The effects of *in utero* irradiation on mutation induction and transgenerational instability in mice

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ABSTRACT

Epidemiological evidence suggests that the deleterious effects of prenatal irradiation can manifest during childhood, resulting in an increased risk of leukaemia and solid cancers after birth. However, the mechanisms underlying the long-term effects of foetal irradiation remain poorly understood. This study was designed to analyse the impact of *in utero* irradiation on mutation rates at expanded simple tandem repeat (ESTR) DNA loci in directly exposed mice and their first-generation (F_1) offspring. ESTR mutation frequencies in the germline and somatic tissues of male and female mice irradiated at 12 days of gestation remained highly elevated during adulthood, which was mainly attributed to a significant increase in the frequency of singleton mutations. The prevalence of singleton mutations in directly exposed mice suggests that foetal irradiation results in genomic instability manifested both *in utero* and during adulthood. The frequency of ESTR mutation in the F_1 offspring of prenatally irradiated male mice was equally elevated across all tissues, which suggests that foetal exposure results in transgenerational genomic instability. In contrast, maternal *in utero* exposure did not affect the F_1 stability. Our data imply that the passive erasure of epigenetic marks in the maternal genome can diminish the transgenerational effects of foetal irradiation and therefore provide important clues to the still unknown mechanisms of radiation-induced genomic instability. The results of this study offer a plausible explanation for the effects of *in utero* irradiation on the risk of leukaemia and solid cancers after birth.

1. Introduction

The developing embryo is especially sensitive to ionising radiation, exposure to which results in foetal mortality and increases the risk of leukaemia and solid cancers after birth [1–2]. However, to date the mechanisms underlying the health risks associated with prenatal irradiation remain poorly understood. Given that the majority of embryonic cells are actively proliferating, exposure during the early stages of development could lead to a substantial accumulation of radiation-induced mutations across all tissues, thus contributing to the risk of cancer after birth. It is therefore clear that the analysis of mutation induction *in utero* can provide important insights into the long-term effects of foetal exposure to mutagens, including ionising radiation. It should however be stressed that at present the genetic effects of prenatal irradiation remain poorly characterised. To date, the analysis of both gene mutations and chromosome aberrations in the somatic tissues of adult mice irradiated *in utero* has resulted in a highly controversial set of data. A number of studies have shown that the frequency of chromosome aberrations and gene mutations in haemopoietic cells remained elevated during adulthood following foetal irradiation [3–5], while in others no measurable increases in translocation frequencies were found in the lymphocytes of *in utero* exposed adults [6,7]. Although relatively few studies have analysed the impact of *in utero* exposure on the developing germline, preliminary evidence suggests a low efficiency for radiation-induced mutations in foetal gonads [8,9]. One major reason for the apparently conflicting results describing the effects of foetal irradiation is due to the lack of a sensitive *in vivo* technique that efficiently detects spontaneous and radiation-induced mutations in both the germline and somatic tissues. Further analysis of *in utero* mutation induction, using an appropriate detection system, therefore offers an opportunity to gain novel insights into the effects of prenatal exposure to mutagenic agents. One such system, that we have used extensively, utilises mouse expanded simple tandem repeat (ESTR) loci as a biomarker of mutation induction in germline and somatic cells following adult exposure to many mutagenic agents, including ionising radiation [10-13]. These studies have shown that ESTR loci provide a sensitive experimental system for monitoring germline mutation in mice, permitting evaluation of mutation induction at low doses of exposure and in very small population samples.

In addition to the studies of mutation induction in the germline and somatic tissues of directly exposed organisms, considerable progress has been made in the analysis of the transgenerational effects of parental irradiation, which are manifested in the offspring of treated parents (reviewed in Refs. 14-17). It was established that mutation rates in the germline and somatic tissues of non-treated offspring of adult male exposed to ionising radiation or chemical mutagens remain highly elevated [17–21]. These data suggest that transgenerational genomic instability may be a contributory factor in the elevated cancer risk and enhanced tumour progression observed in the offspring of exposed fathers. They also indicate that a transgenerational genomic destabilisation of the genome can be attributed to an as yet unknown radiation-induced signal in the paternal genome which is inherited through sperm in an epigenetic fashion. In previous studies we have evaluated the transgenerational effects of irradiation of different stages of mouse spermatogenesis and found that mutation rates remained equally elevated in the offspring conceived from 1 to 8 weeks after paternal exposure [18–20]. These results imply that a radiation-induced instability signal is retained during spermatogenesis, even after several rounds of DNA replication. The persistence of such a signal in the adult germline raises questions regarding the effects of *in utero* exposure on transgenerational instability. It should however be stressed that the massive epigenetic reprogramming occurring in the developing germline [22] could potentially erase all

epigenetic marks of foetal radiation exposure, thus preventing the manifestation of genomic instability in subsequent generations.

Using a combination of approaches to assess ESTR mutation rate, here we have studied the effects of *in utero* irradiation on mutation induction and transgenerational instability in mice.

2. Materials and Methods

2.1. Animals

BALB/c mice were obtained from Harlan (Bicester, UK) and housed at the Division of Biomedical Services, University of Leicester. Eight-week-old pregnant females (12 days of gestation) and 7-week-old adult males were given whole-body acute irradiation of 1 Gy of X-rays delivered at 0.5 Gy min^{-1}, (250 kV constant potential, HLV 1.5 mm Cu, Pantak industrial X-ray machine, Connecticut, USA). Eight-week-old *in utero* irradiated and sham-treated male and female mice were mated to non-irradiated BALB/c partners (Fig. 1A). Tissue samples were taken from all irradiated mice and from the 8-week-old male offspring of *in utero* irradiated parents. All animal procedures were carried out under the Home Office project license No. PPL 80/1564.

2.2. ESTR mutation detection in pedigrees

Genomic DNA was prepared from tails by SDS lysis, proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. All parents and offspring were profiled using two mouse-specific hypervariable single-locus ESTR probes Ms6-hm and Hm-2 as described previously [10]. DNA samples were resolved on a 40-cm long agarose gel and detected by Southern blot hybridization. Autoradiographs were scored by two independent observers. As in a number of previous studies [11-14, 23], only bands showing a shift of at least 1 mm relative to the progenitor allele were scored as mutants. DNA fragment sizes were estimated by the method of Southern [24], with a 1 kb DNA Ladder (Invitrogen, Paisley, UK) included on all gels.

2.3. ESTR mutation detection by single-molecule PCR

To minimise the risk of contamination, DNA samples from sperm, bone marrow (BM) and brain were prepared in a laminar flow hood as previously described [11,19]. Briefly, the sperm pellet was suspended in 1 x SSC and somatic cells lysed by addition of SDS to 0.15%. The lysate was centrifuged and the resulting sperm pellet re-suspended in 1 ml 0.2 x SSC, 1% SDS, 1M 2-mercaptoethanol and digested with 200μg ml^{-1} proteinase K (Sigma) for 1 hour at 37°C. DNA was recovered after phenol/chloroform extraction. DNA from BM and brain was similarly prepared by SDS lysis, proteinase K digestion, phenol/chloroform extraction and ethanol precipitation.

Approximately 5 μg of each DNA sample was digested with 20 U *Mse*I for 2 h at 37^{o}C; *Mse*I cleaves outside the ESTR array and distal to the PCR primer sites used for PCR amplification and was used to render genomic DNA fully soluble prior to dilution. Each digested DNA sample was diluted to approximately 10 ng ml^{-1} in 5 mM Tris-HCl (pH 7.5) in the presence of 5 μg ml^{-1} carrier salmon sperm DNA (Sigma, Dorset, UK) prior to mutation analysis.

The frequency of ESTR mutation was evaluated using SM-PCR as previously described [11,19]. The *Ms6-hm* ESTR locus was amplified in 10 μl reactions using 0.4 μM

flanking primers HM1.1F (5′-AGA GTT TCT AGT TGC TGT GA-3′) and HM1.1R (5′-GAG AGT CAG TTC TAA GGC AT-3′). Amplification was performed using 0.035 U μl^{-1} of the Expand High Fidelity PCR System (Roche, Mannheim, Germany), 1 M Betaine and 200 μM each dNTP. Amplification was carried out at 96°C (20 sec), 58°C (30 sec), 68°C (8 min) for 30 cycles on a PTC-225 DNA Engine Tetrad (BioRad, Hemel Hempstead, UK). To increase the robustness of the estimates of individual ESTR mutation frequencies, on average 140 amplifiable molecules were analysed for each tissue for each mouse.

PCR products were resolved on a 40-cm long agarose gel and detected by Southern blot hybridization as previously described [10]. Following Southern blot hybridization, autoradiographs were scored by two independent observers. The frequencies of ESTR mutation and standard errors were estimated using a modified approach proposed by Chakraborty and co-workers [25].

3. Results

3.1. Experimental design

As in a number of our previous studies [18–21], we have used BALB/c inbred mice, to evaluate the effects of *in utero* irradiation on ESTR mutation induction and transgenerational instability. BALB/c pregnant mice (Theiler stage 20, 12 days of gestation) were exposed to 1 Gy of acute X-rays. At this stage of mouse development, primordial male and female germ cells are already in the genital ridge area and undergo active mitotic proliferation before entering meiotic or mitotic arrest at about 13.5 days post coitum [26]. The majority of somatic cells also undergo active mitotic proliferation in 12-day mouse embryos [27].

Using a pedigree-based approach, we first evaluated ESTR mutation rates in the germline of *in utero* irradiated males and females. The *in utero* irradiated mice (generation F_0) were mated to non-exposed partners and ESTR germline mutations were scored in their first-generation (F_1) offspring (Fig. 1A). To compare the effects of *in utero* paternal irradiation with adult exposure, 7-week-old male mice were given 1 Gy of acute X-rays and mated to control females 8 weeks after irradiation, thus ensuring that their offspring were derived from exposed spermatogonial stem cells [28]. It was possible to establish the parental origin of mutant bands detected at the *Ms6-hm* and *Hm-2* loci due to the extensive multiallelism and heterozygosity seen at these two loci in inbred BALB/c mice. The number of mutations scored in the F_1 offspring of irradiated males and females was divided by the total number of offspring to give an estimate of germline mutation rates for the germline of F_0 parents. A control group consisted of 242 offspring of age-matched non-irradiated BALB/c mice.

To further study the effects of *in utero* irradiation we used a single molecule-PCR (SM-PCR) approach to assess mutation frequency at the *Ms6-hm* ESTR locus. The SM-PCR technique involves the dilution of bulk genomic DNA followed by the amplification of multiple samples, each containing approximately one ESTR molecule [11]. For each tissue, PCR analysis was conducted on multiple samples, and PCR products were resolved on a 40-cm long agarose gel and detected by Southern blot hybridization (Fig. 1B and 1C). PCR products showing a shift of at least 1 mm relative to the progenitor allele were scored as mutant *Ms6-hm* molecules.

A major advantage of this approach is that both somatic and germline mutation frequencies can be assessed in the same individual. The frequency of ESTR mutation was evaluated in DNA samples extracted from sperm, BM and brain of 8-week-old adult male mice, which were irradiated *in utero*. To compare the effects of *in utero* irradiation with those

during adulthood, 7-week-old adult male mice were exposed to 1 Gy of acute X-rays and their tissue sampled 1 and 8 weeks after exposure. These samples provided sperm DNA which was derived from exposed sperm and A_s spermatogonia, respectively [28]. Due to the 7-week age difference between the two groups of adult irradiated males, age-matched control tissues were collected from non-irradiated 8- and 15-week old male mice. SM-PCR was also used to assess somatic mutation frequencies (for brain and BM) in age-matched control and *in utero* irradiated females.

To evaluate the transgenerational effects of *in utero* irradiation, tissue samples were collected from the 8-week-old male F_1 offspring of irradiated parents from different litters (Fig. 1A). Using SM-PCR, the frequency of ESTR mutation was established in DNA samples prepared from sperm, BM and brain.

3.2. Mutation induction in the germline of in utero irradiated mice

A summary of the mutational data is presented in Table 1. *In utero* irradiation resulted in a substantially elevated mutation rate in the germline of exposed males and females mated during their adulthood. Radiation-induced mutation rates in the paternal and maternal germline did not significantly differ (Fisher's exact test, P=0.96). The germline ESTR mutation rate of male mice irradiated during adulthood exceeded that of the *in utero* exposed males, although the difference between the two groups was not significant (P=0.22).

We next determined the spectra of ESTR mutations. This analysis was restricted by the resolution of agarose gel electrophoresis as the smallest mutational changes detectable at the *Ms6-hm* (~2.5 kb) and *Hm-2* (~3.5 kb) loci were 2 and 5 repeats, respectively. As the mutational spectra for male mice irradiated either *in utero* or during adulthood did not significantly differ (Kolmogorov–Smirnov two sample test, P=0.78), these data were aggregated. The combined distributions of length changes at ESTR loci were indistinguishable between irradiated and control parents (Fig. 2). As the mutational spectra of paternal and maternal mutation did not significantly differ, our data therefore imply that the mechanisms underlying spontaneous and radiation-induced ESTR mutation in the paternal and maternal germline are likely to be similar.

3.3. Mutation induction in the germline and somatic tissues

Using SM-PCR, the frequency of ESTR mutation at the *Ms6-hm* locus was evaluated in DNA samples extracted from brain, BM and sperm. In some control and irradiated animals the presence of germline or somatic mosaicism was observed, where a similar mutation was detected in multiple DNA samples taken from one or more tissues (Fig. 1B, 1C). Given that mosaicism at the *Ms6-hm* locus most likely results from the propagation of a single mutational event occurring during the first few cell divisions following fertilisation [29], all instances of mosaicism were counted as a single independent mutation event and the total number of unique mutations was used to estimate ESTR mutation frequency.

As the frequency of ESTR mutation in all tissues of 8- and 15-week-old control males did not differ (sperm, P=0.81; BM, P=0.96; brain, P=0.69), the data for these two groups were combined to form a single control group. It should be noted that the frequency of ESTR mutation in sperm, determined by the SM-PCR technique (~0.04), significantly differs from the pedigree data (25 paternal mutations at the *Ms6-hm* locus among 242 control offspring, 25/242=0.10; Table 1). The main reason for this discrepancy is attributed to the presence of hidden mosaic mutations in the pedigrees. Using the Poisson distribution, the conditional probability of detecting a mosaic mutation in just one littermate, when it will wrongly be scored as a singleton mutation, can be estimated as $P[x=1|x>0] = \lambda e^{-\lambda} / (1 - e^{-\lambda})$, $\lambda = mk$,

where *m* and *k* are the frequency of mosaic mutations in the paternal germline and the litter size, respectively. Therefore, the expected frequency of mutations found only once among littermates is $u + mP[x=1|x>0]$, where *u* is the frequency of singleton mutations in the paternal germline. According to the SM-PCR data for sperm, $u = 0.04$ and $m = 0.05$, and the mean litter size for controls is ~6. Given this, the expected frequency of ESTR mutations found only once in control litters is 0.09, which more than twice exceeds the SM-PCR estimate and, in the meantime, is remarkably close to that from the pedigree data. We therefore conclude that more than a half of ESTR 'singleton' mutations found in the BALB/c litters in fact belong to mosaics. The estimates also show that the presence of germline mosaicism can only be unambiguously detected by profiling of unrealistically high numbers of offspring of a single male (>90). In the meantime, ~140 amplifiable molecules were analysed in sperm samples extracted from each control male, which provide unbiased estimates of the frequency of singleton and mosaic ESTR mutations.

ESTR mutation frequencies in the adult males and females irradiated *in utero* were similarly elevated across all tissues (Table 2). In contrast, male mice irradiated during adulthood and sampled 1 week after exposure did not show any measurable changes in the frequency of ESTR mutation. A significant increase in ESTR mutation frequency was found in the germline of male mice, sampled 8 weeks after adult irradiation. Furthermore, in this group of mice a marginal elevation in the frequency of ESTR mutations was observed in the BM DNA; whereas the frequency in the DNA extracted from brain samples did not differ from the controls.

A comparison of the pattern of ESTR mutation induction in the tissues of adult male mice irradiated either *in utero* or during adulthood provides further support for our previous results describing the stage-specificity of mutation induction at these loci [10,12,30]. In these studies we showed that elevated paternal germline mutation rates were only observed following the exposure of replication-proficient spermatogonia and stem cells, whereas the same treatment of post-meiotic spermatids did not result in any measurable induction of ESTR mutations. These data imply that ESTR mutation induction in the male germline occurs mostly, if not exclusively, in replication-proficient mitotic cells. The data presented here fully support these observations as no alterations in ESTR mutation frequency were observed in the germline of male mice, irradiated during adulthood and sacrificed 1 week after exposure, since the samples assessed at this stage were derived from non-dividing mature sperm cells. In contrast, the frequency of ESTR mutation measured 8 weeks after adult irradiation is significantly elevated, which reflects the exposure of replication-proficient A_s spermatogonia (Fig. 3). This study has also assessed ESTR mutation frequencies in the somatic tissues of prenatally exposed individuals and clearly demonstrates that replication-proficiency is a pre-requisite for somatic ESTR mutation induction, as highly elevated mutation frequencies of were observed in the brains of the *in utero* irradiated males, whereas adult exposure did not alter the ESTR mutation rate observed in this tissue (Fig. 3). Given that the brain cells do not undergo mitotic proliferation in adults, these results clearly show that ESTR mutation induction only occurs in replication-proficient brain cells. The irradiation of male mice as adults resulted in a relatively small change in ESTR mutation frequency in BM. This modest alteration may be explained by the fact that the percentage of actively dividing stem cells in mouse BM is very low (~ $9.1/10^5$ cells in BALB/c mice, Ref. 31) and therefore the DNA samples studied here were enriched for the genomes of the non-dividing supporting cells. Considering these data and the results of our recent study addressing the stage-specificity of spontaneous mutation in the mouse germline [32], it would appear that both spontaneous and radiation-induced ESTR mutations occur almost exclusively in replication-proficient cells and may be attributed to replication slippage.

SM-PCR was also used to determine the frequency of ESTR mutation in the somatic tissues of female mice, and statistically significant increases were detected in the DNA extracted from both brain and BM when compared to an age-matched control group (Table 2). The levels of mutation induction were similar to those observed in the *in utero* exposed males (brain, P=0.34; BM, P=0.98).

3.4. Transgenerational effects of foetal irradiation

The frequency of ESTR mutation was evaluated in the F_1 male offspring of *in utero* irradiated males and females (Table 2). In the offspring of irradiated males, highly significant increases in ESTR mutation frequency were found across all tissues. In contrast, the frequency of ESTR mutation in the F_1 offspring of irradiated females was very close to that in controls. We therefore conclude that transgenerational genomic instability is only manifested in the F_1 offspring of *in utero* irradiated males, whereas the maternal exposure does not affect the ESTR mutation rates in their offspring.

4. Discussion

The analysis of the long-term effects of *in utero* irradiation has established that (i) ESTR mutation frequencies in the adult males and females irradiated *in utero* are similarly elevated in the germline and somatic tissues; (ii) the efficiency of *in utero* irradiation on ESTR mutation induction in the male germline is close to that following adult exposure; (iii) paternal *in utero* irradiation results in transgenerational instability manifested in the germline and somatic tissues of their F_1 offspring; (iv) maternal *in utero* exposure does not affect the F_1 stability. These results offer further insights into the unknown mechanisms underlying mutation induction at ESTR loci and transgenerational genomic instability. Additionally, our data provide important information about the susceptibility of early stages of mouse development to ionising radiation.

The elevated frequency of ESTR mutation detected in the germline and somatic tissues of adult mice following *in utero* irradiation can be attributed to the clonal propagation of mutations induced in the directly exposed cells in the developing foetus. The embryonic stage at which irradiation was performed corresponds to the major period of organogenesis where many of the foetal cells undergo active mitotic proliferation, therefore a substantial accumulation of mosaic mutations may be expected in the adult tissues of exposed individuals. However, our data are not consistent with this hypothesis; we have observed that the mean frequency of mosaic mutations in all tissues from *in utero* irradiated mice was very similar to that in controls (0.0221 ± 0.0034 and 0.0278 ± 0.0029, respectively; t=1.27, P=0.20). In contrast, a highly significant increase in the frequency of singleton mutations was found in the exposed group (0.0935 ± 0.0072 *vs.* 0.0340 ± 0.0032; t=7.53; $P << 10^{-6}$). Our data clearly demonstrates that *in utero* exposure results in long-term changes that affect the genome stability of the cellular descendants of directly irradiated foetal cells. An accumulation of ESTR mutations in multiple tissues detected in adult mice following foetal irradiation can be explained by ongoing genomic instability occurring in dividing tissues. Given that BM stem cells and A_s spermatogonia both undergo active mitotic proliferation in adult mice, our data also indicate that genomic instability induced in foetal tissues can manifest in adulthood. These data are in line with the results of previous studies indicating that foetal irradiation can affect the frequency of unstable chromosome aberrations in the haemopoietic cells of adult mice [3,4]. Taken together, these data provide a plausible explanation for the elevated risk of childhood leukaemia and solid cancers in humans following *in utero* irradiation [1,2].

In this study we address for the first time the issue of ESTR mutation induction in female mice. Our results clearly show that the pattern of mutation induction in the maternal germline and somatic tissues is very close to that of the *in utero* irradiated males. Given that at this stage of the mouse development both male and female germ cells undergo active mitotic proliferation [26], the apparent similarity can be explained by the targeting of replication-proficient cells in the primordial germline and somatic tissues. As the frequency of singleton mutations is highly elevated in the somatic tissues of the *in utero* irradiated males and females, the observed changes in the germline can also be attributed to genomic instability. The apparent discrepancy between our data and those obtained previously using the specific-locus test [9] may be explained in part by the fact that in these studies females were exposed to acute X-rays on 17.5 and 18.5 days post coitum. As at the later stages of oogenesis the primordial oocytes are already meiotically arrested and do not undergo any further rounds of mitotic divisions [26], and therefore radiation-induced genomic instability may not be fully manifested in these non-replicating cells during adulthood.

Here we have also analysed the effects of *in utero* irradiation on transgenerational instability manifested in the F_1 offspring of exposed males and females. Previous work has established that the transgenerational changes affect mutation rates at tandem repeat DNA loci and protein-coding genes, as well as the frequency of chromosome aberrations the offspring of male mice irradiated during adulthood (reviewed in Refs. 15, 16). It was also shown that the destabilisation of the F_1 genome occurs regardless of the stage of paternal exposure, including irradiated transcriptionally inert sperm [20], as well as transcriptionally active spermatids [18] and spermatogonia [17-19]. Using the same SM-PCR technique, we previously analysed the frequency of ESTR mutation in the offspring of BALB/c males conceived 1 week (sperm) and 6 (spermatogonia) weeks after paternal exposure [19,20]. Fig. 4A compares our current and previous data. As the magnitude of the increases in ESTR mutation frequency in the three groups of F_1 offspring does not significantly differ, we therefore conclude that the efficiency of *in utero* exposure on transgenerational instability is close to that observed following adult irradiation. It should be stressed that during this stage of mouse development (12 days of gestation) the primordial germ cells undergo massive epigenetic reprogramming, including the erasure of parentally derived imprints and X-chromosome reactivation [22,33]. Given the wealth of experimental evidence suggesting that radiation-induced genomic instability is an epigenetic phenomenon [15,16], our results imply that some as yet unknown epigenetic marks of foetal irradiation can survive this genome-wide reprogramming and become manifested as genomic instability during adulthood. Since the elevated frequency of ESTR mutation observed in adult mice irradiated prenatally is most probably attributed to ongoing genomic instability, our data also suggest that similar mechanisms may underlie the long-term changes affecting the stability of directly exposed animals and their offspring.

In contrast to paternal irradiation, maternal prenatal exposure does not affect the genomic stability of their F_1 offspring. This was surprising considering the similarity in the elevation of ESTR mutation frequencies in the germline and somatic tissues of directly exposed males and females (Fig. 4B), and the fact that our previous study showed similarly elevated mutation rates in the germline of the F_1 male and female offspring of irradiated fathers [18]. Besides, according to our previous data there is no difference in the transmission of instability through the male or female germline of F_1 and F_2 offspring of irradiated male mice [18]. It would appear that, although the epigenetic marks of foetal irradiation can equally survive developmental reprogramming in both sexes and lead to genomic instability across multiple tissues during adulthood, the inability of irradiated females to pass an instability signal to their offspring may be related to early post-fertilisation events. It has been established that within four hours of fertilisation the paternal genome undergoes active DNA

demethylation and other epigenetic changes, whereas in the maternal genome this process is passive and continues over several cell divisions [34,35]. The mechanisms underlying the active and passive erasure of epigenetic marks could differentially affect the fate of instability signals induced in the germline of irradiated parents. The active demethylation of the paternal genome may preserve more epigenetic modifications than the passive process occurring in the maternal pronucleus. It should be noted that our data are consistent with the results of recent studies showing transgenerational effects of prenatal exposure to the endocrine disruptor vinclozolin, where transmission was observed exclusively through the male germline [36,37].

In conclusion, our study has shown that the effects of foetal irradiation can manifest during adulthood, resulting in genomic instability occurring in the germline and somatic tissues of directly exposed males and females. These data therefore provide a plausible explanation for the high susceptibility of early developmental stages to ionising radiation, including the late effects of prenatal exposure on the risk of childhood leukaemia and solid cancers in humans. The results of our study are also important in furthering our understanding of the as yet unknown mechanisms of radiation-induced transgenerational instability in mammals. First of all, they demonstrate the high efficiency of *in utero* paternal exposure for the manifestation of genomic instability in the F_1 germline and somatic tissues, thus implying that epigenetic marks of foetal irradiation can survive the genome-wide reprogramming and remain intact in the germ cells of adult male mice. As genomic instability equally manifests in the *in utero* exposed males and their offspring, our data suggest that similar mechanisms may underlie the phenomena of radiation-induced and transgenerational genomic instability. In addition, the results of this study demonstrate for the first time the important role of early post-fertilisation events on the differential manifestation of transgenerational instability in the offspring of exposed males and females. These data imply that the passive erasure of epigenetic marks in the maternal genome can diminish the transgenerational effects of foetal irradiation. Future work should address in detail the effects of early post-fertilisation events on the manifestation of transgenerational effects in the offspring of irradiated parents.

Conflict of interest statement

None

Acknowledgments

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Figure legend

Fig. 1. Experimental design and mutation detection at the *Ms6-hm* locus by SM-PCR. (A) Design of *in utero* study. Irradiated pregnant female is in grey; *in utero* exposed parents (F_0) are in black; their non-exposed offspring (F_1) and control parents with no history of irradiation are in white. (B) Representative image of a singleton ESTR mutation. (C) Representative image of a mosaic ESTR mutation. Mutants are indicated with arrowheads.

Fig. 2. Spectra of germline ESTR mutations in controls and exposed mice. (ANOVA: sex P=0.22; irradiation P=0.92; interaction P=0.87). The progenitor allele was assumed to be the parental allele closest in size to the mutant allele.

Fig. 3. Frequency of ESTR mutations in the germline and somatic tissues of BALB/c male mice irradiated prenatally or during adulthood.

Fig. 4. Transgenerational effects of paternal and maternal exposure to ionising radiation. (A) Frequency of ESTR mutations in the F_1 offspring of BALB/c male mice conceived 1 (sperm) and 6 (spermatogonia) weeks after irradiation or following *in utero* exposure. All males were exposed to 1 Gy acute X-rays; data for the spermatogonia and sperm irradiation are taken from [19,20]. (B) Comparison of the effects of paternal and maternal *in utero* irradiation on mutation induction and transgenerational instability.

Fig 1

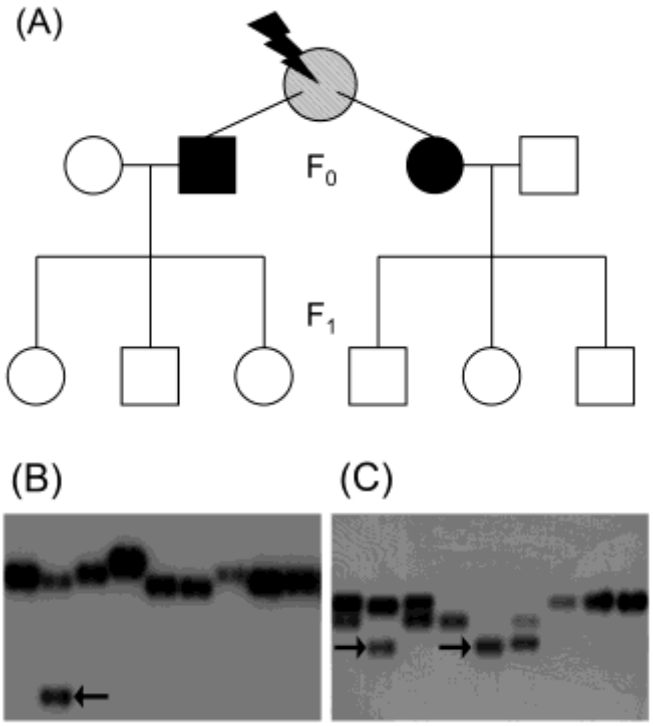
(A)
F0
F1
(B)
(C)

Fig 2

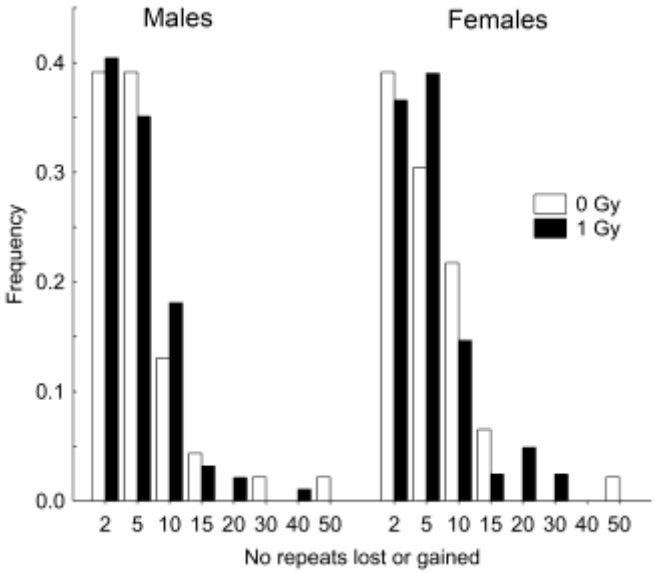
Males
Females
0 Gy
1 Gy
Frequency
0.0
0.1
0.2
0.3
0.4
2 5 10 15 20 30 40 50
2 5 10 15 20 30 40 50
No repeats lost or gained

Fig 3

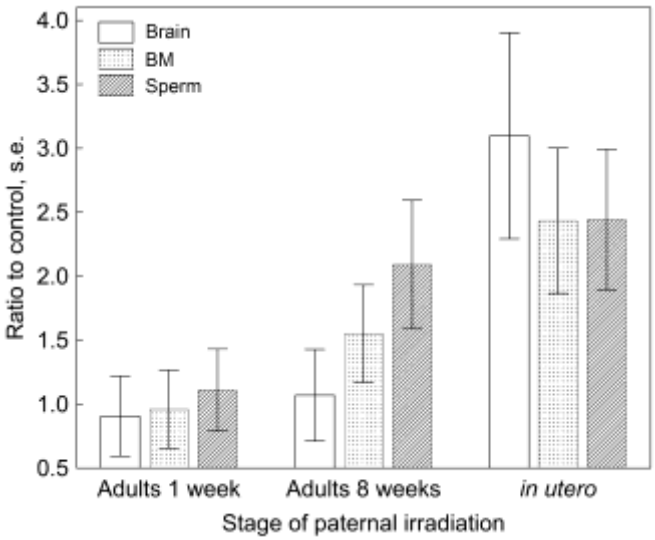
Brain
BM
Sperm
Ratio to control, s.e.
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
Adults 1 week
Adults 8 weeks
in utero
Stage of paternal irradiation

Fig 4

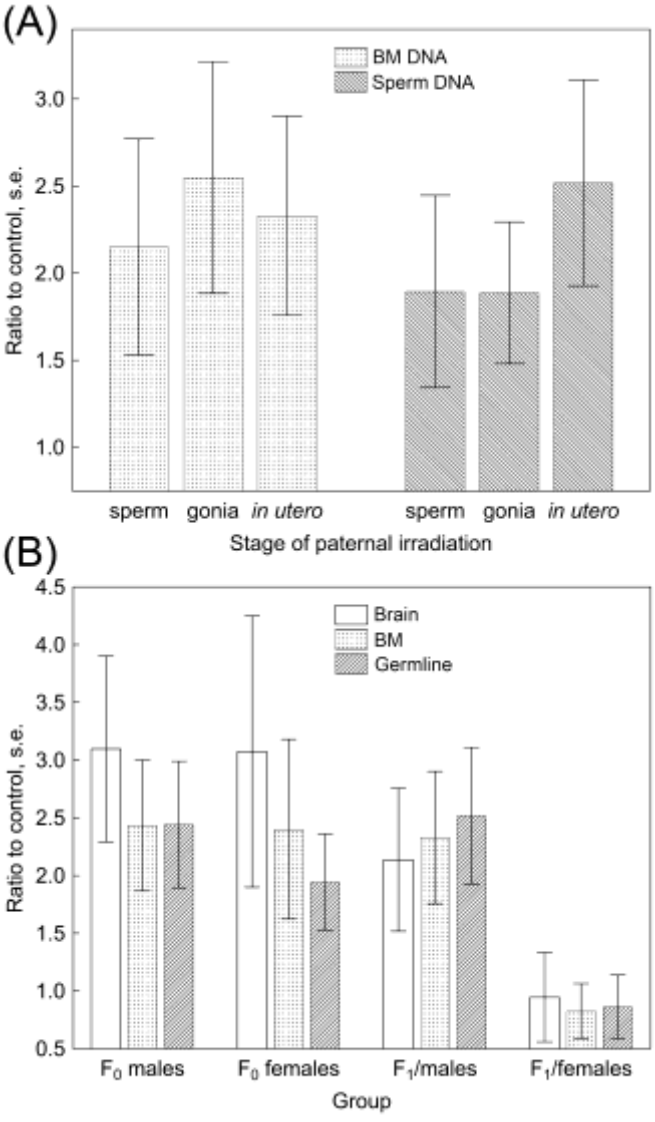
(A)
BM DNA
Sperm DNA
Ratio to control, s.e.
3.0
2.5
2.0
1.5
1.0
sperm
gonia
in utero
sperm
gonia
in utero
Stage of paternal irradiation
(B)
Brain
BM
Germline
Ratio to control, s.e.
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
F0 males
F0 females
F1/males
F1/females
Group

Table 1
ESTR mutation rates in control and irradiated mice (pedigree data).

	No offspring	No mutations[a]	Rate	Ratio[b]	*P*[c]
Control ♂	242	42 (25+17)	0.0868	-	-
In utero exposed ♂	99	40 (20+20)	0.2020	2.33	8.72×10^{-5}
Adult ♂, 8 weeks post	97	50 (30+20)	0.2578	2.97	3.82×10^{-8}
Control ♀	242	46 (23+23)	0.0950	-	-
In utero exposed ♀	111	41 (23+18)	0.1847	1.94	0.0016

[a] Number of mutations detected at *Ms6-hm* and *Hm-2* loci is given in parenthesis.
[b] Ratio to control group.
[c] Probability of difference from control group (Fisher's exact test, two-tailed).

Table 2
ESTR mutation frequency in controls, irradiated parents and their offspring (SM-PCR data).

Tissue, group	No mutations[a]	Frequency ± s.e.	Ratio[b]	t[c]	P[c]
Control ♂					
Brain	28 (851)	0.0329 ± 0.0064	-	-	-
Bone marrow	40 (993)	0.0403 ± 0.0066	-	-	-
Sperm	39 (958)	0.0407 ± 0.0067	-	-	-
In utero exposed ♂					
Brain	38 (373)	0.1019 ± 0.0176	3.10	3.69	**0.0002**
Bone marrow	41 (418)	0.0981 ± 0.0163	2.44	3.28	**0.0011**
Sperm	49 (493)	0.0994 ± 0.0152	2.44	3.52	**0.0004**
Adult ♂, 1 week post					
Brain	13 (436)	0.0298 ± 0.0085	0.91	0.29	0.7708
Bone marrow	14 (362)	0.0387 ± 0.0106	0.96	0.13	0.8975
Sperm	19 (420)	0.0452 ± 0.0107	1.11	0.36	0.7206
Adult ♂, 8 weeks post					
Brain	14 (398)	0.0352 ± 0.0096	1.07	0.20	0.8439
Bone marrow	30 (480)	0.0625 ± 0.0119	1.55	1.63	0.1035
Sperm	37 (435)	0.0851 ± 0.0149	2.09	2.72	**0.0066**
F_1 ♂ of exposed ♂					
Brain	23 (327)	0.0703 ± 0.0153	2.14	2.26	**0.0242**
Bone marrow	34 (362)	0.0939 ± 0.0172	2.33	2.92	**0.0036**
Sperm	41 (400)	0.1025 ± 0.0172	2.52	3.44	**0.0008**
Control ♀					
Brain	10 (387)	0.0258 ± 0.0084	-	-	-
Bone marrow	14 (341)	0.0411 ± 0.0113	-	-	-
In utero exposed ♀					
Brain	28 (353)	0.0793 ± 0.0159	3.07	2.98	**0.0030**
Bone marrow	39 (395)	0.0987 ± 0.0170	2.40	2.82	**0.0049**
F_1 ♂ of exposed ♀					
Brain	11 (354)	0.0311 ± 0.0096	0.94	0.16	0.8736
Bone marrow	19 (570)	0.0333 ± 0.0079	0.83	0.68	0.4977
Sperm	14 (399)	0.0351 ± 0.0096	0.86	0.48	0.6317

[a] Total number of amplifiable molecules is given in brackets.
[b] Ratio to control group.
[c] Student's test and probability for difference from control group. Significant values are given in bold.