THE ROLE OF P53 IN RADIATION-INDUCED ESTR MUTATIONS

EXAMINING THE ROLE OF P53

IN RADIATION-INDUCED MUTATIONS AT ESTR LOCI

By

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Abstract

It is well known that ionizing radiation is genotoxic, and can trigger heritable mutations in the germ cells of an animal. Recently, researchers have used hypervariable expanded simple tandem repeat (ESTR) regions of DNA to explore this phenomenon. ESTRs facilitate the examination of induced genetic mutations using relatively low radiation doses and fewer mice than more traditional approaches. Numerous studies have examined the responses of ESTRs to radiation in the germ line; however the mechanism behind germ line mutations at ESTR loci is poorly understood. Current hypotheses propose that error-prone DNA repair, which allows for misalignment of DNA strands through replication slippage produces in changes in ESTR size. P53 is involved in DNA replication as well as repair of DNA damage, apoptosis and other cancer-related processes. We use p53-deficient heterozygous male mice to examine the role of p53 in germ line mutations at ESTR loci. Males were irradiated with a variety of dose combinations both prior to and post-meiosis, and were mated to unirradiated wildtype females. DNA from the adults and offspring was analyzed for mutations at ESTR loci using DNA fingerprinting. Surprisingly, the study found no significant differences in germ line mutation rate between any treatment groups, including the 0Gy and 1Gy treatments. I discuss the possibility that these results are due to the p53 deficiency of the males, and that p53 homozygosity is necessary for radiation-induced germ line mutations at ESTR loci to occur. I conclude that further studies need to be done, including a control study using wildtype males of the same background strain as that of the p53 deficient line in order to verify our results.

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Introduction

Radiation-Induced Adaptive Response

It is well known that high doses of ionizing radiation to living tissues causes damaging effects. This provides the basis for the development of the now widely applied linear-no-threshold (LNT) model for the biological effects of toxicants such as ionizing radiation (Pollycove and Feinendegen, $2001_{a,b}$). The LNT model predicts that adverse effects increase linearly with exposure. Very little low dose data (i.e. below about 1.3 Gy) was available during the development of the LNT model (Pollycove and Feinendegen, $2001_{a,b}$). As such, it was assumed that biological effects at low doses would follow the same linear, no threshold relationship seen for high doses. Therefore, the dose-response curve was extrapolated from the high dose data through the zero point, predicting that low doses of toxicant such as radiation have some negative biological effect and that a zero dose is always preferred over a minimal exposure (Pollycove, 1998).

The LNT model, with its low dose extrapolation, was accepted in 1959 by the International Commission on Radiological Protection (ICRP) as the basis for judging biological effect. It continues to be the accepted model by such regulatory organisations despite growing evidence that it is not accurate for low-dose effects (Pollycove, 1998). Radiation-induced adaptive response (AR) refers to the phenomenon whereby harmful effects (either endogenous or radiation-induced) are mitigated by exposure to a low dose of radiation. The adapted cells/organism may show responses such as increased survival,

increased resistance to disease, and/or increased genomic instability than those that are not given the "priming dose" (reviewed in: Pollycove and Feinendegen, $2001_{a,b}$; Wolf, 1994, 1996, 1998). Such responses directly oppose the LNT model for low doses of ionizing radiation.

Numerous studies demonstrate the existence of radiation induced AR. Studies using tissue culture (e.g. Redpath *et al.*, 2001; Broome *et al.*, 2002) single-celled organisms such as bacteria and yeast (e.g. Samson and Cairns, 1997; Boreham and Mitchel, 1994), as well as whole organism mouse models (e.g. Mitchel *et al.*, 2003;Wojcik and Tuschl, 1990) and even retrospective studies on exposed human populations (e.g. Monsieurs *et al.,* 2000; Thierens *et al.*, 2002) all provide an idea of the general properties of the phenomenon. For example, past studies have shown that radiation induced AR in mammalian cells generally occurs with an optimal priming dose below 0.1Gy (Redpath *et al.,* 2001) with an optimal priming dose rate of 0.01Gy/min (Cortes, 1994). Also, studies indicate that doses higher than 0.2Gy are not able to induce an AR, but erase any adapted condition (Sasaki, 1995) and that AR occurs only in metabolically active cells but not in dormant G_0 cells (Shadley *et al.*, 1987; Wang *et al.*, 1991). Radiation induced AR is best expressed 4-6 hours after irradiation with a priming dose (Sasaki, 1995), but can last up to 40 days (Cai and Wang, 1995). Recently our laboratory has demonstrated that AR can take place germ line of irradiated males by looking at radiation-induced mutation rates in the unirradiated offspring (Somers *et al.*, *accepted [June, 2004] for publication in Mutat. Res*).

Although AR to ionizing radiation has been studied since the 1950s, the cellular mechanism behind the phenomenon is not well understood (Dacquisto, 1959; Sasaki *et al.*, 2002). It is thought that DNA repair plays a major role in AR, perhaps with a repair system being induced by a priming dose and remaining active for a sustained period, thereby allowing the cell to be better prepared for the damaged induced by the subsequent challenge dose (Sasaki *et al.*, 2002). This hypothesis, however, has not been proven.

ESTRs

Tandem repeat DNA loci have been used over the past decade as biomarkers to study induced germ line mutations for both laboratory (e.g. Dubrova *et al.*, $1998_{a,b}$; Dubrova *et al.*, 2000; Niwa *et al.*, 1996) and field applications (e.g. Somers *et al.*, 2002; Somers *et al.*, 2004). Expanded simple tandem repeat (ESTR) loci are found in non-coding regions of the genome and have been shown to be particularly useful in the study of transmissible radiation-induced mutations in the murine germ line (Yauk, 2004). Formerly classified as minisatellites, ESTRs are now distinctly recognized as being long, homogenous arrays (up to 16 kb), of short repeat units composed of less than 10 base pairs each (Yauk, 2004). ESTRs exhibit elevated levels of instability as gains and losses of repeat units occur at high spontaneous and induced rates. This characteristic has made ESTRs very useful markers of radiation-induced germ line mutations in mice. ESTR studies require substantially fewer mice and much lower doses than traditional models such as the Russell 7-locus test (Yauk, 2004). Further, estimates of doubling dose for exposure to radiation give similar values for studies using ESTR loci and more traditional

approaches, which could indicate a correlation between ESTR instability and mutation rates at coding loci (Dubrova *et al.*, 1998_a).

Past studies using ESTR loci as genetic markers have demonstrated that radiation-induced mutations at these loci are dose dependent, with mutation rates increasing in a linear fashion for radiation of both high and low linear energy transfer (LET; Dubrova *et al.*, $1998_{a,b}$; Dubrova *et al.*, 2000). The stage of spermatogenesis at which the developing sperm are irradiated also appears to influence the germ line mutation rate. Most studies show that irradiation of pre-meiotic diploid cells results in a significant increase in germ line mutations, whereas post-meiotic irradiation of haploid cells produces no dose effect (Niwa *et al.,* 1996, Dubrova *et al.*, 1998_a). It has also been discovered that elevated germ line mutation rates at ESTR loci are transgenerational in that both first and second generation offspring of irradiated males show increased frequencies of ESTR mutations (Barber *et al.*, 2002). More recently, germ line mutations at ESTR loci have been found to be susceptible to adaptive response, where male mice that received 0.5 or 1Gy alone exhibited germ line mutation rates significantly above control whereas mice that received a 0.1Gy priming dose prior to 1Gy did not (Somers *et al.*, *accepted [June, 2004] for publication in Mutat. Res.*).

While plenty is known about the response characteristics of ESTRs to radiation in the male germ line, relatively little is understood about the mechanisms of spontaneous or induced ESTR mutation. It is currently thought that mutations at tandem repeat loci occur through a process called replication slippage or copy-choice recombination (Viguera, Canceili and Ehrlich, 2001). This process takes place during normal or repair-

induced DNA replication when DNA polymerase is halted and subsequently dissociates from the newly synthesized strand, which separates from template strand and realigns with a different repeat unit before polymerase reloads and resumes. This causes the formation of a folded or loop structure in either DNA strand, resulting the insertion or deletion of any number of repeat units upon the completion of replication, depending on where the newly synthesized strand realigns (Viguera, Canceili and Ehrlich, 2001). Such folded structures have been shown to occur *in vitro* at one ESTR locus (Fukuda *et al.*, 2002; Katahira *et al.* 1999).

p53

p53 is a tumour suppressor gene that has been shown to be a critical regulator of genomic integrity. The p53 protein is widely implicated in cancer susceptibility as well as DNA repair processes, DNA damage recognition and apoptosis. In humans, mutant alleles of p53 are found in the majority of tumours, and germ line mutation of one p53 allele results in Li-Fraumeni syndrome, whereby individuals are highly prone to a variety of cancer types (Fei and El-Deiry, 2003). Thus, it is reasonable to suspect that p53 plays an important role in cellular processes such as AR and ESTR mutation induction, which are expected to involve induced DNA repair. This, however, has never been tested.

B6.129S2-Trp53^{tm1Tyj}/J Mice

Protein-deficient transgenic mouse models provide a useful *in vivo* model for examining the role of specific proteins in damage responses. For this study, we have used a transgenic mouse model that carries a mutant p53 allele. This mutation, which deletes approximately 40% of the p53 coding region, completely blocks production of the

p53 protein (Jacks *et al.*, 1994). Homozygous null mutants are viable and undergo apparently normal embryonic and postnatal development. They are, however, highly predisposed to malignancy, and have greatly accelerated tumorigenesis compared to heterozygous or wildtype mice. The null mice only live for about 6 months, and are not reliably fertile (Jacks *et al.*, 1994). We required a p53-deficient mouse model that would withstand irradiation then 10 weeks of housing and still provide large numbers of offspring; therefore it was not feasible to use p53 null mice.

Mice that are heterozygous for the p53 mutation develop sarcomas and lymphomas of the breast and brain. These are also the predominate tumour types in humans with Li-Fraumeni syndrome. Heterozygous mice live for about 9 months before they start to develop cancers, and may live more than 17 months. These mice are also reliably fertile. The researchers that developed this mutant strain examined effect of the p53 null mutation on the synthesis of p53 protein using fibroblasts isolated from trp53+/+, trp53+/- and trp53-/- mice. They found that heterozygotes consistently produced approximately half of the p53 that a wildtype mouse produces, that a null mouse does not produce any p53 and that no truncated proteins are produced (Jacks *et al.*, 1994). Alsbeih *et al.* (2004) repeated this test using only null and wildtype fibroblasts, and found that the p53 was transcriptionally active because it led to activation of Cdknla (p21, the subsequent protein in the p53 response pathway) in the wildtype cells but not the null cells. We chose to use the trp53+/- mice in our study because they could withstand our treatment protocol and still reproduce while carrying a mutation that renders them deficient in p53 protein production. Further, by using heterozygous mice,

we could produce both wildtype and trp53-/+ offspring, which can be compared and used as a built in control for the 1-week breeding.

The difficulty with using trp53 heterozygous mice is that there is very little known about the concentrations of p53 that are required for responses within various tissues. This is most likely dependent on multiple factors such as the tissue type, level of signal or damage, and response pathway. To date, there appear to be no published studies that examine the amount of p53 necessary to allow for damage responses to take place within irradiated germ cells. However, given that heterozygosity for the p53 allele results in increased sensitivity to radiation-induced cancers and increased numerical and structural chromosome abnormalities (Boyle *et al.*, 2002), we can reasonably assume that a 50% deficiency in p53 has a measurable effect on germ cell stability. Indeed, the results of our study indicate that a 50% reduction in p53 production may prevent ESTR mutation induction, and that further analysis is necessary to verify this finding.

Materials and Methods

Irradiation, Dosimetry and Breeding

All mice were purchased from Jackson Laboratories, Bar Harbour, ME. Male mice were C57Bl/6J *trp53+/-* (Strain name: B6.129S2-*Trp53*^{tm1Tyj}/*J* Stock number: 002101). Males were irradiated at AECL Chalk River Laboratories using two ^{60}Co gamma sources with dose rates of 0.5mGy/minute for the priming doses and 300mGy/minute for the 1Gy doses. Dose rates are calculated daily using a dosimetry table based on the known decay of the gamma sources, and measured monthly using a Keithley Therapy dosimeter. When the dosimetry was calculated, dose rate was

measured in R/min and converted to cGy/min based on the ICRP soft tissue factor 1R=0.968cGy. All dose measurements were calculated to be accurate within a 5% margin of error for the depth of the cages and to account for the position of mice during exposure.

For the priming dose exposures, mice were exposed using the Gammabeam 150C in the irradiation hall specifically designed for low dose exposures at AECL. Racks containing cages of individual mice were wheeled into the facility and placed at a calculated distance from the source (3.946m) based on the dose rate determined at 3 metres (0.065cGy/min) from the source in order to experienced the desired dose rate of 0.05cGy/min. The source was exposed for 20 minutes and 200 minutes for the 0.01Gy and 0.1Gy doses respectively.

For the 1Gy exposures the mice are exposed in the Gammacell 200. The mice are placed in small cylindrical containers (~5 inches tall) with breathable lids. The containers fit into a rack that is lowered into the cell for exposure. Any exposure during lowering and raising of the rack unit (called Dead-Time dose) is accounted for in the total exposure time based on the calculated dose rate.

All females were C57Bl/6J and were not irradiated. Mice were irradiated, housed, bred and harvested according to CCAC approval at Biological Research Facility at AECL Chalk River Laboratories, Chalk River, Canada. Spermatozoa-Irradiated mice were placed in their mating groups immediately after irradiation. This does not allow time for spermatogenesis, therefore eggs are fertilized with sperm that received a radiation dose as haploid spermatozoa cells, allowing us to examine the effects of

irradiated spermatozoa on germ line mutations (Fig. 1, arrow b). These males were 9 weeks old at the time of mating (Table 1). There is no age-matched 0Gy control for the spermatozoa-irradiated groups. Instead, Group SG6 (Table 2) is used as a control for both breeding times.

To examine the germ line effects of irradiating diploid spermatogonial stem cells, spermatogonia-irradiated mice were held for 10 weeks before mating (Table 2). The cells were dividing spermatogonia at irradiation, enabling us to examine the effect on germ line ESTR mutation rates when the cells are allowed to go through both mitosis and meiosis after irradiation (Fig. 1, arrow a). The male trp53+/- mice that were used as unirradiated control mice for both breeding times were 19 weeks of age at the time of mating. Given that the 10-week age difference represents a small fraction of the mouse lifespan, it is assumed that there is no age-related effect on ESTR mutations or on fertility for these relatively young male mice.

At the desired mating time, each male was placed in a cage with 4 randomly assigned C57Bl/6J wildtype, untreated females. Males were removed after two weeks and females were separated into individual cages. Females were checked daily. At approximately 1 day prior to delivery, pups were removed via caesarean section and females were euthanized via cervical dislocation. Tissue samples were taken from parental tails and whole pups and stored in cell lysis buffer at 4° c.

DNA Fingerprinting

Tissue samples were digested with Proteinase K in lysis buffer at 37°c overnight. DNA was extracted using standard phenol chloroform protocol and dissolved in TNE_2.

Isolated DNA was quantified and 9 μg was restriction digested with HAE III and precipitated using sodium acetate and 95% ethanol. 4-6 μg of DNA was size-fractioned along a 28 cm 0.8% agarose gel in 1x TBE buffer for 48 hours at 2.14V/cm and transferred by Southern Blotting to a nylon membrane (Hybond XL, Amersham Biosciences).

We used synthetic single locus ESTR probes, Ms6-Hm (Kelly *et al.* 1989) and Hm-2 (Gibbs *et al.*, 1993) and one multi-locus probe, MMS10, which detects a highly variable rodent-specific family of multiple loci (Bois *et al.*, 1998). Probe DNA was heat denatured, then labelled by random primer extension with ^{32}P using a (Rediprime DNA labelling kit, Amersham Biosciences) and allowed to hybridize to the blot at 65°c overnight. Unbound DNA was washed away with 65° 0.1x SSC plus 0.01% SDS (single locus probes) or 2x SSC plus 0.1% SDS (multi-locus probe) for 20 minutes twice. Blots were then sealed in plastic, exposed to phosphor screens, which were scanned using a Molecular Dynamics Phosphor Imager, and finally visualized using Image Quant softwear. Blots were stripped of probe DNA between hybridizations by shaking in 42° 0.5M NaOH followed by boiling 0.1% SDS for 20 minutes each.

Each sample was run with 30ng of in-lane lambda-DNA size standard as described in Galbraith *et al.* (1991). This creates a number of reference bands that ESTR bands can be scored against using a hand-traced overlay, which reduces scoring errors resulting from lane inconsistencies or differential migration. ESTR bands were hand scored. Band shifts of at least 0.5 mm from the size of parental bands were recorded, and mutation rate data were calculated using only shifts greater than or equal to 1 mm (Fig. -

2). Any bands that were detected by both single and multi-locus probing were only included in the single-locus mutation calculations. For single locus probes, a maximum of two bands (one from each parent) can be scored per pup per probe; however, two bands could not always be scored due to problems with band clarity, resulting in variable numbers of bands assayed by each probe. Some blots were not of good enough quality to score for MMS10, but still showed clear bands for the single locus probes, resulting in fewer litters and pups scored for the MMS10 probe. Mutations that were shared among littermates (clustered mutations) and somatic mutations (extra non-parental single-locus bands) were not included in the analysis. Due to adequate heterozygosity of the single locus alleles, the parental mice did not usually share alleles. This made it possible to determine the parental origin of bands detected by single locus probes, permitting the calculation of mutation rates for the paternal and maternal germ lines. Scoring was done blind to treatments and an independent observer scored a random sub-sample of gels.

Genotyping

All genotyping was done by AECL Chalk River Laboratories according to the following protocol: The Trp53 genotypes of the mice were determined using polymerase chain reaction (PCR). The primer sequences used for amplification of the wild-type Trp53 allele were from exon 6 (EX658: 5'-CAGCGTGGTGGTACCTTATGA-3') and exon 7 (W3': 5'-TATACTCAGAGCCGGCCT-3' ;Jacks *et al.*, 1994). The PCR product length was ~470 base pairs. The deletion allele was identified using the *neo* generic primers suggested by Jackson Laboratories, (IMR013: 5'-

CTTGGGTGGAGAGGCTATTC-3' and IMR014: 5'-AGGTGAGATGACAGGAGATC-3'). The *neo* PCR product was ~280 base pairs long.

The 25 μL PCR incubation mixture contained: 100mM Tris, pH 8.3; 10mM $MgCl_2$; 50mM KCl; 0.01% gelatin (Sigma); all four deoxynucleotides (Life Technologies), each at 200μM; 25pmol of each of the four primers (Life Technologies); 1.25 units of Ampli-TAQ Gold DNA polymerase (Perkin-Elmer) and ~ 15ng of template DNA. A 20μL mineral oil overlay was used to reduce evaporation during cycling. PCR was carried out in a PTC-100 thermal cycler (MJ Research). The cycling conditions were: 95°C for 5 minutes; 40 cycles of 95°C for 30 seconds, 51°C for 30 seconds and 72°C for one minute; 72°C for 5 minutes. The reaction products were visualized using ethidium bromide fluorescence following electrophoresis in a 1.5% agarose gel. Digital images were captured using a CCD video camera and Image Pro computer software.

Mutation Rate Calculations and Statistical Analyses

Per band mutation rates were calculated by dividing the number of mutations scored (≥ 1mm shifts) by the total number of offspring bands scored per treatment group. Data were tested using a two-tailed Fisher's exact test for pair-wise comparisons. Lack of statistical significance in uncorrected *P*-values eliminated the need to Bonferroni correct for multiple pair-wise comparisons. 95% confidence intervals for proportions were derived from the Poisson distribution (Sachs, 1983). Paternal per-family mutation rates for single locus ESTR probes (*Ms6Hm* + *Hm2)* combined, and the multi-locus probe MMS10 were compared using a separate *one-way* ANOVA test per probe type

(Appendix I). Per-family mutation rates were log-transformed in order to meet the assumptions of the ANOVA. The log-transformed data passed Bartlett's Test for Equal Variances and were tested for heteroscedasticity by plotting the residuals of the log-transformed data by treatment group.

Mean litter sizes and standard deviations were calculated for each treatment group. Litter sizes were compared between treatment groups and between breeding times using a *one-way* ANOVA. The litter size data were cosine transformed in order to meet the assumptions of the ANOVA. The cosine transformed data passed Bartlett's Test for Equal Variances and were tested for heteroscedasticity by plotting the residuals of the cosine-transformed data by treatment group. There were insufficient degrees of freedom to perform a Main Effects ANOVA to test the effect of both breeding time and treatment on litter size.

Pup genotype ratios were tested using a chi-square analysis with the expected values calculated as 50% of the total pups obtained per treatment group. Due to the increased probability of making a Type 1 error with multiple pair-wise comparisons, *P*-values were Sequential Bonferroni corrected for multiple pair-wise comparisons (Holm, 1979).

Testing Mutation Detection Power

Given that the *Hm2* loci in the C57Bl/6J mouse line that we were using were very large (>11 kb), we were concerned that there was insufficient migration of *Hm2* fragments to detect a significant increase in mutation frequency. In order to test this, 15 samples that were originally scored as showing band shifts of 0.5mm (i.e. did not meet

the criteria to be considered a mutation, but still showed a deviation from the parental band) for the *Hm2* locus were chosen from random treatment groups. These samples were run alongside their parental DNA on a 0.8% 42cm agarose gel at 1.66V/cm for 48 hours, Southern Blotted and probed for *Hm2* and the lambda in-lane marker and scored using the 1mm band shift criterion. The 42cm gel trays used for this test were custom made for fingerprint profiling mice with large differences in the sizes of the *Ms6Hm* and *Hm2* alleles. Somers *et al.* (*accepted [June, 2004] for publication in Mutat. Res.*) were able to show a significant increase in germ line mutation in males exposed to 1Gy of gamma rays using this equipment, voltage/cm and scoring criteria.

Results

Spermatogonia-Irradiated Treatments

For the spermatogonia-irradiated treatments, there were no statistically significant differences in overall (maternal and paternal combined for both genotypes) mutation rates by treatment group compared to the unirradiated control group as determined by individual probes or all probes combined (Table 3). Similarly, there were no statistical differences in mutation rates between any treatment group and the unirradiated control group when mutation rates are divided by parental origin of bands (Table 4). When paternal mutation rates were separated by pup genotype, 0.1+1Gy produced a statistically higher per band mutation rate than the 0Gy control for the trp53+/+ pups (two-tailed Fisher's Exact P=0.023) and 0.01+1Gy produced a significantly higher per band mutation than the 0Gy control for trp53+/- pups (two-tailed Fisher's exact P=0.03; Table 7);

however, both *P*-values are rendered insignificant upon Sequential Bonferroni correction for multiple pair-wise comparisons. For maternal mutation rates, there were no statistically significant differences in mutation rates between any treatment groups for either genotype (Table 7).

Spermatozoa-Irradiated Treatments

For the spermatozoa-irradiated treatments, there were no statistically significant differences in overall (maternal and paternal combined for both genotypes) mutation rates by treatment group compared to the unirradiated control group as determined by individual probes or all probes combined (Table 5). Similarly, there were no statistical differences in mutation rates between any treatment group and the unirradiated control group when mutation rates are divided by parental origin of bands (Table 6). When mutation rates were separated by pup genotype, a two-tailed Fisher's Exact Test for pair-wise comparisons determined that the per band mutation rate for paternally-derived bands in trp53+/- pups whose fathers were irradiated with 0.01Gy priming dose 24 hours prior to a 1Gy challenge dose and bred immediately thereafter was significantly lower than that for trp53+/- pups whose fathers were given a 0.1Gy priming dose 24 hours prior to a 1Gy challenge dose (p=0.01; Table 8). This, however, was rendered insignificant upon Sequential Bonferroni-correction for multiple pair-wise comparisons. There were no statistically significant differences between any treatment groups for maternal mutation rates separated by pup genotype (Table 8).

Pup Genotype Ratios

Uninterrupted Mendelian inheritance predicts that a heterozygous x homozygous mating should produce 50% homozygous offspring and 50% heterozygous offspring. We compared the number of trp53+/+ pups to the number of trp53+/- pups for each treatment group and for all treatments combined using a Chi-square test, where the expected value was calculated as half of the total number of pups obtained (Table 9). We found that there were significantly more trp53+/- pups in three groups: the unirradiated control (P= 0.022), spermatozoa-irradiated 0.01+1Gy (P= 0.033) and spermatogonia-irradiated 0.1+1Gy (P= 0.041). However, when the P-values were adjusted for multiple pair-wise comparisons using a Sequential Bonferroni correction, all three p-values were no longer >0.05 (Table 9). When the data for the spermatozoa-irradiated groups were pooled, there was no bias towards either pup genotype (P=0.252). When the data for all spermatogonia-irradiated groups were pooled, there was a highly significant bias towards heterozygous pups, which withstood Bonferroni correction (P=0.014; Table 9).

Litter Sizes

Mean litter sizes ± standard deviations are presented in Table 10. Litter sizes were compared among treatment groups both between and within breeding times using multiple *one-way* ANOVA analyses (Table 11). Litter size data were cosine transformed in order to meet the assumptions of the ANOVA. Analyses by treatment group determined that there were no statistical differences in litter size among spermatozoa-irradiated treatment groups (P= 0.37641) or among spermatogonia-irradiated treatment groups (P= 0.84848). A *one-way* ANOVA by breeding time determined that

spermatozoa-irradiated groups had significantly smaller litters than spermatogonia-irradiated groups and the unirradiated control (P= 0.00005).

ESTR Size and Mutation Detection

15 pups that had originally been scored as having band shifts of 0.5 mm (i.e. didn't meet the ≥1mm scoring criteria for mutations) were run using longer gel trays that had previously been used in a study that showed a significant increase in mutation rate with a 1Gy pre-meiotic dose (Somers *et al.*, *accepted [June, 2004] for publication in Mutat. Res.*). 14 out of 15 samples could be confidently scored, and 11 out of 15 of the re-run samples did not show a detectable mutation upon re-analysis (Table 12). There were three samples (1175, 1703 and 132) that were determined to carry mutations from the 48cm gel. These additional mutations do not affect the original findings when added to the data set.

Discussion

Adaptive Response

Given that p53 is considered the guardian of the genome for its role in many cell damage responses, p53 is often suspected for playing an essential role in the adaptive response mechanism (Sasaki *et al.*, 2002). Indeed, Sasaki *et al.* (2002) found that trp53-/- mouse embryonic fibroblasts were unable to undergo x-ray induced adaptive response, whereas their trp53+/+ counterparts were fully capable of the affect. This, however, was determined *in vitro* using a somatic cell line, which may bear little relevance to a whole-

organism *in vivo* study of germ line mutations. We hypothesized that if p53 is integral to the mechanism for radiation-induced germ line mutations, pre-irradiation of male mice with a 0.01 or 0.1Gy priming dose would produce less of an adaptive response effect than in a fully functional trp53+/+ mouse. 1Gy alone, however, did not result in any increase in mutation rate for either breeding time in our study, so any protective effect of pre-irradiation could not be detected.

We also included two groups that received 0.01Gy or 0.1Gy alone, without a challenge dose of radiation. Mitchel *et al.* (2003) found that low dose irradiation of cancer-prone trp53+/- mice acted to protect against spontaneous lymphomas and spinal osteosarcomas by increasing the latency period of the cancers. For paralysis due to spinal osteosarcomas, 0.01Gy dose significantly increased the latency by 58%. A 0.1Gy dose initially increased latency by 37%, but this was soon nullified by the accelerated occurrence of later-appearing tumours. For death due to lymphomas, both the 0.01 and the 0.1Gy dose significantly increased latency ($P<10^{-4}$) compared to unirradiated controls.

The researchers suggest that low doses of radiation act to inhibit mechanisms associated with genomic instability (Mitchel *et al.*, 2003). With the groups that received only 0.01 or 0.1Gy, we were able to test whether a low, priming dose of radiation could act to decrease the spontaneous rate of germ line ESTR mutations. Since ESTRs are known to show high levels of genomic instability compared to other regions of the genome, we hypothesized that this effect might also be observed with germ line ESTR mutations. If this were true, it could indicate a link between protection against ESTR

mutations and protection against cancer, perhaps suggesting a role of ESTRs in radiation-related cancer induction.

We found that there were no significant differences in mutation rate between either the 0.01 or 0.1Gy group and unirradiated control ($P>0.14$; Table 3), indicating that a priming dose alone is not able to induce an adaptive response to spontaneous germ line mutations at ESTR loci in trp53+/- mice. This may be because spontaneous ESTR mutations are not susceptible to a radiation-induced adaptive response. Or, it could be due to the p53 deficiency of the mice, which would suggest that p53 is indeed involved in the adaptive response to germ line mutations.

ESTR Size and Mutation Detection

ESTR loci can range in size from 3-16kb (Yauk, 2004). For isogenic or inbred mice, allele sizes for *Ms6-hm* and *Hm-2* loci are consistent across individuals, whereas these loci can be highly variable in size in an outbred strain (Somers *et al.*, *accepted [June, 2004] for publication in Mutat. Res.*). Allele size can influence the calculated mutation rate in two ways: 1) Larger alleles tend to show more instability (Buard *et al.*, 1998). This is thought to be because longer arrays have more repeat units and therefore present more opportunity for replication slippage events (Yauk, 2004). 2) Larger alleles migrate more slowly than small alleles through an agarose gel. This means that for a smaller allele, an insertion or deletion of a given number of repeat units would show more differential migration and therefore would be more likely to meet the minimum scoring criteria (i.e. ≥ 1mm shift from the parental band) than a larger allele. That is, our

scoring technique is more sensitive to mutations of smaller ESTRs. Thus, it is important to run gels for enough time to allow for sufficient migration of larger alleles in order to be able to obtain adequate data to detect statistically significant differences in mutation rates. This poses a problem because while larger alleles need more time/voltage to migrate sufficient distance, smaller alleles may migrate off of the gel. It is necessary to use a long enough gel to detect mutations of large alleles but not lose smaller alleles, and a small enough gel to still be able to physically handle it without too much risk of cracking or ripping the agarose.

The *Hm2* loci in the C57Bl/6J mouse line that we used were very large (>11 kb). Using custom made 42 cm gel trays, we tested whether the lack of increase in mutation rate following the 1Gy treatment in our study was a result of insufficient detection of mutations at *Hm2* loci by re-running 15 samples that had originally been scored as having 0.5 mm band shifts. The results indicate that band shifts were inconsistent for the same samples run on short and long gels. Therefore, it does not appear that running longer gels would have allowed for significantly more sensitive detection of mutations. Also, it is apparent that scoring for 0.5mm band shifts is not a reliable criterion for mutation detection.

Spermatogonia-Irradiated Treatments

Mutation Rates by Genotype

By separating mutation rates by parental origin and then by pup genotype, the sample size (i.e. total number of bands scored) is essentially divided into 4, thus significantly reducing statistical power. Further, separating mutation rates by genotype

doubles the number of treatment groups and therefore pair-wise comparisons possible, increasing the necessity for Bonferroni correction in order to reduce the chance of making a type 1 statistical error. We have seen elevated mutation rates above the unirradiated control for the trp53+/+ 0.1+1Gy group and the trp53+/- 0.01+1Gy group that are statistically significant prior to Bonferroni correction (P=0.023 and P=0.03 respectively; Table 7). However, given that these are rendered insignificant after Bonferroni correction, it seems reasonable to assume that these differences are simply a result of random chance and do not reflect biological processes that are not taking place in the unirradiated control.

Accepting the Bonferroni-corrected P-values and dismissing significant uncorrected P-values appears to make sense biologically because irradiating males 10 weeks prior to breeding allows time for spermatogenesis to take place. This ensures that fertilizing sperm were irradiated as spermatogonial stem cells. Spermatogonia are diploid, meaning that each homologous chromosome has equal probability of being damaged by radiation, regardless of whether it carries a functional p53 allele. It has demonstrated that in order for mutations at ESTR loci to occur, germ cells must be diploid and therefore pre-meiotic (Dubrova *et al.*, 1998_b). This means that once the germ cells reach the haploid stage, any ESTR mutations are already established. Each spermatozoa, therefore, should have equal probability of carrying ESTR mutations, regardless of p53 status. Thus, given what is currently known about the ESTR mutation mechanism, it does not appear to make biological sense to separate mutation rates of spermatogonia-irradiated treatment groups by genotype, and our Bonferroni-corrected P-

values for these data seem reasonable. Nevertheless, the mechanism for radiation-induced ESTR mutation induction is still poorly understood; the possibility of an unexpected result makes it necessary to examine all data despite pre-conceived notions of biological processes and expected results.

Paternal Mutation Rates

Previous studies have been consistent in showing a statistically significant dose response for radiation-induced mutations at ESTR loci in the paternal germ line when germ cells are irradiated prior to meiotic division (Dubrova *et al.*, $1998_{a,b}$; Dubrova *et al.*, 2000; Barber *et al.*, 2002; Somers *et al., accepted [June, 2004] for publication in Mutat. Res.*). The results of the present study are surprising in that we have seen no statistical differences in paternal mutation rates between any treatment groups. It is especially curious that there is no difference between the control group and the 1Gy treatment, as this treatment has been repeated numerous times within the literature using a variety of dose rates, radiation types and mouse strains, showing a significant dose response.

Living systems have developed several mechanisms for coping with the damaging effects of radiation. For example, antioxidant enzymes, repair and stress protein systems, and apoptosis all act to control cell damage and therefore long-term effects of radiation. Initiation of these systems takes place via a range of triggers that can be dependent on biological differences such as genetic background and organism health, or physical factors such as dose, duration of exposure and dose rate (Magae *et al.*, 2003). Further, overall effect on endpoints such as cell survival, oncogenesis, mutation or chromosome stability is likely due to a combination of these biological and physical factors, ultimately

making it difficult to tease out causal relationships and response trends. Nevertheless, it is important to try to examine every possible factor carefully in order to get a clear idea of what is actually influencing the results. The following sections discuss whether the lack of dose response in paternal mutation rates seen in our study reflects something within our protocol such as the genetic background of the trp53+/- mice, dose, dose rate or a subtle difference in technique. Also discussed is whether the p53 heterozygosity of the male mice prevented an increase in ESTR mutations, indicating an essential role of p53 in germ line mutations at these loci. It is apparent that in order to confidently interpret the results of the study, a control study using wildtype C57Bl/6J mice is required.

Genetic Background: C57Bl/6J mice

Genetic background appears to play an important role in determining mutation rates at ESTR loci. Both *Ms6-hm* and *Hm-2* show differences in mutation rates between inbred mouse strains (Barber *et al.*, 2002). Barber *et al.* (2002) demonstrated that rates of spontaneous and radiation-induced mutation rates differed considerably among three mouse strains examined, with BALB/c > CBA/H > C57Bl/6. From these data, it appears that C57Bl/6 mice may be somewhat resistant to ESTR mutations. It is unlikely, however, that this would contribute to the lack of dose response seen in our study. A genetic predisposition to lower spontaneous and induced mutation rates does not infer radioresistance, or resistance to radiation-induced responses. These mice still show a highly significant 4.2 fold increase in germ line mutation following a dose of 0.4Gy of

neutrons, indicating that this strain is indeed capable of radiation-induced ESTR mutations (Barber *et al.*, 2002).

Bhilwade *et al.* (2004) examined the relative radiosensitivity of 7 popular inbred mouse lines to radiation-induced micronuclei in bone marrow eurythrocytes. This appears to be the only published study to date that compares radiosensitivity to DNA damage across several inbred mouse lines. C57Bl/6 mice were found to show significantly increased levels of micronuclei, even at the lowest dose of 0.125Gy (γ-rays 0.46Gy/min), and were 4th out of the 7 strains tested for mean response to dose. The study clearly shows that radiation sensitivity is indeed genotype dependent, and that C57Bl/6 mice are fully capable of a dose-response to ionizing radiation (Bhilwade *et al.*, 2004). This, along with Barber *et al.*'s (2002) study indicate that does not appear that the genetic background of the mice plays a substantial role in the lack of mutation induction seen in our study.

Radiation Quality

Radiation quality is another factor that may contribute to the germ line mutation response. The only other published study that uses C57Bl/6 mice to study germ line mutations uses neutrons, which are much more densely ionizing (i.e. they have a higher LET) than gamma rays (Barber *et al.*, 2002). Generally, high LET radiation is capable of inducing endpoints associated with genomic instability at much lower doses than low LET radiation such as X-rays and gamma-rays (Smith *et al.*, 2003). This could explain why Barber *et al.* (2002) see a highly significant 4.2 fold increase in germ line mutation

rate following 0.4Gy of neutrons whereas we see no increase after 1Gy of gamma irradiation. In the same study, however, the authors irradiated 5 CBA/H males with 2Gy of x-rays and compared their germ line mutation rates to the 5 CBA/H males irradiated with 0.4Gy of neutrons and found very similar mutation rates of 0.192 and 0.209 respectively, indicating that mutation rate is not largely affected by radiation quality (Barber *et al.*, 2002). Further, there are a number of studies that report a significant increase in germ line mutation frequency following 1Gy of gamma rays or x-rays, using a variety of mouse strains and dose rates (Dubrova *et al.*, $1998_{a,b}$; Dubrova *et al.*, 2000; Somers *et al.*, *accepted [June, 2004] for publication in Mutat. Res.*). Thus, it seems unlikely that radiation quality plays a large role in our results.

Dose Rate

Researchers have often debated how dose rate ultimately affects a biological system (Vilenchik and Knudson, 2000). There are numerous published studies that examine radiation-induced biological effects at a variety of dose rates in an attempt to derive a generalized mathematical model that predicts dose rate effects. Almost all of these studies, however, were conducted using *in vitro* assays with a variety of cell types from commercially bought, genetically altered cell lines to isolated human lymphocytes. These models have little relevance to our study given that they were derived from somatic cells and were not conducted under *in vivo* conditions that allow for a whole-organism response. Given the importance of preserving genomic integrity across generations, germ cells may be expected to have evolved especially stringent and

efficient repair mechanisms compared to somatic cells, and therefore should have different responses to dose-rate than somatic tissues. Thus, models that predict dose rate responses for somatic tissues are perhaps not applicable to genetic tissues.

Dubrova *et al.* (1998_b, 2000) compared radiation-induced mutation rates at ESTR loci in the paternal germ line induced by low-dose rate exposure to γ-radiation and high-dose rate fission neutrons. They found that with a very low dose rate of 0.166mGy/min, a pre-meiotic total dose of 0.5Gy and 1Gy caused a statistically significant increase in the paternal mutation rate from unirradiated controls. The dose-response fit a linear regression relationship between number of mutations scored and total radiation dose. Further, comparison with their previous data indicated that there are no substantial differences in mutation induction by acute exposure of 0.5Gy/min for the same protocol. The researchers also found that chronic irradiation with high LET fission neutrons is much more effective than acute or low-dose exposure for induction of ESTR mutations in the paternal germ line (Dubrova *et al.*, 1998_b; Dubrova *et al.*, 2000).

In the present study, we have used to different dose rates: 0.5mGy/min for the priming doses and 300mGy/min for the challenge dose. For the challenge dose, both the dose and the dose rate are well above those used by Dubrova *et al.* (1998_b, 2000) Thus, it is unlikely that the lack of dose response seen in our 1Gy treatment groups is a result of the dose rate used.

The only published study that examines adaptive response in the mammalian germ line was previously done by our group, using dose rate of 360mGy/min for both the 0.1Gy priming dose and the 1Gy challenge dose (Somers *et al.*, *accepted [June, 2004] for*

publication in Mutat. Res.). This is a much higher priming dose rate than that used in the current study. There is some evidence that the optimal dose rate for an adaptive response in somatic cells is approximately 0.01Gy/min, and that protection against induced mutations declines with further decrease in the dose rate of the priming dose (Cortes, 1994). This, however, was determined by measuring micronucleus formation in human lymphocytes, and therefore should be interpreted and extrapolated with caution. Nevertheless, if this is true for germ cells, it is possible that our priming dose rate may have been too low to induce a detectable adaptive response either to the 1Gy challenge dose or to endogenous DNA damage.

The Role of p53 in ESTR Mutation Induction

The current model for the ESTR mutation mechanism proposes that DNA repair that is induced by radiation damage (e.g. DNA adducts and strand breaks) allows for replication slippage to take place, resulting in insertion and deletions of repeat units. One problem with this model is that the number of radiation-induced damage evens predicted for a given radiation dose cannot account for the number of induced mutations at ESTR loci for that dose (Niwa and Kominami, 2001; Dubrova *et al.*, 2000). It is estimated that an 8-μm diameter spherical cell would experience approximately 12 tracks of radiation per 1Gy of ^{252}Cf neutrons (Dubrova *et al.*, 2000). This, combined with the small target size of up to 16kb for ESTRs Ms6-Hm and Hm-2, cannot account for the 4-6 fold increases in paternal mutation rates seen in the literature if this direct radiation-induced model for mutation at these loci is assumed (reviewed in: Bridges, 2003; Niwa, 2003;

Dubrova, 2003). This suggests that the radiation-induced elevated mutation rates at ESTR loci result from non-targeted radiation damage elsewhere in the genome or cell.

A more recently proposed hypothesis suggests that a damage response signal acts within the cell in response to radiation-induced damage either within that cell, or via a bystander effect from a neighbouring cell. The signal is thought to trigger a stall or pause in DNA replication at *all* DNA polymerase sites (Barber *et al.*, *accepted [12 May, 2004] for publication in Mutat. Res.*; Yauk, 2004). This allows for replication slippage to take place at all stalled polymerase sites, and misaligned repeat units result in changes in strand length upon completion of DNA replication (Barber *et al.*, *accepted [12 May, 2004] for publication in Mutat. Res.*; Yauk, 2004). Such global stalling of DNA polymerase molecules could theoretically account for mutations at ESTR sites that were not directly hit.

The above model depends on intact DNA damage response systems to trigger global stalling of DNA polymerase. As such, transgenic mouse lines deficient in essential damage response proteins can be effectively used to test the hypothesis. Recently, Barber *et al.* (*accepted [12 May, 2004] for publication in Mutat. Res.*) used severe combined immunodeficient (*scid*) mice and poly(ADP-ribose) polymerase (PARP-1) deficient mice to test whether deficiencies in these DNA-damage response genes can effect spontaneous and induced ESTR mutation rates in their germ lines. Key to this study was the use of isogenic wildtype control mice, which allows for the comparison of spontaneous and induced mutation rates between protein-deficient and wildtype mice.

The first important finding of this study was that the spontaneous mutation rates for both *scid* and PARP-$1^{-/-}$ mice were significantly higher than their wildtype counterparts, with 1.86 and 2.52 fold increases respectively (Barber *et al., accepted [12 May, 2004] for publication in Mutat. Res.*). Since the deficient and wildtype strains are isogenic, this can be directly attributed to the *scid* and PARP-1 mutations. Given that both transgenic strains are defective in early responses to DNA damage, it is hypothesized that they experience delayed repair due to the inability to recognize endogenous DNA damage. This allows for damage to accumulate in the tandem repeat sequences, which in turn could result in replication fork pausing. This could allow for replication slippage events to take place, ultimately elevating insertion and deletion frequencies and therefore mutation rates (Barber *et al., accepted [12 May, 2004] for publication in Mutat. Res.*).

The researchers' second important finding is that irradiation of *scid* and PARP-$1^{-/-}$ mice with 1Gy X-rays delivered at 0.6Gy min^{-1} did not result in significant increases in mutation rates whereas it did in their wildtype counterparts (Barber *et al., accepted [12 May, 2004] for publication in Mutat. Res.*). Again, this can be directly attributed to the *scid* and PARP-1 genes. The authors hypothesize that this is a result of high cell killing effects in the germ line of deficient mice due to the preferential killing of irradiated germ cells that show substantially delayed replication. This hypothesis is supported by reduced litter sizes for the deficient strains compared to their wildtype counterparts (Barber *et al., accepted [12 May, 2004] for publication in Mutat. Res.*).

Our results are similar to the above study in that we have not seen an increase in germ line mutation rate following a 1Gy dose of radiation. Without the wildtype control comparison, however, we do not know whether this is a result of our protocol or of the p53 protein status of these mice. Nevertheless, given that p53, like the *scid* and PARP-1 genes, is an essential component in early DNA-damage response pathways, we suspect that we are seeing a similar effect.

p53 plays an integral role in the G1/S cell cycle checkpoint (Iliakis *et al.*, 2003). The G1/S checkpoint functions to recognize damaged DNA, and delay the cell cycle so that the damage can be repaired before progressing into the replication, or DNA synthesis stage of the cell cycle (Iliakis *et al.*, 2003). During the G1/S checkpoint, DNA repair takes place via non-homologous end joining (NHEJ), which acts as a quick, emergency response system that hastily repairs lesions such as single and double strand breaks before the cell cycle progresses. Thus, without p53, and therefore without a functional G1/S checkpoint, it can be assumed that NHEJ does not take place, and damaged DNA gets passed on through the cell cycle. According to the hypothesis proposed by Barber *et al.*, (*accepted [12 May, 2004] for publication in Mutat. Res.*) such delayed repair is what allows for accumulated DNA damage and therefore promotes global stalling of DNA polymerase molecules later in the cell cycle, which intern promotes instability at ESTR loci. Thus, perhaps p53 as well as other proteins that are directly involved in early DNA damage responses are required in order for radiation-induced ESTR mutations to occur in the germ line. If this is the case, perhaps we are not seeing elevated mutation rates with a

1Gy treatment because the trp53+/- males in our study are unable to produce enough p53 to allow for the response to take place.

Epigenetic Effects

There is some evidence that irradiation of germ cells induces epigenetic effects. Barber *et al.* (2002) demonstrated that radiation-induced ESTR mutations exhibit transgenerational instability, whereby germ line mutations were detected in F_1 offspring of irradiated F_0 fathers. The transgenerational capability of radiation-induced germ line ESTR mutations implies that that there exists a heritable signal that triggers mutations at ESTR sites that is not a change in actual DNA sequence.

DNA methylation and chromatin condensation are two commonly proposed epigenetic factors induced by radiation (Pogribny *et al.*, 2004). These conditions that are commonly caused by environmental agents are replicated with DNA and are therefore passed on through generations. They can cause changes in gene expression or problems during replication (Dubrova, 2003). If this is the case with germ line ESTR mutations, it is conceivable that the epigenetic signal acts to trigger the global stalling of DNA polymerase molecules that is proposed by Barber *et al.* (*accepted [12 May, 2004] for publication in Mutat. Res.*), thus accounting for the transgenerational nature of germ line ESTR mutations.

Spermatozoa-Irradiated Treatments

The published data for radiation-induced germ line mutations at ESTR loci in males that are irradiated at post-meiotic stages of spermatogenesis are inconsistent both within and across studies (Sadamoto *et al.*, 1994, Niwa *et al.*, 1996, Dubrova *et al.*, 1998_b). There are a number of discrepancies among some of the earlier published studies for the sensitivity of post-meiotic germ cells to ESTR mutations (Sadamoto *et al.*, 1994; Niwa *et al.*, 1996), although more recently, a thorough investigation appears to have clarified that ESTR mutation induction only occurs in pre-meiotic germ cells (Dubrova, 1998_b). If induced ESTR mutations take place by replication slippage, we should not expect an increase in mutation rates in the irradiated spermatozoa because mature sperm do not undergo DNA replication or cell division, and are not capable of DNA repair (Ahmadi and Soon-Chye, 1999). As such, there is no opportunity for replication slippage to take place and ESTR mutation induction should not occur.

It is also well documented that elimination of highly damaged cells via apoptosis does not occur in mature sperm (Ahmadi and Soon-Chye, 1999). This means that any radiation-induced damage is carried through to fertilization, which has been confirmed by previous reports (Ahmadi and Soon-Chye, 1999; Shimura *et al.*, 2002_a). Thus, unless DNA-repair-induced replication slippage can take place after fertilization, but before the first cell division, we should not see elevated ESTR mutations in spermatozoa-irradiated pups. Any mutations taking place after the first cell division would appear as a third, non-parental band and be scored as a somatic mutation and excluded from the analysis.

Although it has been shown that normal damage-induced cell cycle checkpoints and apoptosis are absent or restricted in embryonic stem cells until the implantation stage of embryogenesis, there is evidence that some repair can take place shortly after fertilization but before the first cell division (Shimura *et al.*, 2002_a). This occurs during a damage-induced S-phase delay of the first cell cycle following fertilization (Shimura *et al.*, 2002_b). This process has been shown to be dependent on p53 status. Shimura *et al.* (2002_b) demonstrated that spermatozoa-irradiated (6Gy X-rays) zygotes showed no G1/S delay regardless of p53 status, but trp53+/+ mice took about 2 hours longer to complete S-phase. Microinjection of GST-p53 fusion protein into the cytoplasm of trp53-/- spermatozoa-irradiated zygotes resulted in the restoration of the S-phase delay. Further, the delayed zygotes were able to synthesize the normal amount of DNA, whereas those incapable of the delay had less than normal DNA content (Shimura *et al.*, $2002_{a,b}$).

If the p53 dependent S-phase delay were taking place in the spermatozoa-irradiated zygotes in our study, we would expect a difference in radiation-induced ESTR mutations between trp53+/- and trp53+/+ pups. That is, the homozygous pups would presumably be more capable of the delay than the p53-deficient heterozygotes. Therefore, if ESTR mutations are indeed induced during erroneous repair, the trp53+/+ pups should have elevated mutation rates compared to the p53 deficient trp53+/- pups. We did not find this. It should be noted, however, that Shimura *et al.* (2002_b) saw suppression of DNA synthesis during an induced S-phase delay in trp53+/- spermatozoa-irradiated zygotes. This indicates that perhaps the reduced amount of p53 produced by

heterozygous zygotes is sufficient to induce the S-phase delay. Unfortunately, without trp53-/- zygotes in our study, we cannot make this distinction.

Maternal Mutation Rates

In the current study, irradiated males are mated to unirradiated females. As such, one would not expect to see elevated rates of mutations in the maternal germ line as a direct response to radiation. There is, however, conflicting evidence that radiation-damaged mature spermatozoa induce mutations in the maternal germ line. Dubrova's group has never found such an increase (Dubrova *et al.*, 1998_a). They hypothesize that this is because in order for ESTR mutations to take place, cells must be dividing and capable of DNA repair (Dubrova *et al.*, 1998_a). In mammals, oogenesis takes place while the female is still in eutero (Jackson Laboratory, 1966), and therefore in order for genotoxin-induced ESTR mutations to take place in the maternal germ line, the pregnant maternal grandmother would have to be exposed (see Dubrova, 1998_a).

Niwa and Kominami (2001), however, found a significant 2-fold increase in germ line mutation frequency at ESTR loci in unirradiated maternal alleles when fertilized with irradiated sperm. These researchers later speculate that this may be a result of pronuclear cross talk in mouse zygotes (Shimura *et al.*, $2002_{a,b}$). This is a proposed system whereby irradiated sperm can trigger damage recognition responses that can be seen in the unirradiated maternal pronucleus, triggering ESTR instability in the maternal DNA (Shimura *et al.*, 2002_b). This response is not seen in p53 deficient model systems, suggesting that the cross-talk is p53 dependent (Shimura *et al.*, 2002_b). If this is the case,

we expect an increase in maternal mutation rates in trp53+/+ spermatozoa-irradiated pups compared unirradiated controls and compared to the p53-deficient trp53+/- siblings. We did not see such differences, therefore pronuclear cross talk did not influence ESTR mutations in our study.

Litter Sizes

We found no differences in litter sizes for exposed and non-exposed mice bred 10 weeks following treatment, and no difference in litter size between treatment groups for males bred 1-week following treatment (Table 11). We did, however, find that males bred 1-week following irradiation produced significantly smaller litters than unirradiated males and those mated 10-weeks following treatment (P=0.00005; Table 11). We can assume that this is not an age-related decline in fertility because the older mice produced larger litters, which is opposite from what would be expected from older mice (Jackson Laboratories, 1966). One major difference between the 1-week and 10-week breeding times is that mature spermatozoa are unable to undergo apoptosis whereas developing spermatogonia are readily apoptotic through both p53-dependent and p53-independent pathways (Beumer *et al.*, 1998; Hasegawa *et al.*, 1998). This means that radiation-damaged spermatogonia can be eliminated by apoptosis whereas damaged spermatozoa cannot. Also, dividing spermatogonia are capable of DNA repair whereas spermatozoa, being haploid with DNA tightly condensed into chromatin, cannot undergo any repair of DNA damage (Beumer *et al.*, 1998). Instead, radiation-damaged spermatozoa, regardless

of the degree of damage, survive to be ejaculated and have the ability to fertilize the oocyte (Ahmadi and Soon-Chye, 1999).

Embryonic development and survival, however, are highly related to the extent of radiation damage in spermatozoa (Ahmadi and Soon-Chye, 1999). Ahmadi and Soon-Chye measured the extent of DNA damage in mature spermatozoa following 0, 5, 10, 50 and 100Gy of gamma irradiation to semen of 10-12 week old C57BlxCBA male mice. Fertilization of oocytes was not significantly affected by radiation dose ($P>0.01$), but blastocyst development declined severely with dose, ranging from 49.8% in control mice to 20.3%, 7.8%, 3.4%, and 2.3% with sperm exposures of 2, 10, 50 and 100Gy respectively (Ahmadi and Soon-Chye, 1999). Thus, the reduced litter sizes for the spermatozoa-irradiated litters in our study could be explained by decreased survival of blastocysts fertilized with radiation-damaged sperm.

Genotype Ratios and Apoptosis

P53 has been widely implicated in the mechanism of apoptosis in highly damaged cells following ionizing radiation (reviewed in: Fei and El-Deiry, 2003). If p53 is essential in eliminating severely damaged spermatogonial cells, we should see highly elevated germ line mutation rates at ESTR loci in our study given that the male mice are p53 deficient. That is, given that the trp53+/- males in our study have half of the normal p53, fewer extremely damaged cells would killed by apoptosis than in trp53+/+ males. Therefore, DNA damage would persist, some being fixed into mutations and passed through the germ line resulting in elevated ESTR mutation rates.

The exact role of p53 in apoptosis, however, is not clear as a number of studies have shown that radiation-induced apoptosis can occur in p53 deficient model systems, that p53 dependent apoptosis is highly tissue specific, and that it is dependent on the level of accumulated damaged DNA or the level of genotoxic agent (Schwartz *et al.*, 1999; Lips and Kaina, 2001; Fei and El-Deiry, 2003; Offer *et al.*, 2002; Ohnishi *et al.*, 2000). There is also evidence of the existence of p53-independent mechanisms for induction of apoptosis in radiation-damaged cells (Kato *et al.*, 2002). This makes it impossible to predict how apoptosis could affect ESTR mutation rates in our study.

It is possible to use the pup genotype ratio data to infer some information about the role of p53-dependent apoptosis in irradiated germ cells. We tested whether the pup genotype ratios follow the 50:50 ratio that would be expected by uninterrupted Mendelian inheritance and found that there was a highly significant overall bias towards heterozygous offspring in the spermatogonia-irradiated treatments, which withstood Sequential Bonferroni correction (corrected P=0.014; Table 9). This could indicate that p53-dependent apoptosis acted to eliminate trp53+/+ offspring in these treatment groups at some point either pre- or post-fertilization. It is unclear whether this is a radiation-induced phenomenon because this trend is also seen the 0Gy control, although it did not withstand Bonferroni correction. Therefore, it seems that this is a p53-dependent apoptotic system that acts to eliminate cells that experience endogenous and/or radiation-induced damage.

Prior to meiosis, all developing germ cells were identical in terms of p53 status. As such, any p53-dependent system should affect all cells equally. Thus, it appears that

in order to affect pup p53 genotype ratios, any p53-dependent apoptosis would have to take place after meiosis, when cells differed in p53 genotype. Given that there was no bias towards either genotype in the spermatozoa-irradiated treatments (P=0.378; Table 9), and that it is well known that mature spermatozoa are not capable of apoptotic responses (Beumer *et al.*, 1998), it is apparent that this p53-dependent response must have taken place prior to the spermatozoa stage or after fertilization. If, however, apoptosis had taken place after fertilization, we would have seen significantly smaller litters for spermatogonia-irradiated treatments, which is opposite from our actual results. Thus, it appears that there exists a p53-dependent apoptotic system that acts to eliminate damaged developing sperm, and that this system takes place during the spermiogenesis stage of spermatogenesis.

Conclusion

The spermatogonia-irradiated mutation rate results of this study could have significant implications for the understanding of the mechanisms responsible for germ line mutations at ESTR loci. Given that it is unlikely that genetic background, radiation quality, dose or dose rate play a large role in our results, it is conceivable that we have not seen the expected elevation in germ line mutation rate following a 1Gy exposure to dividing spermatogonia because p53 homozygosity is required for such mutations to take place. If this is the case, it would provide significant insight to the mechanism of ESTR mutation induction. Also, it is well established that p53 is significantly involved in cancer susceptibility as well as other endpoints that are associated with genomic

instability such as genetic disease (Jacks *et al.*, 1994). Understanding the role p53 in the ESTR mutation pathway could provide insight to the relationship between ESTR instability and such endpoints. Finally, understanding the mechanism of ESTR mutation induction could allow for further understanding of the genetic dangers and/or benefits of radiation exposure, as well as exposure to other genotoxic agents that are capable of inducing mutations at ESTRs.

To implicate p53 as the primary cause of our results, we need to compare the results from the trp53+/- mice to an isogenic wildtype control in a post-hoc experiment that repeats every aspect of the current experiment (see Appendix II). Also, studies using micro-array analysis of protein expression following irradiation of germ cells at various stages of development would provide further insight to the role of p53 in ESTR mutation induction.

Appendix I

Per-Family Mutation Analyses

There has been some debate as to whether treating individual pups as distinct units is biologically and/or statistically valid. Given that all pups within a family come from the same paternal germ lines, some researchers speculate that mutation rates should be calculated on a per-parent basis rather than a per-pup basis in order to accommodate for any inherent differences in individual parents that may affect germ line mutation rates. This means that all pups within a family are condensed down to one per-family mutation rate for each parent, drastically reducing the sample size per treatment group, especially in our protocol which mates 4 females to each male in order to reduce the

number of transgenic mice needed for the experiment. Based on previous studies, we would most likely expect to see a significant increase in paternal mutation rates in our study. Therefore, a *one-way* ANOVA analysis was done comparing per-male mutation rates by treatment group for each probe type. The results are consistent with per-band mutation rates in that there are no significant differences between any treatment groups for either breeding time (Table 13).

Appendix II

Wildtype Control Study

When the above study was designed, it was thought that by using mice that are heterozygous for the p53 gene, the study had an elegantly built-in control group. Given that both wildtype (trp53+/+) and trp53+/- offspring were expected, it was assumed that there was no need for a separate batch of wildtype males to be incorporated into the study as controls and that we could simply compare mutation rates for the two pup genotypes. As the study progressed, it was realized that the built-in control might not be valid for the spermatogonia-irradiated pups since the fertilizing sperm was diploid at irradiation. Since ESTR mutations are thought to take place prior to chromosome separation at meiosis, each homologous chromosome would theoretically have equal chance of carrying mutations regardless of p53 status. This means that in theory, all mature sperm have the same probability of carrying mutations despite differences in p53 status. Thus, it was necessary to design a post-hoc control for the 10-week breeding component of the study using wildtype C57Bl/6 mice.

It would have been ideal to repeat the entire study (or at least the 0Gy and 1Gy groups) using both trp53+/- mice and wildtype controls in order to guarantee identical conditions for both groups. This, however, was not feasible in terms of the resources available. Instead, we designed a study that acts both as a post-hoc control for the p53 project, and is also a separate study in itself. Using only trp53+/+ males, we've added a 2Gy wildtype treatment group in order to test the radiosensitivity of C57Bl/6 mice and to produce somewhat of a dose-response curve for radiation-induced germ line mutations in the wildtype mice. We've also included a wildtype adaptive response group, which receives a 0.1Gy priming dose 24 hours prior to a 1Gy challenge dose, replicating Somers *et al.*'s (*accepted [June, 2004] for publication in Mutat. Res.*) study. Finally, a wildtype priming dose alone (0.1Gy alone) group has also been included, which was lacking in the previous study, and should provide further insight to the adaptive capabilities of germ line mutations at ESTR loci (Table 14).

If we see a significant increase in germ line mutation rate at ESTR loci in wildtype males following a 1Gy treatment, as is expected given the published data for this type of study, it could clarify our results in a number of ways. Firstly, we could assume that neither strain-specific ESTR resistance nor strain-specific radio-resistance caused our previous results. Secondly, insufficient dose or dose-rate could be eliminated as causes. Finally, we could be fairly assured that the results are not due to a flaw in our protocol given that the control mice were and will be treated as identically as possible to the original samples. This means that if the wildtype control study does show a

significant dose-response, we can be much more confident in indicating p53 as an essential player in the ESTR mutation mechanism.

Currently the wildtype samples are ready to be analyzed. The males were irradiated in April, 2004, and were housed in individual cages for 10 weeks. They were then randomly assigned to 4 females, left in their breeding groups for 2 weeks, and females were separated into individual cages. Females were euthanized at approximately day 18 of gestation, and pups were removed via caesarean section. Tissue samples were taken from parental tails and whole pups stored at 4°c to await analysis. Every effort was taken to ensure that these mice were treated identically to those in the previous study in order to constitute a valid control.

Wildtype Litter Sizes

Mean litter sizes ± standard deviations are presented in Table 15. There are significantly smaller litters for the spermatozoa-irradiated trp53+/- males than for the spermatogonia-irradiated trp53+/- and trp53+/+ males (Table 16; P=0.00028). Litter sizes for the wildtype males did not differ significantly from those of the trp53+/- spermatogonia-irradiated males (Table 16; P=0.90021). This is different from Barber *et al.*'s (*accepted [12 May, 2004] for publication in Mutat. Res.*) study, which showed that litter sizes in radiation-exposed PARP-$1^{-/-}$ and *SCID* mice were significantly smaller than to their non-exposed and wildtype counterparts. They attribute the decreased litter sizes in the DNA repair-deficient mice to increased radiosensitivity resulting in increased cell death of their germ cells. Given that we do not see the same trend with the p53 deficient

and isogenic wildtype control mice, we can speculate that p53 heterozygosity does not have a significant effect on radiation-induced cell death of dividing germ cells.

Appendix III

Glossary of Radiation Biology Terms

Adaptive Response – Radiation-induced adaptive response refers to the phenomenon whereby harmful effects (either endogenous or radiation-induced) are mitigated by exposure to a low, priming dose of radiation. The adapted cells/organism may show responses such as increased survival, increased resistance to disease, and/or decreased genomic instability than those that are not given the priming dose.

Doubling Dose – The amount of radiation required to double the incidence of a somatic or genetic effect.

Germ line Effects – Germ line effects are seen in the offspring of the individual that receives the radiation dose.

Gray (Gy) – The gray is the unit that describes the amount of energy absorbed by some material. It is equal to one joule of energy deposited in one kilogram of material, relating to any type of radiation or material.

Linear Energy Transfer (LET) – The rate of energy deposited along the track of an ionizing particle per unit track length (usually expressed in keV/μm)

Minisatellite – Minisatellites are repetitive regions of non-coding DNA that consist of repeated units of 10-100 base pairs. Individual minisatellites can be 100-2000 base pairs long. They are inherently unstable and are generally susceptible to higher rates of mutation than other sequences of DNA.

Microsatellite – Microsatellites are short (generally less than 100 base pairs) repetitive regions of non-coding DNA that consist of repeated units of 1-4 base pairs.

Relative Biological Effectiveness (RBE) – A measure of how damaging a given type of radiation is when compared to an equivalent dose of x-rays.

Roentgen (R) – The roentgen is a unit used to measure radiation exposure by gamma or X-rays in air. One R of X-rays or gamma rays is required to produce ions carrying one electrostatic unit of electric charge in $1cm^3$ of dry air under standard conditions.

Sievert (Sv) – The sievert is used to describe the equivalent dose, which relates the absorbed dose in human tissue to the effective biological damage of the radiation. 1Sv =

absorbed dose (Gy) x Q (the quality weighting factor that is unique to the type of incident radiation).

Somatic Effects – Somatic effects are seen in the individual that receives the radiation dose.

Teratogenic Effects - Teratogenic effects are non-hereditary effects that are induced in the offspring of the individual that receives the dose. Irradiation must take place during the gestation period.

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Tables

Table 1. Treatment Groups for Spermatozoa-Irradiated Mice[a]

Group Number	Male Age at Treatment	Male Age at Mating	Female Age at Mating	Priming Dose	Challenge Dose
S1	9 weeks	9 weeks	8 weeks	0Gy	1Gy
S2	9 weeks	9 weeks	8 weeks	0.01Gy (10mGy)	1Gy
S3	9 weeks	9 weeks	8 weeks	0.1Gy (100mGy)	1Gy

[a]There is no age-matched 0Gy control for the spermatozoa-irradiated groups. Instead, Group SG6 (see table 2) is used as a control for both breeding times.

Table 2. Treatment Groups for Spermatogonia-Irradiated Mice

Group Number	*Male Age at Treatment*	*Male Age at Mating*	*Female Age at Mating*	*Priming Dose*	*Challenge Dose*
SG1	9 weeks	19 weeks	10 weeks	0Gy	1Gy
SG2	9 weeks	19 weeks	10 weeks	0.01Gy (10mGy)	1Gy
SG3	9 weeks	19 weeks	10 weeks	0.1Gy (100mGy)	1Gy
SG4	9 weeks	19 weeks	11 weeks	0.01Gy (10mGy)	0Gy
SG5	9 weeks	19 weeks	12 weeks	0.1Gy (100mGy)	0Gy
SG6 (control)	9 weeks	19 weeks	11 and 12 weeks	0Gy	0Gy

Table 3. Sample sizes and overall (maternal and paternal combined) mutation rates for spermatogonia-irradiated treatment groups (i.e. males were bred 10 weeks following irradiation).

Treatment	*Probe*	*# of Litters*[a]	*# of Pups*	*# of Mutant Bands*	*# of Bands Scored*	*Mutation Rate*[b]	*Ratio to 0Gy Control*	*P-value*[c]
Control[d]	Ms6-Hm	16	89	12	142	0.085		
	Hm-2	16	89	9	161	0.056		
	Single			21	303	0.069		
	MMS10	10	57	8	548	0.015		
	Total			29	851	0.034		
1Gy	Ms6-Hm	14	78	17	153	0.111	1.306	0.245
	Hm-2	14	78	9	146	0.062	1.107	0.374
	Single			26	299	0.087	1.206	0.183
	MMS10	14	78	20	772	0.026	1.733	0.124
	Total			46	1071	0.0429	1.263	0.120
0.01+1Gy	Ms6-Hm	14	79	13	138	0.094	1.106	0.319
	Hm-2	14	79	15	143	0.105	1.875	0.118
	Single			28	281	0.100	1.450	0.114
	MMS10	14	79	13	607	0.021	1.400	0.247
	Total			41	888	0.046	1.353	0.092
0.1+1Gy	Ms6-Hm	16	90	16	160	0.100	1.176	0.288

	Hm-2	16	90	10	121	0.083	1.482	0.267
	Single			26	281	0.093	1.348	0.154
	MMS10	13	73	8	476	0.017	1.133	0.380
	Total			34	757	0.045	1.321	0.115
0.01Gy	Ms6-Hm	14	80	10	149	0.067	0.788	0.306
	Hm-2	14	80	7	153	0.046	0.804	0.378
	Single			17	302	0.056	0.812	0.218
	MMS10	11	63	8	634	0.013	0.867	0.379
	Total			25	936	0.027	0.78	0.149
0.1Gy	Ms6-Hm	14	77	11	127	0.087	1.024	0.343
	Hm-2	14	77	7	127	0.055	0.982	0.406
	Single			18	254	0.071	1.023	0.262
	MMS10	16	89	22	773	0.028	1.867	0.080
	Total			40	1027	0.039	1.14	0.171

[a]4-6 pups were arbitrarily chosen from each litter for analysis. [b]Calculated as number of mutant bands/number of bands scored. [c]Probability value as determined by a two-tailed Fisher's Exact test comparing irradiated groups to the unirradiated control. [d]The unirradiated control males used for both the spermatozoa-irradiated and spermatogonia-irradiated comparisons are age-matched to the spermatogonia-irradiated group (i.e. they are 10 weeks older than the males bred immediately after treatment).

Table 4. Sample sizes, maternal and paternal mutation rates for spermatogonia-irradiated treatment groups (i.e. males were bred 10 weeks following irradiation).

Group	*Mutant Bands*	*Bands Scored*	*Mutation Rate*[a]	*Ratio to Control*	*P-value*[b]
PATERNAL					
Control[c]	13	157	0.083		
1Gy	15	153	0.100	1.205	0.286
0.01+1Gy	16	143	0.111	1.337	0.230
0.1+1Gy	14	145	0.097	1.169	0.295
0.01Gy	10	156	0.064	0.771	0.289
0.1Gy	9	134	0.067	0.807	0.317
MATERNAL					
Control[d]	8	146	0.055		
1Gy	11	146	0.075	1.364	0.302
0.01+1Gy	12	138	0.087	1.582	0.228
0.1+1Gy	12	136	0.088	1.600	0.221
0.01Gy	7	146	0.048	0.873	0.403
0.1Gy	9	120	0.075	1.363	0.324

[a]Calculated as number of mutant bands/number of bands scored. [b]Probability value as determined by a two-tailed Fisher's Exact test comparing irradiated groups to the unirradiated control. [c]The unirradiated control males used for both the spermatozoa-irradiated and spermatogonia-irradiated comparisons are age-matched to the spermatogonia-irradiated group (i.e. they are 10 weeks older than the males bred immediately after treatment).

Table 5. Sample sizes and overall (maternal and paternal combined) mutation rates for spermatozoa-irradiated treatment groups (i.e. males were immediately following irradiation).

Treatment	*Probe*	*# of Litters*[a]	*# of Pups*	*# of Mutant Bands*	*# of Bands Scored*	*Mutation Rate*[b]	*Ratio to 0Gy Control*	*P-value*[c]
Control[d]	Ms6-Hm	16	89	12	142	0.085		
	Hm-2	16	89	9	161	0.056		
	Single			21	303	0.069		
	MMS10	10	57	8	548	0.015		
	Total			29	851	0.034		
1Gy	Ms6-Hm	14	77	19	136	0.140	0.167	0.130
	Hm-2	14	77	9	127	0.071	1.268	0.338
	Single			28	263	0.106	1.536	0.085
	MMS10	13	68	16	562	0.028	1.867	0.100
	Total			34	825	0.041	1.205	0.154
0.01+1Gy	Ms6-Hm	16	95	14	160	0.088	1.035	0.323
	Hm-2	16	95	11	150	0.073	1.304	0.306
	Single			25	310	0.081	1.174	0.215
	MMS10	12	71	11	477	0.023	1.533	0.100
	Total			36	787	0.046	1.352	0.103
0.1+1Gy	Ms6-Hm	14	83	13	149	0.087	1.023	0.329

	Hm-2	14	83	8	112	0.071	1.268	0.350
	Single			21	261	0.080	1.160	0.228
	MMS10	10	58	9	397	0.023	1.533	0.258
	Total			30	658	0.0456	1.341	0.116

[a,b,c,d] Footnotes are as in table 3.

Table 6. Sample sizes, maternal and paternal mutation rates for spermatozoa-irradiated treatment groups (i.e. males were bred immediately following irradiation).

Group	*# of Mutant Bands*	*# of Bands Scored*	*Mutation Rate*[a]	*Ratio to Control*	*P-value*[b]
PATERNAL					
Control[c]	13	157	0.083		
1Gy	15	132	0.114	1.370	0.228
0.01+1Gy	12	161	0.075	0.904	0.318
0.1+1Gy	11	140	0.079	0.952	0.322
MATERNAL					
Control[d]	8	146	0.055		
1Gy	13	131	0.100	1.812	0.158
0.01+1Gy	13	149	0.087	1.582	0.218
0.1+1Gy	10	121	0.083	1.510	0.271

[a,b,c]Footnotes are as in table 4.

Table 7. Sample sizes, paternal and maternal mutation rates by pup genotype for spermatogonia-irradiated treatment groups.

Group	*Pup Genotype*	*# of Mutant Bands*	*# of Bands Scored*	*Mutation Rate*[a]	*95% CI (lower, upper)*[b]
PATERNAL					
Control[c]	Trp53+/+	4	51	0.078	0.02, 0.2
	Trp53+/-	9	100	0.9	0.04, 0.17
1Gy	Trp53+/+	12	80	0.15	0.07, 0.26
	Trp53+/-	8	73	0.11	0.05, 0.22
0.01+1Gy	Trp53+/+	16	74	0.216	0.12, 0.35
	Trp53+/-	16	68	0.235	0.13, 0.38
0.1+1Gy	Trp53+/+	14	45	0.311	0.17, 0.52
	Trp53+/-	14	99	0.141	0.08, 0.24
0.01Gy	Trp53+/+	10	72	0.139	0.07, 0.26
	Trp53+/-	10	82	0.122	0.06, 0.23
0.1Gy	Trp53+/+	8	61	0.131	0.06, 0.26
	Trp53+/-	7	73	0.1	0.04, 0.2
MATERNAL					
Control[d]	Trp53+/+	8	48	0.167	0.07, 0.33
	Trp53+/-	8	92	0.087	0.04, 0.28
1Gy	Trp53+/+	11	76	0.145	0.07, 0.26
	Trp53+/-	11	70	0.157	0.08, 0.28
0.01+1Gy	Trp53+/+	12	71	0.169	0.09, 0.3
	Trp53+/-	12	65	0.185	0.09, 0.32
0.1+1Gy	Trp53+/+	12	46	0.261	0.13, 0.46

	Trp53+/-	12	89	0.135	0.07, 0.24
0.01Gy	Trp53+/+	7	70	0.1	0.04, 0.21
	Trp53+/-	7	74	0.095	0.04, 0.2
0.1Gy	Trp53+/+	9	53	0.17	0.08, 0.32
	Trp53+/-	9	67	0.256	0.061, 0.26

[a]Calculated as number of mutant bands/number of bands scored. [b]95% confidence intervals were derived from the Poisson distribution. . [c]The unirradiated control males used for both the spermatozoa-irradiated and spermatogonia-irradiated comparisons are age-matched to the spermatogonia-irradiated group (i.e. they are 10 weeks older than the males bred immediately after treatment).

Table 8. Sample sizes, paternal and maternal mutation rates by pup genotype for spermatozoa-irradiated treatment groups.

Group	*Pup Genotype*	*# of Mutant Bands*	*# of Bands Scored*	*Mutation Rate*[a]	*95% CI (lower, upper)*[b]
PATERNAL					
Control[c]	Trp53+/+	4	51	0.078	0.02, 0.2
	Trp53+/-	9	100	0.9	0.04, 0.17
1Gy	Trp53+/+	3	55	0.055	0.01, 0.16
	Trp53+/-	9	70	0.129	0.06, 0.25
0.01+1Gy	Trp53+/+	6	60	0.1	0.04, 0.22
	Trp53+/-	4	97	0.041	0.01, 0.1
0.1+1Gy	Trp53+/+	11	83	0.133	0.07, 0.24
	Trp53+/-	11	56	0.196	0.1, 0.35
MATERNAL					
Control[d]	Trp53+/+	8	48	0.167	0.07, 0.33
	Trp53+/-	8	92	0.087	0.04, 0.28
1Gy	Trp53+/+	5	55	0.09	0.03, 0.21
	Trp53+/-	7	71	0.1	0.04, 0.2
0.01+1Gy	Trp53+/+	3	57	0.053	0.009, 0.16
	Trp53+/-	9	88	0.102	0.05, 0.2
0.1+1Gy	Trp53+/+	8	73	0.11	0.05, 0.22
	Trp53+/-	7	46	0.152	0.06, 0.32

[a,b,c]Footnotes are as in table 8.

Table 9. Descriptive Data and Statistical Summaries for Pup Genotype Ratio Analysis

Group	*Treatment*	*Trp53+/+*	*Trp53+/-*	X^2	*P-value*	*Sequential Bonferroni corrected P-value*
1	1Gy	39	54	2.42	0.12	0.36
2	0.01 +1Gy	38	59	4.55	0.033*	0.132
3	0.1 + 1Gy	54	44	1.02	0.312	0.312
1,2,3	All immediate breedings	131	157	2.35	0.126	0.252
4	0.01Gy (10 weeks)	42	50	0.696	0.404	0.808
5	0.1Gy (10 weeks)	39	56	3.04	0.081	0.324
6	0Gy (control)	35	57	5.26	0.022*	0.132
1.1	1Gy (10 weeks)	42	51	0.871	0.351	1.053
2.1	0.01 +1Gy (10 weeks)	47	45	0.0435	0.835	0.835
3.1	0.1 + 1Gy (10 weeks)	38	58	4.71	0.041*	0.205
4,5,6,1.1,2.1,3.1	All 10-week breedings	243	317	9.78	0.002*	0.014*

*Statistically significant deviation from expected.

Table 10. Mean litter size ± standard deviation for each treatment group and for each breeding time.

Treatment	*Breeding Time*	*Mean Litter Size (±SD)*
1Gy	Immediate	6.71 ± 1.41
0.01+1Gy	Immediate	6.61 ± 1.28
0.1+1Gy	Immediate	7.03 ± 1.91
All spermatozoa-irradiated litters	Immediate	6.79 ± 1.55
0Gy	10 weeks	7.9 ± 1.72
1Gy	10 weeks	7.31 ± 2.05
0.01Gy	10 weeks	7.5 ± 1.55
0.01+ 1Gy	10 weeks	7.96 ± 1.0
0.1Gy	10 weeks	8.14 ± 1.17
0.1+1Gy	10 weeks	7.5 ± 1.58
All spermatogonia-irradiated litters	10 weeks	7.68 ± 1.59

Table 11. Summary of *one-way* ANOVA results for comparisons of litter sizes within and between breeding times for trp53+/- males.

Comparison	*df*	*F-value*	*P-value*
Between spermatozoa-irradiated groups	5, 148	0.39963	0.84848
Between spermatogonia-irradiated groups	1, 95	0.98719	0.37641
Between breeding times	1, 250	17.124	0.00005*

*Statistically significant difference among groups being compared

Table 12. Mutation scores for samples run on both 28 and 42 cm gels.

Parent	*Pup*	*28 cm gel score (band shift in mm)*	*42 cm gel score (band shift in mm)*
61	65	-0.5	0
66	67	-0.5	0
92	97	+0.5	0
12	1177	+0.5	Can't score (12 is undigested)
1176	1175	-0.5	+ 1.5
1176	1178	-0.5	+ 0.5
2	1085	+0.5	+ 0.5
52	1703	-0.5	- 1
128	132	-0.5	+1
1849	1852	+0.5	0
54	1854	-0.5	0
302	307	+0.5	0
3	107	+0.5	0
11	362	+0.5	0
359	364	+0.5	-0.5

Table 13. Summary of *one-way* ANOVA results for the effect of irradiation of germ cells on per-male ESTR mutation rates as detected in the offspring DNA.

Breeding Time	*Probe Type*	*df*	*F-value*	*P-value*
Immediate				
	Single Locus	2,15	0.46931	0.63432
	Multi Locus	2,13	0.2494	0.97541
10-weeks				
	Single Locus	5,28	0.46725	0.79726
	Multi Locus	5,29	1.1457	0.36088

Table 14.Treatment Groups and Breeding Dates for the Wildtype Control Study

Group Number	*Priming Dose*	*Challenge Dose*	*Male age at Mating*	*Female age at Mating*	*Mating Date*
7	0Gy	0Gy	18 weeks	9 weeks	11/06/04 - 25/06/04
8	0.1Gy	0Gy	18 weeks	9 weeks	11/06/04 - 25/06/04
9	0.1Gy	1Gy	18 weeks	9 weeks	11/06/04 - 25/06/04
10	0Gy	1Gy	18 weeks	9 weeks	11/06/04 - 25/06/04
11	0Gy	2Gy	18 weeks	9 weeks	11/06/04 - 25/06/04

Table 15. Mean litter sizes with standard deviations for the wildtype control treatment groups.

Treatment	*Breeding Time (post treatment)*	*Mean Litter Size ± SD*
0Gy	10 weeks	7.68 ± 1.46
0.1Gy	10 weeks	7.63 ± 1.56
0.1 + 1Gy	10 weeks	6.85 ± 1.59
1Gy	10 weeks	7.5 ± 1.05
2Gy	10 weeks	7.43 ± 1.56
All wildtype males	10 weeks	7.43 ± 1.47

Table 16. Summary of *one-way* ANOVA results for comparisons of litter sizes within and between breeding times for trp53+/+ and trp53+/- males.

Comparison	*df*	*F-value*	*P-value*
Between all wildtype control treatment groups	4,105	0.37900	0.82320
Between all spermatogonia-irradiated groups	10, 253	0.48321	0.90021
Between all groups	2,359	8.3678	0.00028*

*Statistically significant difference among groups being compared

Figures

Figure 1.

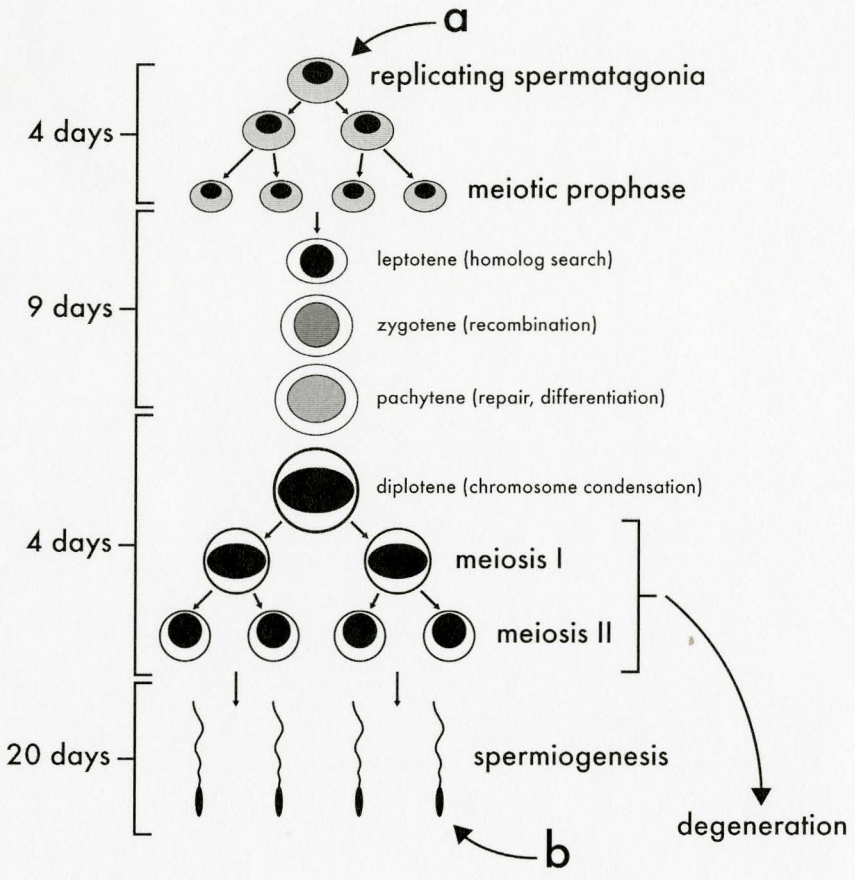

Figure 1. Timeline of mouse spermatogenesis. Diploid spermatogonial stem cells exist before the spermatogonial stage. Mature spermatogonia are produced during spermiogenesis, and it takes up to 7 days for packaging and preparation for ejaculation once mature (adapted from: Swartz *et al.*, 1999). Arrow a: stage at which cells are irradiated for males bred 10 weeks prior to breeding. Arrow b: stage at which cells are irradiated for males bred immediately before breeding.

Figure 2.

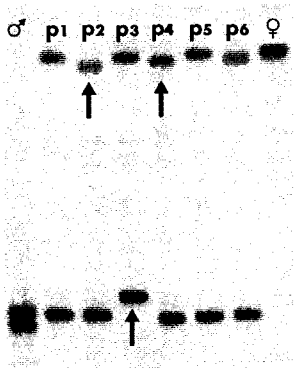

Figure 2. DNA fingerprint of paternal, maternal and pup DNA probed with *Ms6-Hm* single locus probe. Parental lanes are identified by sex, pup lanes are identified as p_{1-6}. Mutant bands (band shifts >1mm) are indicated by arrows.