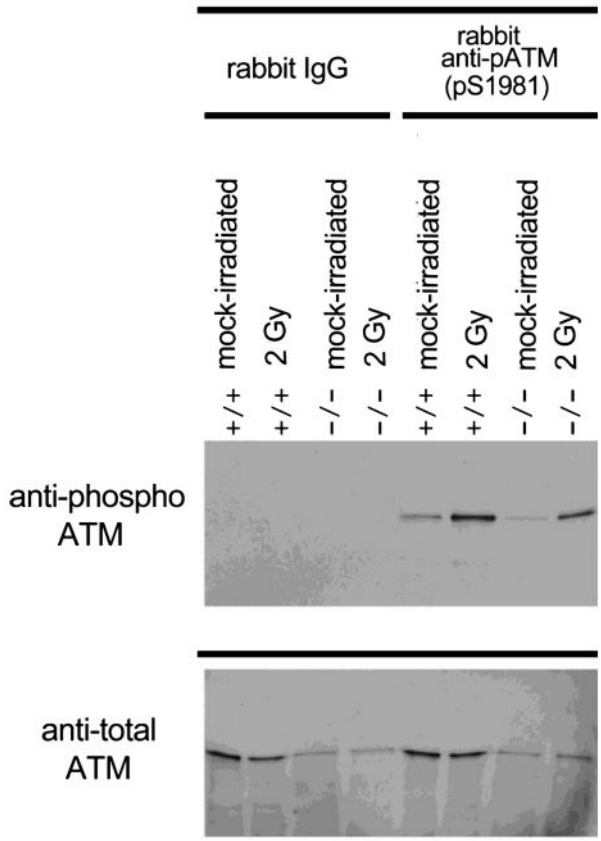

FIG. 6.

Activation of ATM in *Polq*$^{+/+}$ and *Polq*$^{-/-}$ bone marrow stromal cells by ionizing radiation. Immunoprecipitation and immunoblotting were performed to detect the amount of activated ATM (phosphorylated on serine 1987; top panel) and total ATM in the whole cell extracts (lower panel). An anti-phospho-PlK pT210 antibody was used as the IgG control.

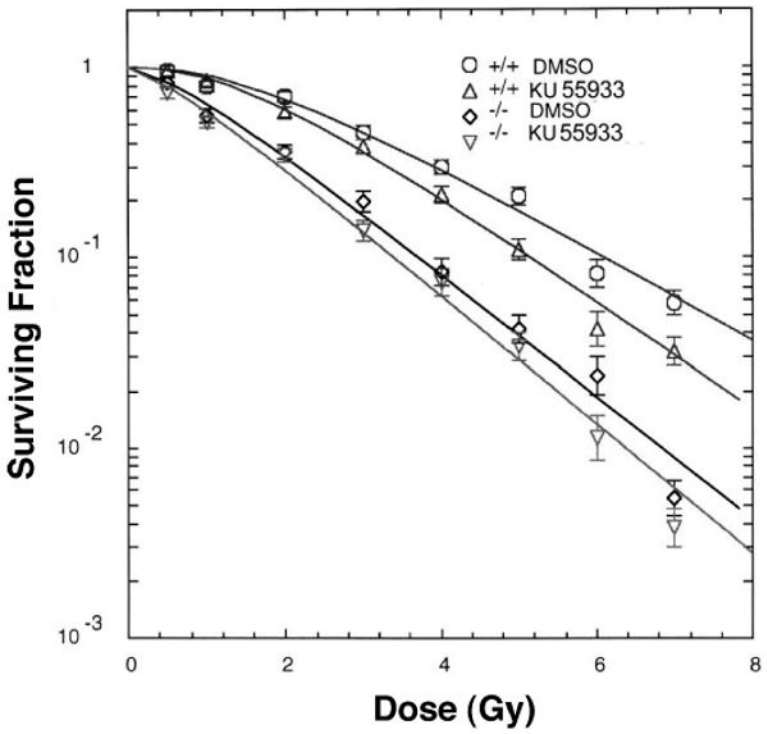

FIG. 7.

Radiation survival curves of long-term bone marrow culture-derived clonal bone marrow stromal cell lines treated with the ATM kinase inhibitor KU55933 for 4 h after irradiation. Points are means ± SE.