Gamma irradiation of Type B spermatogonia leads to heritable genomic instability in four generations of mice

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Mice conceived 6 weeks after paternal exposure to ionizing radiation were fathered by sperm that were Type B spermatogonia at the time of irradiation. Previous studies of these offspring showed that this paternal F₀ germ cell irradiation led to decreased embryonic cell proliferation rates, altered enzyme activities, protein levels and whole-body weights. In the present study, we examined four generations of CD1 mice following paternal F₀ irradiation of the Type B spermatogonia to determine the stability of the heritable effects. Offspring were evaluated for changes in protein kinase C and mitogen-activated protein kinase enzyme activities and Trp53 and p21[waf1] protein levels. Two or more endpoints were significantly altered in all four generations of offspring from the irradiated F₀ sire (P ≤ 0.05). To test the hypothesis that these heritable biochemical effects are random stochastic responses rather than some predictable uniform response, each endpoint was also evaluated in terms of a variability index (VI). Results of VI analyses show that the observed heritable phenotype is unpredictable in magnitude and direction of change for an endpoint between generations and within generations. These results indicate that irradiated spermatogonia develop a capacity to transmit a type of heritable genomic instability to four generations of offspring.

Introduction

The transmission of effects to F₁ offspring from paternal F₀ exposure of premeiotic spermatogonia to ionizing radiation has been well documented. Following paternal F₀ irradiation of Type B spermatogonia with either X rays (1) or γ rays (2), the F₁ embryo exhibits decreased embryonic cell-proliferation rate when challenged by direct cell–cell contact with a normal embryo in an aggregation chimera. Sperm from F₁ males with an F₀ irradiation history have a decreased fertilization capacity in vitro, although there is no apparent effect on litter size in vivo (3). Other studies have suggested that parental exposure of mice to genotoxic agents, including ionizing radiation, may induce tumor formation in the F₁ progeny (4,5). Mice with paternal F₀ germline irradiation history have been observed to have reversions of the p⁵³⁰ mutation (6,7) and significantly higher expanded simple tandem repeat (ESTR) mutation rates at several loci in the F₁, F₂ and F₃ generations (8).

In a previous study using CD1 mice, we demonstrated heritable effects of ¹³⁷Cs γ irradiation of the paternal F₀ Type B spermatogonia on the F₂ generation using preimplantation embryo cell-proliferation rate as our endpoint (9). The results of the study were surprising because there was no apparent degradation between the F₁ and F₂ generations in the frequency or severity of the observed decreased rate of cell proliferation among the preimplantation embryos with a history of paternal Type B spermatogonial irradiation. The lack of degradation in the effect of paternal F₀ germline irradiation between the F₁ and F₂ generations indicates a non-Mendelian mode of inheritance.

In subsequent studies, adult F₂ chimeric males were generated with both a component from paternal F₀ Type B spermatogonial irradiation and a control component (10,11). Since these chimeric male mice produced both sperm with paternal F₀ irradiation history and control sperm, litters that included F₃ offspring with paternal F₀ irradiation history and offspring with no irradiation history were obtained from a single dam. The basal hepatic activities of cytosolic receptor tyrosine kinase (RTK), protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) were altered in the juvenile F₃ offspring with an irradiation history in comparison with the littermate controls, as were nuclear protein levels of Trp53 and p21[waf1] (10). PKC and MAPK enzyme activities and Trp53 and p21[waf1] protein levels were selected for evaluation in these studies because of their involvement in cellular proliferation, radiation response and genomic instability.

In the present study, we used conventional breeding to obtain further information about the stability of these effects across several generations. We examined the same biochemical endpoints in four generations of juvenile offspring following a paternal F₀ Type B spermatogonial irradiation using the outbred CD1 strain of mice. In previous studies, longitudinal evaluation of F₂ chimeric males revealed evidence of chimeric drift with selection against the germline having a history of Type B spermatogonial irradiation (11). Since it is possible that interactions between the germines in chimeric male mice could have contributed to the biochemical differences between offspring arising from the irradiation history and from the control cell lineages, the present study utilized conventional breeding rather than chimeric animals.

Because genomic instability is by definition an unstable response, it was not unexpected that independent responses in different individuals might be observed following destabilization of the genome, rather than some orchestrated change in a whole group of individuals from different irradiated sires or spermatogenic cells. As a result, the data obtained in the biochemical experiments were also evaluated for response variability for these biological effects by generation. This measure of response variability is termed the Variability Index (VI).

Materials and methods

Mice

Animal experimentation was carried out in accordance with the principles of the American Association for Accreditation of Laboratory Animal Care (AAALAC) in fully accredited facilities following the Guiding Principles in

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the Care and Use of Laboratory Animals of the Department of Health and Human Services. The study was reviewed by the UCD Animal Use and Care Administrative Advisory Committee (AUCAAC) and regulations are enforced by regular inspections by AAALAC, the US Department of Agriculture and the Office of the Campus Veterinarian and conform to the provisions of the Animal Welfare Act.

Male mice from a single cohort of 8–12-week-old CD1 mice (Charles River, Portage, MI) were stratified by body weight and randomly assigned to the experimental (10 mice) and the control (10 mice) groups of F0 males. For each timed mating, a single cohort of female CD1 mice was obtained (6–8 weeks old; Charles River, Portage, MI). At each mating, females from the single cohort were randomly assigned to the experimental or the control groups of males. The female mice were stimulated with an intraperitoneal injection of pregnant mare serum gonadotropin (PMSG; Sigma Chemical, St Louis, MO) followed 48 h later with an intraperitoneal injection of a 2 U of human chorionic gonadotropin (hCG; Sigma Chemical). Mice were maintained under a 12 h light/12 h dark photoperiod.

Irradiation and dosimetry

The exposed males received an acute whole body absorbed dose of 1.0 Gy from attenuation 137Cs γ-irradiation. Mice were irradiated using a J.L. Shepherd & Associates (San Fernando, CA) Mark I Model 30 calibrated 137Cs γ-ray (0.662 MeV) irradiator at a dose rate of 0.14 Gy/min. Control mice were sham irradiated within the exposure chamber with the door closed but without γ-rays. The exposure for each group of mice was measured using the average of three or more commercially supplied thermoluminescent dosimeters (TLD-100 LiF powder; Harshaw, OH, supplied and read by Radiation Detection, Sunnyvale, CA). Air dose dosimeter measurements were converted to tissue-absorbed dose estimates using the ratio of the energy mass absorption coefficients of γ photons for tissue and air.

With 4 days after irradiation marked as post-irradiation week 1, each male from the control and irradiated groups was mated with a different CD1 female at post-irradiation weeks 4, 5 and 6. The females from the post-irradiation week 6 mating were allowed to deliver their F1 litters. One F1 male was randomly selected from each F1 litter and, at this early age, each F1 male was mated with a different female to provide the F2 generation of offspring. One F2 male was randomly selected from each F2 litter for mating to obtain the F3 generation of offspring. The same breeding protocol was used to obtain the F4 generation of offspring. Each F1, F2 and F3 male continued to be bred until he had sired one litter from which both the sires of the subsequent generation and 19-day-old offspring could be obtained. New females were mated from the vendor for each generation’s matings in order to preclude inbreeding. Throughout the study F1, F2, F3 and F4 offspring were tracked with respect to their F0 sire to minimize overrepresentation of offspring from any given F0 male among the data. For a given generation, both experimental groups of mice were housed together within the same room.

Sample collection and selection

Offspring were killed by cervical dislocation at 19 days of age and the whole body weight of each animal was measured. Livers were then removed entirely and cleaned, weighed and immediately frozen in liquid nitrogen. Once all livers from all litters for a given generation had been obtained, samples were selected for biochemical assays. For the Set 1 experiment, three animals with an irradiation history were randomly selected from the offspring from the four F0 sire lineages. For the Set 2 experiment, three different animals with an irradiation history were randomly selected from the offspring from each of the four F0 sire lineages. Care was taken that at least one offspring from each of the four F0 lineages was selected and that there was a balanced representation of the two sexes. Since there were four lineages, this means that in some cases there was a representative offspring from the same sire in both Sets 1 and 2, whereas in other instances a representative offspring was included only in Set 1 or Set 2. The nested ANOVA incorporates this sire representation into the data analysis. Control offspring of Sets 1 and 2 were separately selected in the same manner from the five concurrent control F0 lineages. Each of the two sets is an independent experiment with animals randomly selected from among the litters for each generation.

Hepatic protein isolation and protein assays

Nuclear and cytosolic fractions of protein were isolated from the frozen liver samples using standard methods (12). In a blinded study, protein from Set 1 animals (from an irradiation history and three control animals) was processed and analyzed as a group for each kinase activity assay and protein level determination. All assays were repeated at least three times for the set of animals. The same experimental procedures were then used to analyze protein from Set 2 animals.

Enzyme activities were measured using Upstate Biotechnology’s Protein Kinase C Assay Kit and MAP Kinase Assay Kit (Lake Placid, NY) (12).

Standard methods were used to measure nuclear Trp53 and p21Waf1 protein levels (12). In brief, 70–100 μg of nuclear protein was loaded into each lane of a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE) and electrophoresed and then electroblotted to a polyvinylidene difluoride membrane (Millipore, Bedford, MA) and blocked. HERCULES ECL Primary Antibody (Santa Cruz BioTech, Santa Cruz, CA). SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) was used to visualize the protein bands. The same PVDF membrane was stripped and reprobed for Trp53 using anti-p53 primary antibody (Santa Cruz BioTech). Detection was performed as described for p21Waf1. Gels were stained for protein loading using GelCode Blue Stain Reagent (Pierce). Gels and western blot images were scanned and quantified using a UMAX Astra 2000 flatbed scanner (Fremont, CA) and NIH Scion Image computer software. The optical density (OD) of each Tryp53 or p21Waf1 protein band was normalized to the OD of its respective protein lane on the gel to obtain an OD per μg of protein.

Statistical analyses

A minimum of three measurements were made for PKC, MAPK, Trp53 and p21Waf1 for each animal. All these individual values are included in all statistical analyses. For each set, the sample size for all endpoints was three animals per experimental group. Each of these animals was a descendant of either an F0 sire that had been irradiated or an F0 sire that was an unirradiated control. This was, therefore, a nested experimental design (13). In this nested design, the factor under study is F0 irradiation group. The data are nested with respect to the relationship of the experimental animals to the F0 sire and the associated F0 irradiation group.

The results were statistically evaluated using nested-design analysis of variance (ANOVA) with computations performed with a general linear model using SuperANOVA (Abacus Concepts, Berkeley, CA). The significance level was taken as α = 0.05. The main factor was the F0 irradiation group, and the nested factor was experimental animal nested with respect to F0 irradiation group and F0 sire (animal (F0 irradiation group, F0 sire)).

Two conditions that were assumed in this ANOVA are that the random portion of the observed data is normally distributed and that the associated variances are the same for both experimental groups. To ensure that these assumptions were not seriously in error, the data were inspected and when needed, the data were mathematically transformed using logarithmic, reciprocal or square-root functions to meet the ANOVA assumptions.

Post hoc evaluations for the effect of animal variability were conducted where the animal factor was significant, using the Tukey–Kramer test as implemented by SuperANOVA. These results are reported to complement the results of the underlying nested ANOVA as well as to complement subsequent analyses of the data VI.

No statistically significant difference in enzyme activity was observed based on sex, so male and female offspring data were pooled for statistical analysis. Linear regressions of the biochemical data for all endpoints showed that fraction of the total variability explained by possible linear correlation between enzyme activity or protein level and animal body weight was < 4%. In every case the slope of the regression line was not significantly different from zero.

Variability index

For each endpoint, the absolute value of the difference between each observed protein measurement and the average value observed among concurrent controls for the same set was taken to obtain the response VI. Since the resulting VI is a normalized value, all animals from both sets were evaluated together by nested ANOVA for a sample size of six irradiation history animals and six concurrent control animals per generation. The data were again nested with respect to the relationship of the experimental animals to the F0 sire and the associated F0 irradiation group. The data were also transformed as necessary to meet the assumptions of normality and equal variance. In this manner, we considered between-group differences in response for enzyme activities or protein levels, as well as the differences among sires within the F0 irradiation group and among individual animals within a given generation.

If there is no unusual response variability in irradiation history mice, there will be no statistically significant difference between the average VI for the concurrent control group and the average VI for the group with a history of paternal F0 germline irradiation. The significance level for VI ANOVA was taken as α = 0.025 to correct for the bias associated with the statistical uncertainty of the true variances.

The VI ratio (with standard error), used for illustrative purposes, is the ratio of irradiation history group VI to the concurrent control group VI where the expected value is 1. Irradiation history to control variability ratios were used only to graphically represent the data. These ratios were not used in any
statistical analyses. Levels of statistical significance were obtained from nested ANOVA of actual VI values as described above.

Results

From the 10 female mice mated with each of the 10 F₀ males who received a radiation exposure, 5 F₁ litters were obtained, 4 of which had male F₁ offspring. The four paternal F₀ lineages with male offspring were used for transgenerational evaluation of biological endpoints. From the 10 female mice mated with each of the 10 F₀ males who had a sham exposure, 5 F₁ litters were obtained, all of which had male F₁ offspring and were used as concurrent controls in the transgenerational study.

PKC and MAPK enzyme activities

Nested ANOVA analyses showed that the PKC activity was significantly altered in at least one set from all four generations of offspring from the irradiated F₀ sire relative to concurrent controls (Figure 1A). PKC activity was higher in both sets of F₁ generation offspring with an irradiation history compared with concurrent controls and statistically significant for Set 1 ($P = 0.05$). In the F₂ generation, Set 1 was significantly higher compared with concurrent controls, whereas Set 2 was significantly lower in comparison with concurrent controls ($P = 0.0008$ and $P = 0.007$, respectively). PKC activities were lower in both sets of F₃ generation offspring with an irradiation history compared with concurrent controls ($P = 0.01$ and $P = 0.002$). Similar to the F₂ generation, the F₄ generation showed that Set 1 activity levels were significantly higher relative to concurrent controls and Set 2 activity levels were significantly lower relative to concurrent controls ($P = 0.03$ and $P = 0.004$, respectively).

Nested ANOVA indicated that Set 1 animals of the F₂, F₃ and F₄ generations and Set 2 animals of the F₁ generation had significant animal effects. Further analyses using Tukey–Kramer tests indicated that in all cases, the animal(s) contributing to this variability were from an F₀ irradiation history.

Analysis of MAPK activity revealed similar trends to those observed for PKC activity, but with much lower magnitude of effect (Figure 1B). Nested ANOVA analyses showed that MAPK activities were significantly lower compared with concurrent controls in Set 2 of the F₂ generation irradiation history offspring and Set 2 of the F₄ generation ($P = 0.03$ and $P = 0.02$, respectively). The F₁ and F₂ generation Set 2 nested ANOVA also showed significant interanimal variability for MAPK activity. Tukey–Kramer tests indicated that for these sets this variability was a non-specific effect that was not driven by a particular animal or group. The Tukey–Kramer test indicated significant variability in the F₄ generation that was attributed to F₀ irradiation history animals.

Trp53 and p21^{waf1} levels

Nested ANOVA analyses showed that Trp53 protein levels were significantly different in at least one set in all four generations of offspring from irradiated F₀ sires compared with concurrent controls (Figure 1C). Trp53 protein levels were lower in Set 2 of F₁ generation offspring with an irradiation history compared with concurrent controls ($P = 0.01$). In the second generation, Set 1 Trp53 protein levels were

![Image](https://example.com/image1)

**Fig. 1.** Observed levels of PKC, MAPK, p53 and p21^{waf1} in four generations of mice following F₀ spermatogonial irradiation. Each symbol represents a mean with standard error for a given animal endpoint where the measurement was repeated a minimum of three times for each animal. Open symbols represent concurrent control animals and closed symbols represent animals with a history of paternal F₀ germline irradiation. For each generation, the endpoints were evaluated in two separate experiments, labeled Set 1 and Set 2, using three different animals from a paternal germline irradiation history and different three concurrent control animals in each of the two sets. $P$ values are obtained from nested ANOVA of all the individual data measurements. The mean ± standard error are only for graphical representation. $P$ values for each set are directly above the set to which they correspond. (A) PKC enzyme activity as measured in pmol $^{32}$P-labeled phosphate incorporated into the PKC substrate peptide per min per μg of cytosolic protein. (B) MAPK enzyme activity as measured in pmol $^{32}$P-labeled phosphate incorporated into the myelin basic protein per minute per μg of cytosolic protein. (C) Nuclear p53 protein levels as measured by optical density (OD) of each Trp53 protein band normalized to the OD of its respective protein lane on the stained SDS–PAGE to obtain an OD per μg of nuclear protein. (D) Nuclear p21^{waf1} protein levels as measured by optical density (OD) of each p21^{waf1} protein band normalized to the OD of its respective protein lane on the stained SDS–PAGE to obtain an OD per μg of nuclear protein.
significantly lower relative to concurrent controls, whereas Set 2 Trp53 protein levels were significantly higher relative to concurrent controls ($P = 0.0001$ and $P = 0.0005$, respectively). Trp53 protein level was higher in both sets of F3 generation offspring with an irradiation history compared with concurrent controls ($P = 0.03$ and $P = 0.004$). Similar to the F2 generation, the F4 generation showed that Set 1 protein levels were significantly lower relative to concurrent controls, whereas Set 2 protein levels were significantly higher relative to concurrent controls ($P = 0.0001$).

Nested ANOVA analyses also showed significant animal variability for the Set 1 animals of F2, F3 and F4 generations and the Set 2 animals of F2 and F4 generations. Tukey–Kramer tests indicated that in F4 Set 1, the animal variability was attributed to concurrent controls and in F4 Set 1, the animal variability was attributed to both experimental groups. For the other three groups demonstrating significant animal effects, the animal variability was attributed to irradiation history animals.

Analysis of p21waf1 protein levels revealed similar trends to those observed for Trp53 (Figure 1D). Nested ANOVA analysis showed that the F2 generation Set 1 protein levels for irradiation history offspring were significantly lower compared with concurrent controls, whereas Set 2 protein levels were significantly higher compared with concurrent controls ($P = 0.001$ and $P = 0.009$, respectively). The F3 generation Set 1 showed higher p21waf1 protein levels whereas Set 2 showed lower levels compared with concurrent controls ($P = 0.007$ and $P = 0.0001$, respectively). The F4 generation showed the same pattern that was observed in the F2 generation. Set 1 protein levels were significantly lower relative to concurrent controls, and Set 2 protein levels were significantly higher relative to concurrent controls ($P = 0.03$ and $P = 0.0001$, respectively).

Nested ANOVA analyses also showed significant animal variability for the Set 1 animals of F2, F3 and F4 generations and the Set 2 animals of F1, F3, and F4 generations. Tukey–Kramer tests indicated that in F4 Set 1, the animal variability was attributed to concurrent controls and in F3 Set 2, the animal variability was attributed to both experimental groups with an emphasis on irradiation history animals. For the other four groups demonstrating significant animal effects, the animal variability was attributed to irradiation history animals.

Variability index
Analysis of the VI provided an overall measure of the magnitude of animal variability for irradiation history offspring relative to concurrent control offspring. Out of 16 independent biochemical endpoint evaluations of VI, 15 were elevated above concurrent control levels. Ten of these were statistically significant ($P < 0.025$), as measured by nested ANOVA (Figure 2).

Of the four generations of offspring with a history of paternal F0 germline irradiation, all four had significantly higher VIs for PKC activity and p21waf1 protein levels. The F2 and F4 generations had significantly higher VIs for Trp53 protein levels. These VI results are supported by the previously described Tukey–Kramer tests of the protein data. Nested ANOVA also demonstrated significant interanimal variability associated with the VI values for all four biochemical endpoints for one or more generations.

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**Fig. 2.** Variability index (VI) ratio for PKC, MAPK, p53 and p21waf1 in four generations of mice following F0 spermatogonial irradiation. Each bar with standard error represents the ratio of irradiation history VI to the concurrent control VI, where the ideal value is one with the null hypothesis ($N = 6$ animals per group). For each animal a minimum of three measurements were taken for each endpoint. The absolute value of the difference of each observed measurement and the control mean for a given endpoint (the VI) was analyzed by nested ANOVA. The $P$ values shown are unadjusted, with a significance level of $P = 0.025$. Irradiation history/control variability ratios are only used to graphically represent the data. $P$ values were obtained from the analysis of actual VI values, not from the irradiation history/control variability index ratios. (A) PKC, (B) MAPK, (C) p53 and (D) p21waf1.
Heritable genomic instability in mice

Discussion

In this study, biological effects resulting from irradiation of the paternal Fₐ Type B spermatogonia were detected in four generations of offspring. Although statistically significant effects were demonstrated in all four generations, the response of the offspring within a given generation was not always consistent, nor was the response consistent between successive generations. These significant effects were observed despite matings of the F₀, F₁, F₂, and F₃ males with unirradiated females. This study does not identify the underlying mechanism, but our observations provide important information for defining a class of heritable effects from paternal germ line irradiation. The unstable phenotype that results from F₀ germline irradiation is characterized by changes in biochemical endpoints that can differ in magnitude and even in the direction of change from one generation to the next and even among individuals within one generation. Recurring differences between control and irradiation history offspring characterize this phenotype rather than a consistently altered endpoint. Such an unstable phenotype could be the result of genomic instability that was initiated by the F₀ germline irradiation.

The results of the present multigeneration study with conventional breeding confirm our previous reports on the heritability of biochemical effects following irradiation of paternal F₀ Type B spermatogonia using chimeric F₂ sires (10). In both series of experiments, significant differences were detected in the four protein endpoints between the offspring with an irradiation history and the concurrent control offspring. Despite some variation in the observed phenotype, both sets of outcomes are compatible with the mechanisms of genomic instability that we hypothesize are involved in transmission of the heritable phenotype.

It must be appreciated that the experimental designs of the previous study and this study are fundamentally very different. In the present study, control and irradiation history offspring were produced by conventional breedings, unlike the previous experiment in which the same chimeric male sired both classes of offspring by a single dam. In the chimera experiment there was an opportunity for direct interaction between the control cells and cells with an irradiation history in the testes of the chimeric male. Alterations in the germline with an irradiation history may have been enhanced as a result of bystander effects in the testes of chimeric males. Bystander effects in response to ionizing radiation have been characterized in various systems and involve communication between directly irradiated cells and surrounding, unirradiated cells (5,14). The possibility of such interactions in chimeric testes is supported by the evidence of chimeric drift in which the germline with an irradiation history was selected against over time (11). In addition, a previous study clearly demonstrated that the effects of irradiation on embryonic cell proliferation rate were mediated by gap junction intercellular communication (GJIC). When GJIC was blocked, no radiation-induced decrease in cell proliferation rate was observed in chimeric embryos. This important finding by Vance and Wiley (15) made the evaluation of conventionally bred offspring an important confirmation that the heritable biochemical phenotype is not a result of an artificial interaction between control cells and cells with an irradiation history in the chimeric environment. Notwithstanding the apparent enhancement of the heritable phenotype that was observed with transmission by chimeric sires (10), the conventional breeding design of the present study provides a better model for extrapolation of heritable, transgenerational effects of paternal F₀ germline irradiation to exposures and outcomes that are relevant to human health.

We hypothesize that transgenerational genomic instability is induced by paternal F₀ germline irradiation, resulting in a heritable, unstable phenotype. In this transgenerational model, genomic instability represents an independent response in each individual on the biochemical level, rather than an orchestrated change in the whole experimental cohort. This point is emphasized by the results of VI analyses and Tukey–Kramer’s tests of the protein data. Nested ANOVA of the VI suggests that the recurring, but unpredictable differences between concurrent controls and offspring with an irradiation history may be a hallmark for genomic instability that was initiated by the irradiation of the paternal F₀ Type B spermatogonia rather than a biochemical endpoint that is altered in a consistent manner.

Some of the variability among both the irradiation history animals and the concurrent control animals of the present study can be attributed to genetic variability in the outbred CD1 mice. The genetic variability that is inherent in the outbred mice affects the data analysis and somewhat limits precision in determining appropriate biomarkers for heritable transgenerational effects from paternal F₀ germline irradiation. The same limitation on the use of outbred strains was encountered in analyses of heritable effects on ESTR mutation rates in mice exposed to air pollution (16). This limitation might be overcome in future studies by evaluating offspring from a much larger number of F₀ sires following germline irradiation and/or using an inbred strain of mice. However, the fact that heritable transgenerational effects of paternal F₀ germline irradiation can be detected in outbred mice underscores the robustness of these responses. Despite the problems associated with using outbred mice, one of the advantages is the greater potential for extrapolation from the mouse model to the human species. It is important to remember how genetically variable the human population is when evaluating the atomic bomb survivors, those affected by Chernobyl and other exposed cohorts for evidence of heritable effects from parental exposures in humans (4,5,17).

Since gene expression was not evaluated in this study, we cannot say whether the changes in enzyme activity or protein levels that were observed are a result of changes in transcription levels. In the future, this hypothesis will be tested using inbred strains of mice. In addition, without sequence data, we cannot say whether mutations of specific DNA sequences have led to changes in the transcripational regulation of a particular gene or changes in specific activity of the protein product of that gene. It has been suggested that activation of signal transduction cascades following radiation exposure can lead to alternative gene expression, mediating genomic instability (18). Results from a number of laboratories suggest a role for MAPK and PKC in mediating the stress response to ionizing radiation (19,20). This stress response can promote genetic reprogramming that alters the cellular background, possibly predisposing the cell to the onset of genomic instability (18). It is possible that our heritable phenotype is evidence of this altered cellular background (12).

Although changes in gene expression following ionizing radiation exposure are generally transient, the expression of some genes may be permanently altered through epigenetic mechanisms (21–23). Epigenetics are thought to play a role in genomic instability and may play a role in the heritable
transgenerational effects we observed. In fact, upon further study we may find that a better term for the mechanism that underlies the observed heritable phenotype is actually epigenomic instability. Alteration of methylation patterns can influence gene expression and have been shown to be capable of surviving reprogramming of the genome during meiosis (23,24). Furthermore, de novo methylation of DNA or alteration of methylation patterns has been observed following exposure to ionizing radiation (25–31). Germline irradiation of the ancestral Type B spermatogonia may have modified DNA methylation patterns of the paternal allele. It is possible that the altered methylation status of the paternal allele then acted in trans in the germline of the F1 offspring, altering the maternally inherited allele, resulting in non-Mendelian inheritance of an epigenetic change. Increased mutation rates of repeat DNA sequences following radiation exposure have also been demonstrated to alter gene expression and genome stability (32–35). High frequencies of minisatellite DNA instability have been observed in F1 offspring of irradiated mice (4,8). Dubrova and co-workers (36,37) also reported increased transmission of expanded simple-tandem repeat (ESTR) locus polymorphism from irradiated male mice to F1 offspring. This higher mutation rate persisted in the germline of F1 offspring when the F2 generation was examined. These data support the hypothesis that the effect of the F0 germline irradiation acted indirectly on the maternal allele in the F1 offspring prior to transmission to the F2 generation, resulting in non-Mendelian inheritance of ESTR instability initiated by paternal F0 germline irradiation.

Our results and the results obtained by others suggest that the initial paternal germline irradiation is capable of inducing DNA-based changes or a genome destabilization that survives meiosis and can be transmitted across as many as four generations. In this study, we observed significant biological effects across four generations of offspring despite F0, F1, F2 and F3 matings with unirradiated females. These biological effects represent an unstable heritable profile that changes in magnitude and incidence across generations in a manner that is consistent with the initiation of genomic instability in the irradiated ancestral Type B spermatogonia of the F0 male. It has been suggested that radiation-induced genomic instability is an early cellular step in the multistep process that results in radiation-induced carcinogenesis (38,39). As a result, it is important that the transgenerational effects of paternal germline irradiation are further characterized and an attempt made to determine the biological mechanisms that are initiated in the paternal germline following radiation exposure.

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