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Ionizing radiation is a potent inducer of mitotic recombination in mouse embryonic stem cells

Natalia G. Denissova[§], Irina V. Tereshchenko[§], Eric Cui[§], Peter J Stambrook^{§§}, Changshun Shao[§], and Jay A. Tischfield^{§,*}

[§]Department of Genetics, Rutgers University, Piscataway, NJ 08854

^{§§}Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH 45267

Abstract

Maintenance of genomic integrity in embryonic cells is pivotal to proper embryogenesis, organogenesis and to the continuity of species. Cultured mouse embryonic stem cells (mESCs), a model for early embryonic cells, differ from cultured somatic cells in their capacity to remodel chromatin, in their repertoire of DNA repair enzymes, and in the regulation of cell cycle checkpoints. Using 129XC3HF1 mESCs heterozygous for *Apert*, we characterized loss of *Apert* heterozygosity after exposure to ionizing radiation. We report here that the frequency of loss of heterozygosity mutants in mESCs can be induced several hundred-fold by exposure to 5 to 10 Gy of x-rays. This induction is 50 to 100-fold higher than the induction reported for mouse adult or embryonic fibroblasts. The primary mechanism underlying the elevated loss of heterozygosity after irradiation is mitotic recombination, with lesser contributions from deletions and gene conversions that span *Apert*. *Apert* point mutations and epigenetic inactivation are very rare in mESCs compared to fibroblasts. Mouse ESCs, therefore, are distinctive in their response to ionizing radiation and studies of differentiated cells may underestimate the mutagenic effects of ionizing radiation on ESC or other stem cells. Our findings are important to understanding the biological effects of ionizing radiation on early development and carcinogenesis.

Keywords

mouse embryonic stem cells; ionizing radiation; mitotic recombination; loss of heterozygosity

1. Introduction

Cultured mouse embryonic stem cells (mESCs) are a relatively easy to manipulate genetic model of early embryonic cells. Because embryonic cells, and by extension ESCs, are the precursors of all adult tissue lineages, including the germ cells that give rise to the next generation of offspring, one might expect the evolution of robust mechanisms for

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*To whom correspondence should be addressed. **Corresponding Author:** Jay A. Tischfield Department of Genetics, Rutgers University, 145 Bevier Rd, Piscataway, NJ 08854, USA Phone # 1-(732) 445-1027x 40042, Fax # 1-(732) 445-1147 jay@biology.rutgers.edu.

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Conflicts of interest None

maintaining genomic stability. Consistent with this notion, mutation rates measured at two different loci are relatively low in ESCs, ranging between 1.2×10^{-7} and 4×10^{-6} [1, 2]. These rates are 2 to 3 orders of magnitude lower than our group has reported for somatic cells [1–3]. The mechanisms underlying this relative genomic stability are unknown but might include (i) unusually sensitive surveillance systems for removing cells with damaged DNA through apoptosis or terminal differentiation; (ii) particularly robust and high-fidelity DNA repair processes [1, 2, 4].

We use the gene for APRT, a ubiquitously expressed purine salvage enzyme [5], as a reporter to measure mutational responses in a variety of cell types *in vivo*. Mutation in *Aprt* *in vivo* causes loss of APRT activity which allows for subsequent *in vitro* cell selection due to resistance to toxic purine analogs, such as 2-fluoroadenine or 2,6-diaminopurine [6]. The frequency of the APRT-deficient colonies *in vitro* serves as a measure of the fraction of APRT-deficient cells *in vivo* at that point in time [7]. A significant advantage of the APRT system is that, in addition to detecting point mutations and small deletion/insertions, it can also be used to identify large chromosomal events, such as mitotic recombination, chromosomal loss, and large multilocus deletions that all lead to *Aprt* loss of the heterozygosity (LOH) in heterozygous animals [8]. Because mouse *Aprt* maps to the telomeric end of chromosome 8 [9, 10], it is especially useful as a marker for detecting proximal mitotic recombination events between homologs [7].

Using *Aprt* heterozygous mice as a model, we and others have been able to evaluate the effects of various genetic and environmental factors on the occurrence of LOH *in vivo* [11]. In these animals, one *Aprt* allele is non-functional due to knockout insertion of the *neo* marker into exon 3 [12]. When strain 129 mice with the knockout allele are crossed with other strains, such as C3H, many of the polymorphic simple sequence repeat (SSR) markers along chromosome 8 serve as heterozygous reporters for gauging the chromosomal regions where LOH begins [7]. In addition, depending on the choice of mouse strain, the chromosome 8 homologs may be heteromorphic at the centromeric region [13], allowing the cytogenetic tracking of each homolog. Each APRT-deficient clone arising *in vivo* but selected *in vitro* can be assigned to a particular LOH pathway based on the analysis of the *Aprt* gene sequence, SSR markers, and cytological markers [7] (See Supplementary data, Fig.S1).

We previously reported that spontaneous mutation rate in mESCs heterozygous at *Aprt* is significantly lower than in isogenic embryonic fibroblasts (MEFs) [1]. The great majority of spontaneous APRT-deficient mutant mESCs arose as a consequence of LOH. In the current study, we examined the mutational response of mESCs to x-rays, a form of ionizing radiation (IR) and a common genotoxic agent [14, 15]. IR can cause double-strand DNA breaks (DSBs) as well as base damage and single-strand DNA breaks (SSBs), which may subsequently convert to DSBs [16–18]. IR is known to cause LOH in mammalian cells, presumably through improperly repaired DSBs [14, 15, 19–22], large-scale genomic rearrangements [23–28], or through more local alterations such as point mutations [24]. We previously showed that adult cells exposed to x-rays undergo *Aprt* LOH primarily due to point mutations and interstitial deletions, whereas *in utero* exposure primarily results in LOH through mitotic recombination [24, 29]. These differences in mutational spectra are likely due to several factors, including differences in base excision repair capacities [19, 30, 31]. Thus, the spectrum of x-ray-induced mutations can be cell-type specific, though there is clear overlap between cell types. Since mESCs are distinct from adult cells in their cell cycle regulation, chromatin state, and DNA repair [32, 33], one might anticipate that mESCs would have a distinctive response to IR. Indeed, we observed that x-rays were extremely efficient in inducing LOH in mESCs and that mitotic recombination accounted for the majority of mutants, which is in sharp contrast to the mutational response of adult cells.

2. Materials and Methods

2.1. Mouse ESCs and their culture conditions

We used two previously described mouse ES cell lines, 3C4 and 2B5, that were derived from C3Hx129F1 *Aprt*^{+/-} blastocysts [1] (Supplementary data, Fig. S1, panel A). Feeder cell-free cultures mESCs were as described [34]. The mESCs were plated onto gelatinized 100 mm plates in N2B27 medium supplemented with leukemia inhibitory factor (LIF) and bone morphogenic protein 4 (BMP4). Cells were cultured at 37°C in the presence of 5% CO₂. To eliminate pre-existing APRT-deficient cells, mESCs were grown in the medium containing adenine and alanosine (final concentrations 50 µM and 4 µg/ml respectively) for 48 h [35] after which cells were washed with N2B27 medium, fed with fresh N2B27 supplemented with LIF and BMP4 and cultured for 4 more days before harvesting. Cells were seeded at 1–2 E6 cells per 100 mm plate and were grown for 24 h before they were irradiated with a calibrated Faxitron X-ray machine. Immediately after irradiation, medium was replaced with fresh supplemented N2B27 medium. Cells were allowed to recover for 30 h before they were plated for estimation of colony forming efficiency (CFE) and drug selection. For estimating CFE, cells were trypsinized to produce a single cell suspension, counted and seeded at 1,000 cells per 100 mm gelatinized dish. Plates were incubated for 10 days without medium change. For drug selection, mESCs propagated without feeder cells for 9 to 10 passages were seeded at 1 E6 cells per 100 mm gelatinized plate. Twenty-four hours later, 2-fluoroadenine (2-FA) was added to the medium to final concentration of 2 µg/ml [36]. 2-FA selection was for 10 days with medium changes every 3 days. Afterwards, colonies were isolated and placed into individual wells of 96 well plates and 24 h later, 2-FA was added. Cells were harvested as they approached 70 to 80% confluence and DNA extracted for genotyping. The mutant frequency was calculated as a ratio of the number of 2-FA-resistant (henceforth designated “FA-resistant”) colonies divided by the total number of cells plated for selection, correcting for colony forming efficiency [7].

2.2. Assigning the FA-resistant cell colonies to the specific LOH pathway

FA-resistant colonies were characterized based on analysis of the *Aprt* sequence, flanking SSR markers, and cytological markers [7]. Supplementary data Fig S1, panels C through E, outline the process. To determine whether or not the phenotype was caused by a physical loss of the wild-type *Aprt* allele, we used PCR with primers specific for the *Aprt* and *neo* sequences [7]. The FA-resistant colonies were divided into two classes based on the absence (class I) or presence (class II) of the untargeted (without *neo* insert) *Aprt* allele. The class I colonies were further characterized using the SSR markers on both sides of *Aprt* (See Supplementary data, Fig S1). LOH could be due to mitotic recombination (MR), chromosome 8 loss, chromosome 8 loss with reduplication (uniparental disomy, UPD), gene conversion (GC), or interstitial deletion (ID). The initial characterization of the type I FA-resistant clones was based on PCR characterization of flanking SSR markers. Supplementary data Fig S1, panel D illustrates the various outcomes. The loss of the telomeric SSR marker (*D8Mit56*) distal to *Aprt* indicates MR-, UPD- or chromosome 8 loss. These clones were further characterized for the centromeric SSR, *D8Mit155*, and only the clones that originated via MR retained heterozygosity, while the UPD- and chromosome 8 loss- clones were homozygous. The latter were interrogated by FISH, to determine whether there was one or two copies of chromosome 8. In contrast, the clones generated through GC or ID retained heterozygosity at both the centromeric *D8Mit155* marker and the telomeric *D8Mit56* marker, while harboring only the *neo* targeted *Aprt*. Distinguishing GC from ID was based on *neo* copy number as determined by real time quantitative PCR (qPCR see below).

2.3. FISH

Fluorescence *in situ* hybridization (FISH) used whole chromosome 8 painting probes (Cambio, Cambridge, UK) according to the manufacturer's instructions.

2.4. Real time quantitative PCR

The copy number of *neo* in clones with putative gene conversion/interstitial deletion was determined by qPCR using the ABI Prism 7900HT Sequence Detection System as described [37] with autosomal *Col1A* as a normalization reference (Mm00607939_s1 TaqMan assay, Applied Biosystem). PCR was carried out in a 384-well plate. Fifty to 100 ng of the sample DNA, 250 nM of each primer and 250 nM of probe were used in each reaction. The PCR conditions were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was assayed in triplicate. Target and control were run in separate wells. A relative standard curve method was used for discrete quantification of the target locus copy number.

2.5. Statistical analysis

The data on the APRT-deficient FA-resistant mutant frequency were analyzed by the Student T-test.

3. Results

3.1. Spontaneous FA-resistant variants in feeder cell-free and serum-free media

Karyotyping indicated that the majority of 3C4 mESCs were euploid ($65 \pm 21\%$), with $25 \pm 14\%$ of cells exhibiting trisomy 8. To streamline the selection of APRT-deficient clones, we adapted the *Aprt* heterozygous mESCs [1] to feeder-cell free conditions and cultured them in synthetic medium. Immunofluorescence and qPCR analysis revealed that even when cultured in the absence of a feeder cell layer, mESCs expressed markers of pluripotency (Supplementary Figs. S2 and S3). In the absence of a feeder cell layer, APRT-deficient mESCs were able to survive continuous exposure to 2-FA. *In situ* staining of colonies arising in this medium showed that 100% of all ES colonies were alkaline phosphatase (AP) positive, although in some instances differentiated cells were present on the edges of the colonies (Supplementary data Fig. S2, panels G & H).

Consistent with previous observations of mESCs grown on a feeder cell layer in serum-containing medium [1], the frequency of spontaneous FA-resistant mutants in the mESCs population remained low. Four independent experiments, using a total of 2.04 E8 cells, indicated a spontaneous mutant frequency 8.5×10^{-7} . This frequency is similar to that previously reported for these cells [1] (See Fig.1). The mutant colonies recovered from serum-free, feeder cell-free culture also exhibited a mutational spectrum similar to that reported [1] (Tables 1 and 2).

3.2. LOH through mitotic recombination is significantly increased in mESCs surviving IR exposure

IR produced a dose-dependent increase in the frequency of FA-resistant mESC colonies, reaching 380-fold induction at 10 Gy (Fig. 1A). An overwhelming majority lost the wild-type (untargeted) *Aprt*, and were thus designated as class I (Tables 1 and 2). In contrast, only a small fraction of recovered mutants retained the untargeted allele, and were designated as class II (Tables 1 and 2). Analysis of SSR markers flanking *Aprt* indicated that the predominant fraction of the class I colonies arose as a consequence of mitotic recombination (MR) (Fig. 2 and Table 2). Such colonies were homozygous for the distal *D8Mit56* marker in close proximity to the telomere and downstream of *Aprt*, but remained heterozygous for

the marker near the centromere, *D8Mit155* (See Supplementary data, Fig. S1). In unexposed mESCs the median frequency of MR was 4.1×10^{-7} . After exposure to x-rays, this category of mutants increased but remained proportional to the overall frequency of FA-resistant mutants (Fig. 2, Table 2).

3.3. Nondisjunction and whole chromosome loss also produce spontaneous and IR-induced FA-resistant mESC colonies

The second most common group of FA-resistant colonies (17.6 to 48%) showed LOH at both *D8Mit56* and *D8Mit155* (Fig. 2, Table 2, Supplementary data Fig. S1, C and D). These mutants could have arisen either by MR crossover between *D8Mit155* and the centromere, loss of the maternal chromosome 8 harboring wild-type *Aprt* (monosomy 8), or chromosome 8 loss/reduplication, i.e., uniparental disomy (UPD). To distinguish between these alternatives, we characterized 10 such mutant clones by qPCR and FISH. QPCR revealed that 9 out of 10 isolates harbored two copies of *neo*, indicating that FA-resistance arose through MR or UPD. FISH analysis of metaphase chromosomes showed two copies of chromosome 8, each with a small centromere characteristic of mouse strain 129 (data not shown). These colonies, therefore, were the product of chromosome nondisjunction and reduplication that resulted in UPD, since both centromeres were of the same parental origin.

3.4. Interstitial deletion induced by IR in mESCs

The third group of class I mutants (14 to 45%) retained heterozygosity at both *D8Mit56* and *D8Mit155* (Table 2, Fig. 2.). Either interstitial deletion, gene conversion or a double crossover could have produced this pattern. To discriminate between these mechanisms, we determined the *neo* copy number in 55 such FA-resistant clones.

Analysis by qPCR showed that 80% of these mutants (44 out of 55) had only one copy of *neo*, and therefore arose as a consequence of interstitial deletion (Fig. 3). Subsequent genotyping of SSR markers flanking *Aprt* showed that such deletions could extend for several Mb (Table 3). The fraction of clones with LOH attributable to interstitial deletions was the most prominent among FA-resistant mutants isolated from cells irradiated with 2 Gy.

3.5. Inactivation of *Aprt* by intragenic mutation in ESC

Only a small fraction, less than 3.1%, of FA-resistant mutants retained the wild-type *Aprt*. To elucidate the basis for these clones we PCR amplified and sequenced the *Aprt* coding regions [7]. At least one function-disrupting mutation was detected in each of the clones (Table 4).

3.6. Similar mutational response to IR in another mESC line

To establish whether or not the data from the 3C4 mESC line were specific for this particular cell line, we studied the effect of ionizing radiation on a second, independently derived mESC line, 2B5 (Cervantes *et al.* 2002). The spontaneous FA-resistant mutant frequency was 6.0×10^{-7} , similar to that of the 3C4 cells. The mutant frequency after exposure to 10 Gy of IR was 4.7×10^{-4} , also similar to that observed in 3C4 cells. As in the case of 3C4 ESCs, 67% of 38 FA-resistant mutant colonies arose as a consequence of MR and the remainder displayed UPD for chromosome 8; no class II mutants were identified. Thus, the remarkable induction of MR by x-rays is probably a general property of ESCs.

4. Discussion

We previously reported that T-cells and fibroblasts from *Aprt*^{+/-} mice have less than a two-fold increase in *Aprt* mutation frequency following 4 Gy x-rays, and that the majority of

these mutations were point mutations and interstitial deletions [29, 38]. In contrast, MR, the predominant mechanism for spontaneous APRT-deficient mutants in T cells and fibroblasts, was not significantly induced by x-rays in these adult somatic cells. Here we show that mESCs responded to x-rays in a distinctly different manner than do differentiated cells. First, x-rays are a more potent mutagen for mESCs than for differentiated cells. The mutant frequency in mESCs is elevated 10- fold by 2 Gy, and 100-fold by 5 Gy x-rays. Second, x-rays induce a distinctly different spectrum of mutations in mESC compared to adult somatic cells. In somatic cells, x-rays cause the loss of *Appt* function primarily through point mutations, small interstitial deletions, and epigenetic silencing [24, 29, 38]. These events are very rare in mESCs and, remarkably, none of the class II mutant clones was attributable to epigenetic silencing, an observation that may be related to their embryonic nature. It is possible that class II clones in mESCs might have arisen through epigenetic inactivation, but may not survive stringent selection with 2-FA due to residual APRT activity. In experiments with adult cells 2,6-diaminopurine was used for selection [7, 37]. In contrast to point mutation, MR and chromosome loss, with or without reduplication, were highly induced in mESCs following exposure to x-rays. UPD of chromosome 8 in ESC may be caused by a high prevalence of trisomy 8 [39], and this phenomenon may be unique to chromosome 8.

MR entails homologous recombination (HR) between chromosome homologs. HR, which most commonly occurs between sister chromatids, represents one of the two major pathways for repairing DSBs, the other being non-homologous end joining (NHEJ). Evidence suggests that HR may be essential for embryogenesis, since the absence of key HR genes, such as *Rad51*, leads to early embryonic lethality [40–42]. In contrast, the absence of genes critical for NHEJ has a less severe effect on embryogenesis [42]. Several lines of evidence suggest that mESCs may rely on HR rather than NHEJ to repair DSBs. For instance, genes involved in HR are expressed at much higher levels in mESCs compared to differentiated cells [3]. Therefore, the preference for HR for the repair of DSBs in mESCs may account for the preferential induction of MR.

Lack of a G1/S checkpoint in mESCs may also contribute to the preferential induction of MR by x-rays. Differentiated adult cells possess a relatively long G1 phase and a G1/S checkpoint that can be activated in case of DNA damage, leading to G1 cell cycle arrest. This arrest is mediated by p53-dependent induction of p21 and it allows for DNA repair before DNA replication is initiated in S phase. mESCs, in contrast, have virtually no G1 phase and lack the G1/S checkpoint. Therefore, they proceed into S phase even in the presence of IR-induced DNA damage [43, 44]. As a consequence, many IR-induced DSBs must be repaired in S phase by HR or MR, the latter resulting in LOH for heterozygous loci in half of the instances. Recent data from our group indicate that MR may occur in other cell cycle phases in mESCs [3].

Reminiscent of our findings in mESCs, the spectrum of *in vivo* somatic mutations induced by x-rays in fetal cells differs from that observed in adult cells [29]. When adult mice were exposed to 1 Gy x-rays, T-cells displayed an increased frequency of intragenic point mutations, but no increase in MR. However, the same x-ray dose applied to fetuses *in utero*, significantly induced MR but not point mutation. In fetal hematopoietic tissues, a combination of two factors might be responsible for the induction of MR. First, these fetal cells are known to have a relatively high rate of proliferation with a large proportion of cells in S-phase and second, base excision repair (BER) activity is lower in fetal hematopoietic tissues than in adult hematopoietic tissues [29], presumably causing more base lesions to be passed into S phase and resulting in stalled replication forks, which are repaired by HR. Therefore, the types of mutations induced by IR can vary between cell types, depending upon the repertoire of DNA repair enzymes available, the integrity of cell cycle checkpoints and other factors [4].

Despite the high capacity for HR in mESCs, the level of spontaneous LOH through MR in these cells is two orders of magnitude lower than that of fibroblasts or T-cells [1, 2]. Why induction of MR by x-rays is so dramatic in mESCs compared to adult cells, whereas spontaneous MR is so low compared to adult cells, remains unclear. Several possibilities could account for this apparent paradox. First, repair of spontaneous DSBs in mESCs may rely on HR between sister chromatids, a mechanism that cannot be detected by our selection system. In contrast, repair of IR-induced DSBs might rely more heavily on MR, using the homologous chromosome as the undamaged template, a mechanism whose outcome our system can detect. Second, MR, but not sister chromatid exchange, might be more likely to lead to apoptosis of mESCs under normal circumstances, removing such cells from the population. Exposure to IR could, potentially, overwhelm such an apoptotic protective mechanism and allow the persistence of MR cells in the population. Third, MR chromosomes may be more likely to exhibit a different segregation pattern in cells exposed to IR compared to unexposed cells. Only in the case of x-segregation, when recombinant chromosomes are sequestered into different daughter cells, would MR lead to LOH. In contrast, z-segregation, when both recombinant chromosomes are segregated to the same daughter cell, would preserve heterozygosity, although not linkage relationships, and thus would be undetectable in our system [45]. Thus, the choice between two chromosome segregation patterns determines whether or not there is LOH due to MR. It is possible that IR influences the segregation pattern due to fundamental differences in mechanism or timing of each.

mESCs resemble simpler single cell eukaryotes such as yeast in their heavy reliance on HR. Similarities between mESCs and yeast may not be coincidental. In yeast, a single mutant cell may divide to establish an entire population that might have reduced fitness relative to wild-type under some circumstances. Thus, error-free DNA repair is highly selected by evolution. Similarly, a single or small group of mutant embryonic cells can contribute to many or even every tissue type of the adult, including the germ line, resulting in reduced fitness that may be passed to the next generation. In embryonic cells of mammals there is a pressing need for error-free DNA repair to avoid these negative consequences since the population of adult animals is relatively small (relative to yeast populations) and mutations can become fixed in the population through genetic drift or selection. These constraints on DNA repair may explain the evolution of HR as the predominant pathway of DNA repair in mESCs and yeast, while in somatic cells with restricted proliferative potential and little opportunity to affect future generations the less costly NHEJ is utilized [50]. Accordingly, if a few mutant adult somatic cells of an organism die due to harmful mutations from misrepair or induced apoptosis, they are likely to be replaced by normal sib cell or from stem cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. X-rays caused a significant increase of *Aprt* LOH in mESCs. A. The APRT-deficient mutant frequency in mESCs exposed to X-rays. mESC heterozygous at *Aprt* were irradiated with the indicated doses. Cells were plated for selection 30h later and APRT-deficient cells were selected as described in Materials and Methods. Mutant frequency was corrected for colony-forming efficiency. Each dot represents an individual experiment. Horizontal bars indicate the median for each group and asterisks indicate T-test $p < 0.05$. B. Effects of x-rays on colony forming efficiency of mESCs. mESCs were plated 1,000 per 100 mm dish and irradiated 15 h later with the indicated doses. All the plates were re-fed and incubated as described in Materials and Methods. Plates were scored 10 days later and the average number of colonies on plates exposed to the same x-ray dose was normalized.

A.

B.

Fig. 2.

Mitotic recombination accounts for majority of LOH events induced by x-rays in mESCs. The FA-resistant clones that appeared during the selection experiment were collected and genotyped as described in Material and Methods. A. Mutational spectrum. B. The frequency of each category.

Fig. 3. Interstitial deletion events are more commonly induced by x-rays than gene conversions. The mESCs class I APRT-deficient mutants that retained heterozygosity at both *D8Mit56* and *D8Mit155* were subjected to *neo* copy number determination by qPCR. The ratio of the threshold copy number (Ct) for *neo* and that for autosomal *Col1A* was determined for each APRT-deficient mutant of this category. DNA from 6 APRT^{+/-} mice and 6 APRT^{+/+} mice was used to determine the range of the Ct_{neo}/Ct_{Col1A} for cells containing 1 or 2 copies of *neo*, respectively.

Table 1

Classes of FA^R mESC clones as determined by allele-specific PCR

IR (Gy)	Number of cells (E6)	Number of characterized clones		
		Total	Class I	Class II
none	204	61	100%	0%
2 Gy	68	82	97.6%	2.4%
5 Gy	76	158	96.9%	3.1%
10 Gy	56	176	98.3%	1.7%

Table 2Classification of FA^R colonies by allele-specific PCR

	Control	x-ray dose		
		2 Gy	5 Gy	10 Gy
Percentage of FA ^R clones				
Class I clones	100% (61/61)	98% (80/82)	97% (153/158)	98% (3/176)
Mitotic recombination	49 % (30/61)	35% (29/82)	60% (95/158)	51% (90/176)
Uniparental disomy	43% (26/61)	18% (14/82)	23 % (36/158)	48% (48/176)
Gene conversions /Interstitial deletions	8% (5/61)	45% (37/82)	14.0% (22/158)	20.0% (35/176)
Class II clones	0% (0/61)	2% (2/82)	3% (5/158)	2% (3/176)
Mutant frequency (E-7)	8.5	100	900	2900
Class I clones	8.5	97.6	872.1	2850.7
Mitotic recombination	4.1	35	540	1479
Uniparental disomy	3.7	17.6	205.2	1392
Gene conversions /Interstitial deletions	0.7	45	12.6	580
Class II clones	0	2.4	27.9	49.3

Table 3

Intervals of interstitial deletions in representative clones

Clone ID	IR (Gy)	3.4Mb							neo	D8Mit321	D8Mit13	D8Mit14	D8Mit280
		D8Mit155	D8Mit224	Mt3	96.6Mb	118.2Mb	126.3Mb	127.1Mb					
9H4	0							ND	1 copy			ND	
9C4	0							ND	1 copy			ND	
14E2	2							ND	1 copy			ND	
14G2	2							ND	1 copy			ND	
14H3	2							ND	1 copy			ND	
14F6	5			ND		grey		ND	1 copy			ND	
7E2	10								1 copy				
7F4	10			ND					1 copy				

Retention of heterozygosity

Loss of heterozygosity

ND

No data

1 copy

1 neo copy

Table 4

The summary of point mutations in mES clones

IR (Gy)	Mutation	Change	Exon	Note
0	Insertion C	Stop110	3	3 clones
0	Duplication AGTC	Stop110	3	
0	T→G	Y105X	3	
0	16 bp Deletion	Stop149	4	
10	A→G	M1V	1	
10	Insertion CA	Stop150	5	
10	C→A	S175Y	5	6 clones