The Effects of Maternal Irradiation on ESTR Mutation Induction and Transgenerational Instability in Mice

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Abstract

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The main source of data used to assess genetic risks of radiation exposure for humans has been derived from experiments analysing the male germline, while the effects of maternal irradiation remain poorly understood. This project therefore aims to analyse the long term genetic effects of acute maternal irradiation. To investigate the effects of acute irradiation on genome stability in the germline of directly exposed females, adult BALB/c and CBA/Ca mice were exposed to 1 Gy of acute X-rays and mated with control males 2-5 days following exposure, enabling analyses of offspring that were conceived from irradiated dictyate oocytes in maturing follicles. The data revealed that frequency of mutation at expanded simple tandem repeat (ESTR) loci in the germline of directly exposed females did not differ from that in control families. To address the effect of parental irradiation on transgenerational instability, ESTR mutation frequency was also established in the germline and somatic tissues of first-generation offspring of exposed adult males and females using single-molecule PCR. The breeding scheme used implied that the offspring of irradiated males and females were derived from irradiated post-meiotic stages spermatozoa and meiotically arrested dictyate oocytes in maturing follicles, respectively. While the frequency ESTR mutation in the offspring of irradiated males was significantly elevated, maternal irradiation did not affect the F_1 stability. The results of this project therefore show that, in sharp contrast to the paternal exposure to ionising radiation, the transgenerational effects of maternal high-dose acute irradiation are likely to be negligible. The analysis of transcription profiles of first-generation offspring of irradiated males and females reveals drastically different patterns of gene expression profiles in both groups. Specifically, a substantial number of genes significantly deregulated in the offspring of irradiated males belong to functional groups directly involved in maintaining the stability of the genome. In contrast, in the offspring of irradiated females none of the significantly deregulated genes can be implicated in the maintenance of genome stability. The work presented here therefore provide new evidence for striking differences in the manifestation of long-term effects of paternal and maternal acute exposure to ionising radiation in mice.

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Abbreviations

5m-C	5-methyldeoxycytosine
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia and Rad3-related protein
bp	Base pairs
BMP-4	Bone morphogenetic protein-4
BSA	Bovine serum albumin
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-protein kinase
DNA-PK$_{cs}$	DNA-protein kinase catalytic subunit
DNMT1/3a/3b	DNA methyltransferase 1/3a/3b
dNTP	Di-nucleotide tri-phosphates
DSB	DNA double strand break
EDTA	Ethylene diamine tetra-acetic acid
ENU	Ethylnitrosourea
ESTR	Expanded simple tandem repeat
EtBr	Ethidium bromide
EtOH	Ethanol
F_0	parental generation
F_1	First filial generation (F_0 x F_0)
F_2	Second filial generation (F_1 x F_1)
FISH	Fluorescence *in situ* hybridisation
GI	genomic instability
GM10115 cells	Chinese hamster-human hybrid parental cells
Gy	Gray
HDACs	Histone deacetylases
Hm-2	Human microsatellie-2
Hprt	Hypoxanthine guanine phosphoribosyl transferase
HR	Homologous recombination
IAP	Intracisternal A-particle elements

Il-	Interleukin-
IR	Ionizing radiation
Kb	Kilo-base pairs
KO	knockout
LET	linear energy transfer
MBD	Methyl-CpG-binding domain protein
MeCP2	Methyl CpG binding protein 2
MeDIP	Methylated DNA immunoprecipitation
MMR	Mismatch repair
MMS	Methylmethanesulfonate
MNU	Methylnitrosourea
Ms6-hm	Minisatellite 6 hypermutable
NaAc	Sodium acetate
ncRNAs	Non-coding RNAs
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NO	Nitric oxide
OLB	Oligo labeling buffer
OSS	Oligo Stop Solution
p	Pico
PARP-1	Poly (ADP-ribose) polymerase-1
PCR	Polymerase chain reaction
PGCs	Primodial germ cells
Prob.	Probability
PTM	post-translational modification
p^{un}	pink-eyed unstable allele
PWS	Phosphate wash solution
RBE	Relative biological effectiveness
RIGI	Radiation-induced genomic instability

RNAi	RNA interference
ROS	Reactive oxygen species
RT	Reverse transcriptase
SCE	Sister-chromatid exchange
scid	Severe combined immunodeficient
SDS	Sodium dodecyl sulphate
SINE	Short interspersed nucleotide elements
siRNAs	Small interfering RNAs
SLT	Specific locus test
SM-PCR	Single molecule polymerase chain reaction
SSB	Single-strand break
SSC	Sodium chloride and sodium citrate buffer
STR	Simple tandem repeats
Sv	Sievert
***T* stock**	Recessive test stock used for the Specific Locus Test
TBE	Tris borate EDTA
TI	Transgenerational instability
TNF-α	Tumour necrosis factor alpha
U	Unit(s)
UV	Ultraviolet
V	Volt(s)
γH2AX	Phosphorylation of histone 2AX

1 INTRODUCTION

Humans are constantly exposed to naturally occurring ionising radiation (background radiation), mostly from natural sources such as cosmic rays and nuclides present in the earths' crust. Over the last century there has been a dramatic increase in the usage of nuclear technology in a variety of purposes such as production of nuclear weapons, generation of nuclear energy and radioisotope manufacturing (UNSCEAR, 1993). Over this period, radiation has also been increasingly applied in medical purposes including diagnosis and therapy. For example, X-rays are commonly used in radiography and radionuclides for tissue imaging purposes. Also, radiotherapy still represents a main treatment of cancer. This increases the risk of exposure to radiation either occupationally, accidentally or medically. For this reason, it is becoming increasingly difficult to ignore the mutagenic effect of ionising radiation or its potential to increase the incidence of cancer.

In addition to mutation induction occurring in directly exposed somatic cells, ionising radiation can also lead to non-targeted and delayed effects manifested in the progeny of irradiated cells many cell divisions after the initial irradiation insult. These delayed effects were found to be manifested both *in vitro* and *in vivo* (Morgan, 2003a; Morgan, 2003b). Indeed, as growing body of experimental evidence demonstrates the enhanced mutation rate in the descendants of irradiated cells, the results of these studies have raised a possibility that parental irradiation can also destabilise the genome of their non-exposed offspring, thereby affecting their mutation rates and cancer incidence (Barber and Dubrova, 2006; Dubrova, 2003).

Currently, our understanding of the genetic risks of radiation exposure is mostly based on the results from epidemiological studies of the effects of occupational, medical or accidental exposure. These studies provide valuable information on the effects of human exposure to radiation from medical and occupational sources. However, the effects of accidental and environmental exposure to radiation are so far hampered by accuracy of the estimated exposure doses. Besides, in many epidemiological studies, the choice of representative control group properly matched the exposed group in ethnicity and life style can be also problematic. This often leads to a biased data being generated and uncertain outcomes. Taken together, animal experimental data remains the main source of comprehensive information on the consequences of exposure to ionising radiation. Indeed, much of what is known has been extrapolated from animal studies, in particular, from male germline data in mice (UNSCEAR, 2000). To date, the genetic effects of maternal irradiation remain poorly understood. Given the profound differences between male and female germ cell biology, a substantial dissimilarity in the manifestation of mutagenic effect of exposure to ionising radiation in the maternal germline may be expected. It is therefore becoming increasingly important to analyse the effects of radiation exposure on the female germline.

The work presented in this thesis aims to analyse the still poorly understood effects of maternal irradiation on ESTR mutation induction in the germline of directly exposed female mice, and the transgenerational effects of maternal irradiation in their non-exposed first-generation offspring.

1.1 Germline mutation induction in the male germline

Over the past decades several assays have been developed to establish the mutagenic effects of paternal irradiation in mice. The experimental systems used to analyse the effect of ionising radiation on germline mutation induction are either pedigree-based, where the *de novo* radiation-induced and spontaneous mutations are detected in the offspring or assays which are capable of detecting mutations directly in the germ cells of exposed and control males. Ideally, any model system for monitoring mutation induction in the germline should be (i) informative and capable of detecting mutation induction in relatively small numbers of animals and (ii) sensitive enough for detecting mutation induction within a wide range of doses. In the next few paragraphs, the traditional mutation detection assays will be discussed with reference to the two main criteria itemised above.

1.1.1 Traditional phenotypic assays

More than six decades ago, Russell and Kelly developed the specific locus test which is also referred to as a Russell-7-locus test (Russell, 1951). This assay quickly become the most widely used system for the analysis of germline mutation induction by irradiation or chemical mutagens. The specific locus test is based on utilisation of a tester strain of mice that is homozygous recessive for the chosen locus. The test (T) stock loci that are commonly used were established by Russell in 1948 and include seven loci: *agouti*, *tyrosine-related protein*, *pink-eyed dilution*, *tyrosinase*, *mysosine Va*, *morphogenic protein 5*, and *endthelin receptor type b* (Russell *et al.*, 1981). The assay involves mating control or exposed wild-type parents with partners of the test strain and mutation scoring of dominant and recessive mutations occurring in in the germline of wild-type

parents among the offspring. The specific locus test is considered as a reliable system and has been widely used for the analysis of germline mutation induction. However, it is dependent upon the analysis of mutation rate of structural genes whose mutation rates never exceed 10^{-5} per gamete (Schlager and Dickie, 1967), thus requires the profiling of enormous numbers of offspring of parents exposed to very high doses of ionising radiation.

Another assay, the dominant lethal test, is based on the analysis of embryonic mortality in non-treated females mated with either control or exposed males. In this test, the pregnant females are sacrificed on approximately 17^{th} day post-conception, and the number of viable, malformed and implanted embryos, together with the total number of ovulations (corpus lutea) is established. This approach has been used in early studies to assess the mutagenic effects of paternal exposure to ionising radiation (Luning and Searle, 1971; Kirk and Lyon, 1984). Although technically straightforward, the dominant lethality approach is non-informative regarding non-lethal mutations and is also largely influenced by maternal health and environmental factors.

The Induction of dominant visible mutations, such as skeletal malformation in the offspring of irradiated parents, has also been used for the detection of germline mutagenicity (Bartsch-Sandhoff, 1974; Ehling, 1991; Selby, 1979). Given that the development of the skeletal system is influenced by a large number of genes, it could provide a suitable model system for the estimation of the overall genetic damage caused by irradiation (Ehling, 1991). However, the difficulty of describing of internal malformation and the large sample sizes required complicate its usage as a model system (Searle, 1974).

Genetic damage induced by ionizing radiation can manifest as chromosomal alterations which are detectable in the irradiated germline either by conventional cytogenetics or fluorescent in situ hybridisation (FISH) (van Buul, 1983). For instance, reciprocal chromosomal translocations detected in the F_1 offspring of irradiated fathers have been used as an endpoint for the detection of germline induced mutations by ionising radiation (Leonard and Deknudt, 1967; van Buul, 1983), as well as in the sperm samples taken from directly exposed males (Generoso *et al.*, 1984). Interestingly, frequency of structural chromosomal alterations induced in the sperm samples taken from irradiated male mice was found to be dose-dependent (Leonard and Deknudt, 1967), however the reliability of this endpoint for the detection of the effects of chronic exposure is questionable.

Another technique used for the detection of radiation-induced DNA damage is the micronucleus test. Micronuclei are small nuclear-like structures detected in cytoplasm near the nucleus that contain fragmented chromosomes arising during cell division. This technique is widely used *in vitro*, however Pampfer and co-authors (1989) have used it for the analysis of the germline mutagenic effect. The authors analysed micronucleus formation in 2-blastomer stage embryos derived from irradiated spermatozoa and observed that the incidence of micronuclei formation increased in a dose dependant fashion. However, no information can be obtained regarding the rate or type of mutation corresponding to the micronuclei formation.

In 1984 Ostling and Johnson developed the Comet assay which based on gel electrophoresis of individual cells and measuring of the amount of DNA damage. During electrophoresis damaged DNA migrates from the nucleus; the amount of which

is assessed on the fluorescently stained slides. It has been shown (using this technique) that the amount of DNA damage in irradiated cells increases in a dose dependant manner (Schindewolf *et al.*, 2000; Haines *et al.*, 2001). However, similar to the micronucleus test, the comet assay is not informative on mutation rate or type.

Another widely used system for *in vivo* mutation detection employs transgenic engineered mice with the *lacI* reporter system (Kohler *et al.*, 1991).The integrated λ shuttle vector containing the *lacI* gene, encoding the β-galactosidase enzyme, is used for mutation detection. Vectors are recovered from genomic DNA of transgenic mice and plated on the media containing X-gal (β-galactosidase), thus blue clones containing *lacI* mutations are easily detected and can be further characterised by DNA sequencing. This technique was used by Luke and co-authors (1997) for the analysis of transgenerational increases in mutation rate among the first-generation (F_1) offspring of male mice exposed to 4 Gy of γ-rays. The authors observed a substantial increase in the number of mutations in the bone marrow of the F_1 offspring of irradiated males. The main advantage of *lacI* system is its ability to detect both spontaneous and induced mutations in DNA samples extracted from any mouse tissue. However, the efficiency of *lacI* system is limited by its inability to detect large deletions, commonly found within the spectrum of radiation-induced mutations (Hoyes *et al.*, 1998).

The abovementioned assays, particularly the specific locus test, have successfully been used in numerous studies for the analysis of mutation induction in mice. This work has also provided irrefutable evidence that exposure of male mice to ionising radiation results in elevated germline mutation rates (Searle, 1974).

However, most, if not all, of them require the analysis of exceptionally high numbers of mice following paternal exposure to high doses of radiation. Furthermore, due to low sensitivity, they are not suitable for the analysis of the effects of low-dose exposure. In contrast, a new molecular system that utilizes tandem repeat DNA loci fulfils these criteria that will be discussed in the next section.

1.1.2 Tandem repeated DNA loci

Tandem repetitive DNA sequences comprise a large extent of mammalian genomes. These sequences are well studied in the human genome and include a wide range of repeat sizes that varies from a few bases to up to several kilobases (Komissarov *et al.*, 2011). Tandem repeats are categorised into three main classes based upon the size of repeat units and the overall size of the repeat array, , namely satellite DNA, minisatellites and microsatellites. Another class of tandem repeats detected in the mouse genome is known as Expanded Simple Tandem Repeat loci (ESTRs), which possess characteristics of both mini- and microsatellites. Microsatellite, minisatellite and ESTRs are all commonly used for genotyping, as well as for the analysis of germline mutation induction (Table 1.1).

Table 1.1: Characteristics of tandem repeat loci (adapted from Bouffler *et al*., 2006)

	Minisatellites	**ESTRs**	**Microsatellites**
Repeat unit	10 – 60 bp	4 – 10 bp	2 – 6 bp
Array size	0.5 – 15 kb	0.1 – 20 kb	10 bp – 1kb
Array complexity	Heterogeneous	Homogenous	Homogenous
Instability	Very law in soma but high in germline	High in both soma and germline	High in soma
Mutation mechanism	Meiotic recombination (most likely)	Replication slippage (most likely)	Replication slippage

1.1.2.1 Microsatellites

Microsatellites or simple tandem repeats, represent the smallest but also the most ubiquitous group of tandemly repeated DNA in both prokaryotic and eukaryotic genomes (Hancock, 1996; Richard *et al.*, 2008). Their distribution and abundance varies among species. Found in non-centromeric regions either near or within coding genes (Toth *et al.*, 2000), microsatellites consist of 1-6bp repeat units arranged in arrays ranging from 0.1 to 1kb with minimal variation in repeat units (Toth *et al.*, 2000). In eukaryotes, the mutation rates of microsatellite loci ranges from 10^{-4} to 10^{-3} per locus per generation (Weber and Wong, 1993).

It is generally accepted that length changes in microsatellites occurs via a replication-dependant process namely, replication slippage (Ellegren, 2004). Replication slippage involves the transitory detachment of the newly synthesised strand from the template strand followed by misaligned reassembly (Levinson and Gutman, 1987; Ellegren *et al.*,

2004). Evidence for replication slippage as a mechanism of spontaneous microsatellite mutation comes from the work of Brinkman *et al.* (1998). They demonstrated high spontaneous mutation rates in the male germline compared to that of the female germline, and additionally, the germline mutation rate was higher in older men. These findings show a strong positive correlation between the number of cell divisions prior to meiosis and the mutation rate of microsatellites indicating that microsatellite mutation is replication based process (Brinkman *et al*., 1998).

In addition, Heyer *et al.* (1997) found that neither intra-allelic exchange nor recombination to be implicated in the microsatellite mutation process. The authors described marked similarities in both the pattern and rate of mutation of autosomal and Y-linked microsatellites. These similarities could not be expected if the microsatellites mutate by recombination events that would be absent on the Y-chromosome. However, in some circumstances, the repeat units of microsatellite could form secondary structures such as hairpin loops which may promote their instability via recombination or interference of enzymes of the DNA replication machinery (Mitas, 1997).

1.1.2.2 Minisatellites

Minisatellites are tandem repetitive DNA loci consisting of 6–100 bp units arranged into 0.5–30 kb long arrays. Thousands of minisatellite loci are scattered throughout the mammalian genomes and they are generally GC rich. They frequently show variability in repeat copy number and therefore allele length (Armour *et al.*, 1990; Wong *et al.*, 1987). These loci mutate almost completely in the germline during meiosis and likely via recombination/inter-allelic gene conversion-like process driven by recombination hotspots in the vicinity of alleles. In contrast, their mutational events in somatic cells are

rare and simple and likely to arise via replication slippage (Jeffreys *et al.*, 1994, 1997; Buard *et al.*, 1998; Buard *et al.*, 2000; Tamaki *et al.*, 1999). Minisatellites possess high spontaneous germline mutation rates, which range from 0.5 to 13% per gamete for some minisatellite loci, making them very useful markers of monitoring germline mutation induction in humans (Jeffreys *et al.*, 1998; Vergnaud and Denoeud, 2000).

1.1.2.3 Expanded simple tandem repeats

ESTRs are tandem repetitive DNA loci that were previously classified as minisatellites, but later, given a number of substantial differences in their biology, renamed (Yauk, 2004). In contrast to minisatellites, ESTRs possess short core sequence units (4–6 base pairs, similar to microsatellites) that are arranged into very long tandem arrays, up to 20 kb (similar to minisatellites). Furthermore, ESTRs are comprised almost exclusively of homogeneous arrays, with longer arrays exhibiting the highest rates of mutation compared to the more complex variant repeat distribution that makes up common minisatellite alleles (Bois *et al.*, 2001). In addition, ESTRs are highly mutable in somatic cells and are likely to have different mechanisms of mutation from the GC-rich minisatellite loci (Bois *et al.*, 1998, 2001; Amarger *et al.*, 1998). The biology of ESTRs has been addressed in many studies, with the two most unstable characterized in detail, namely the *Ms6-hm* and *Hm-2* loci, consisting of GGGCA and GGCA repeat units, respectively (Kelly *et al.*, 1989; Gibbs *et al.*, 1993).

1.1.2.4 Tandem repeated loci as a tool for detection of germline mutation induction

Tandem repeat DNA sequences, such as minisatellite DNA, expanded simple tandem repeats (ESTRs), and microsatellites have been used for detection of germline mutation induction in humans and mice (Yauk, 2004; Dubrova *et al.*, 1993, 1998, 2002; Barber *et al.*, 2002). These loci have high spontaneous rates of mutation which greatly facilitates the analysis of mutation induction in relatively small of numbers of exposed families. As tandem repeated DNA markers also show substantially high mutation frequencies of induced mutation, mutation induction at these loci cannot not be attributed to their direct targeting by ionising radiation. It was therefore suggested that mutation induction at these loci is attributed to the non-targeted mechanisms (explained on page 24) (Bouffler *et al.*, 2006). Mutations at these loci are manifested as gains or losses in repeat units and easily detectable either by pedigree screening or by PCR analysis (Niwa, 2003; Yauk, 2004). These characteristics of tandem repeated DNA loci make them an attractive system for monitoring mutation induction in the germline.

1.1.2.5 Minisatellite mutation induction in human

Germline mutations induced in the minisatellite loci have been analysed in the accidentally irradiated families from Japan and the former USSR using the pedigree based approach (Kodaira *et al.*, 1995; Dubrova *et al.*, 1996, 1997, 2002). For example, two studies were carried out on the atomic bomb survivors at Hiroshima and Nagasaki in 1995 and 2004. Both studies failed to establish significant increases in minisatellite mutation rates in the germline of irradiated parents (Kodaira *et al.*, 1995; Kodaira *et al.*, 2004).

On the other hand, Dubrova *et al.* (1996) estimated the frequency of minisatellite mutation in families inhabiting the heavily polluted areas in Belarus following the Chernobyl accident and found a two-fold increase in minisatellite mutation ate in the germline of exposed parents compared with non-exposed population. The authors found that in exposed families, the mutation rate correlated with the level of caesium-137 surface contamination. In 1997, Dubrova *et al.* confirmed these results by profiling a larger set of minisatellite loci, reporting a similar two-fold increase in the germline of irradiated families. However, since the control used by these two studies was an ethnically-different population from the UK, the results did not provide clear cut evidence of germline mutation induction following radiation exposure.

To further verify their work, Dubrova *et al.* (2002b) repeated the analysis using well matched control and exposed groups that were composed of children born in the same area (Kiev and Zhitomir regions of Ukraine) using the same minisatellite loci used in the previous studies. The authors showed a statistically significant (1.6-fold) increase in the paternal mutation rate in the exposed families from Ukraine, whereas maternal mutation rate of the exposed and the non-exposed mothers was very similar (Dubrova, *et al.*, 2002b).

Minisatellite mutation induction has also been analysed in families of Chernobyl clean-up workers. The results of these studies do not provide any evidence for minisatellite mutation induction in the germline of irradiated fathers (Livshits *et al.*, 2001; Kiuru *et al.*, 2003; Slebos *et al.*, 2004). However, in the study of Slebos *et al.* a marginally significant increase in mutation rate at the of the tetranucleotide microsatellite locus *D7S1482* was detected in the families of Chernobyl liquidators. It should be noted that

analysis of microsatellite mutation in another Belarusian cohort of liquidators failed to confirm this result (Furitsu *et al.*, 2005).

In contrast, Dubrova *et al.* (2002a) found a two fold increase in minisatellite mutation rate in the germline of human families exposed to radioactive fallout in the vicinity of the Semipalatinsk nuclear weapon test site. This substantial increase corresponded with the paternal exposure to higher doses of ionising radiation following the earlier atmospheric explosions, and dropped to 1.5 fold in the F_1 parental generation exposed to lower doses.

In conclusion, it should be stressed that the results of the abovementioned studies are highly inconclusive and so far have not provided enough evidence for minisatellite mutation induction in the germline of irradiated humans. However, it would appear that minisatellite loci do provide a suitable system for the detection of germline mutations in humans and has the advantage of utilising relatively small sample sizes. Indeed, using this system the first experimental evidence that ionising radiation could increase germline mutation rates of exposed humans was obtained (Dubrova *et al.*, 2002a and b; Bouffler *et al.*, 2006).

Considering the biology of minisatellite loci, the nature of radiation exposure and the lack of reliable dose and/or dose response data, as well as the conditions used in each experimental design, may help in understanding the discrepancies in the experimental data. For example, given that human minisatellite loci mutate almost exclusively in the germline during meiosis, it is likely that radiation induced mutagenic effects occur in premeiotic diploid germ cells and then manifest as minisatellite instability during

meiosis (Bouffler *et al.*, 2006). If so, then mutation induction at these loci is determined by the timing of exposure. For example, exposure of post-meiotic germ cells should not affect minisatellite mutation rate in the germline of irradiated parents. Indeed, Dubrova *et al.* (2002b) failed to detect any significant changes in the germline of irradiated mother, none of which had been exposed at pre-meiotic stages of oogenesis. To this end it should be noted that in the abovementioned studies on the families of atomic bomb survivors very high proportion (~50%) of children were born to exposed mothers, which may potentially explain their negative results.

A direct comparison of the results of these studies is also complicated by the fact that exposure of irradiated families dramatically differ with respect of dose, dose-rates and sources (external for the Japanese cohort and mixed for the post-Chernobyl families). These differences could explain the conflicting body of data such as the non-significant change in germline mutation rates obtained from the analysis of Japanese bomb survivors (Kodaira *et al.*, 1995; Kodaira *et al.*, 2004), and the data on elevated minisatellite mutation rate in the germline of irradiated fathers exposed to either the Chernobyl radioactive contamination (Dubrova *et al.*, 1996, 1997 and 2002b), or to the fallout following nuclear weapon tests (Dubrova 2002a).

1.2 ESTR mutation induction in the germline of irradiated male mice

Over the last decades mouse ESTRs have been used extensively for the analysis of germline mutation induction following exposure to a variety of environmental mutagens such as radiation and chemicals. This section will discuss the stage-specificity of ESTR mutation induction and the effects of exposure from different sources of radiation.

1.2.1 ESTR response to irradiation in specific stages of spermatogenesis

It has been established that the time elapsed between irradiation of male mice and mating with a non-exposed female greatly affects ESTR mutation induction in the germline of the exposed male. By changing breeding schedules it is possible to target a certain stage of spermatogenesis (see Table 1.2). For example, when the irradiated males are mated with non-exposed females 6 weeks following irradiation their offspring derive from irradiated diploid pre-meiotic spermatogonia, whereas mating three weeks post-irradiation result in offspring derived from irradiated post-meiotic spermatids (Adler, 1996). However, the data on stage-specificity of ESTR mutation induction in the male germline still remain controversial.

In 1993, Dubrova *et al.* obtained the first experimental evidence showing that acute irradiation significantly increases ESTR mutation rate in the germline of irradiated male mice. The authors analysed DNA fingerprints of the offspring of ^{60}Co γ-irradiated male mice exposed at the diploid, pre-meiotic spermatogonial cell stage (6 weeks after irradiation). According to the results of this study, paternal acute exposure to 0.5 and 1 Gy of γ-rays can significantly increase ESTR mutation rate in the germline of irradiated males. However, no other stages of mouse spermatogenesis were examined.

Table 1.2: Stages of spermatogenesis (adapted from Searle, 1974)

Stage of development	**Days taken to reach ejaculate**	**Mating scheme**	**Relation to meiosis**
Primordial germ cells	Over 42	> 6 weeks	Pre-meiotic
As stem cells	Over 42		
Type A spermatogonia	Over 42		
Intermediate spermatogonia	35-37	5 weeks	
Type B spermatogonia	34-36		
Primary spermatocytes	23-33		Post-meiotic
Secondary spermatocytes	21-22	3 weeks	
Spermatids	7-21		
Spermatozoa	0-7	1 week	

The first study to attempt to establish the stage-specificity of ESTR mutation induction was carried out by the Niwa's group (Sadamoto *et al.*, 1994; Fan *et al.*, 1995). The authors demonstrated substantially elevated ESTR mutation rates in the germline of male mice mated either one week or 2-3 weeks post-irradiation thus, following exposure to the post-meiotic sperm and spermatids, respectively. According to the results of these studies the effects of exposure to stem cells (mating 10-11 weeks post-irradiation) are likely to be negligible. The same authors later confirmed these results by analysing ESTR mutation induction in the germline of male mice exposed to high-LET fission neutrons (Niwa *et al.*, 1996).

In contrast, Dubrova *et al.* (1998) analysed the offspring of male mice derived from irradiated male germ cells at different developmental stages: spermatogenic stem cells

(mating 10 weeks post-irradiation), spermatogonia (mating 6 weeks post-irradiation), and spermatids (mating 3 weeks post-irradiation). They observed a highly significant elevation of ESTR mutation frequency (~4 fold increase) in the offspring derived from either irradiated premeiotic diploid spermatogenic stem cells or spermatogonia, whereas among the offspring derived from post-meiotic spermatids, it was close to that in controls.

Several studies have confirmed the findings of Dubrova *et al.* (1998). For example, Somers *et al.* (2004) have also found similar results following irradiation of out-bred Swiss-Webster mice. In addition, the same finding was consistently reported using similar breeding schedules (Barber *et al.*, 2000; Barber *et al.*, 2002; Barber *et al.*, 2009; Dubrova *et al.*, 2000). Furthermore, using a novel single molecule PCR technique that utilises ESTR loci, Yauk *et al.* (2002) demonstrated elevated mutation frequency in the sperm of male mice irradiated at premeiotic diploid spermatogonia.

One possible explanation for this conflicting evidence comes from the efficiency of repair systems during each stage of spermatogenesis. For instance, the spermatid stage undergoes the most rapid changes in terms of DNA repair efficiency, so that the early spermatids maintain their capacity to repair DNA damage. On the other hand, the DNA repair system is completely suppressed in late spermatids, especially when their nuclei become more condensed by replacement of histones on to protamines (Russell *et al.*, 1991; Bouffler *et al.*, 2006; Niwa, 2006). This means that the early spermatid stage is capable of repairing DNA damage before it is manifested as ESTR mutation, whereas the late spermatids are unable to repair DNA damage that then passes through sperm and manifests as a mutation during fertilisation (Fan *et al.*, 1995; Niwa, 2006).

Comparing the findings of Dubrova *et al.* with that of the Niwa's group regarding the post-meiotic spermatid stage sensitivity we see that the Dubrova's group targeted the repair proficient early spermatid stage by mating males with control females 3 weeks post-irradiation. On the other hand, Niwa *et al.* tested the late spermatids that lack a functional repair system. The discrepancy between the two findings could be attributed to the differential capability of each stage to repair radiation induced damage before manifestation as ESTR mutation. Accordingly, one can explain the high ESTR mutation rates observed following irradiation of post-meiotic spermatids by Niwa group but not seen by the Dubrova group (Niwa, 2006).

Another explanation for these discrepancies could arise from the difference in the endpoint used for detection of ESTR mutation scoring as well as the criteria for scoring mutant alleles used in each laboratory. For example, the system for ESTR mutation detection used by the Niwa laboratory is dependent upon genomic southern blotting. According to this system the mutant alleles are those that show size differences of at least 40-200 bp from either of parental alleles (Sadamoto *et al.*, 1994; Fan *et al.*, 1995), not necessarily the same criteria used by other groups. Indeed, other methods such as SM-PCR system can detect ESTR mutants that shift by only two repeat units from parental alleles (Yauk *et al.*, 2002).

Critical evaluation of the growing body of data shows it to generally support the high mutability of ESTRs following irradiation of pre-meiotic diploid spermatogonia not post-meiotic spermatids (Bouffler *et al.*, 2006; Yauk, 2004; Niwa, 2003; Somers, 2006).

1.2.2 Radiation quality, dose response, dose rate and doubling dose

Ionising radiation is either high linear energy transfer (high-LET) or low linear energy transfer (low-LET) according its ability to deposit energy through systems it traverses. High-LET radiation transfers high energy per unit path length compared to the low-LET. The former includes particulate radiation such as neutrons, α-particles, electrons and heavy ions and the later includes the electromagnetic radiation such as X and γ-rays. The deposition of energy by low-LET ionising radiation occurs in widely dispersed pattern taking form of single excitations, ionisations or clusters of small amounts of energy (~2keV). In contrast, the high-LET forms denser tracks of energy deposition reaching ~10KeV (Goodhead, 1988). The pattern of energy deposition can therefore profoundly affect the pattern and complexity of radiation-induced DNA damage.

Many studies have attempted to assess the effects of ionising radiation, from different sources, on the germline of exposed male mice. In most of these experiments the male mice was either acutely exposed to low-LET radiation (Dubrova *et al.*, 1993; Yauk *et al.*, 2002; Dubrova *et al.*, 1998; Dubrova *et al.*, 2000; Barber *et al.*, 2000; Barber *et al.*, 2002; Barber *et al.*, 2009; Somers *et al.*, 2004) or high-LET radiation (Barber *et al.*, 2002, Dubrova *et al.*, 2000a; Niwa *et al.*, 1996). All of these studies, regardless of the source of radiation, have reported elevated ESTR mutation frequencies in the germline of male mice exposed at premeiotic stage of spermatogenesis. For example, the ESTR mutation frequencies have been analysed in the germline of male mice exposed to varying doses and dose rates of high-LET fission neutrons and low LET-X-rays and γ-rays. In one study, Dubrova *et al.* (1998) observed ESTR mutations to be induced in a linear, dose dependant manner in the germline of male mice acutely irradiated at

premeiotic stage with doses up to 1Gy. They also reported highly significant increases in ESTR mutation rates in male mice exposed to either 0.5 Gy or 1 Gy of acute X-rays. Interestingly, this linear dose-response has also reported following acute γ- exposure using single molecule PCR analysis. Recently, Mughal *et al.* (2012) observed that ESTR mutation induction in the germline of male BALB/c mice showed linear dose-response within the dose range of 10 to 100 cGy.

Unexpectedly, chronic exposure to either low-LET γ-rays at a dose rate of $1.66x10^{-4}$ Gy min^{-1} (administered over 100 hours) or high-LET fission neutrons delivered at 0.003Gy min^{-1} gives the same linear, dose dependant response as that of acute exposure (Dubrova *et al.*, 2000b). These findings are inconsistent with studies carried out using the specific locus test which revealed that the efficiency of acute low-LET irradiation substantially exceeds that for chronic (Lyon *et al.*, 1972; Russell and Kelly, 1982a; Russell and Kelly, 1982b). The reduced mutation induction efficiency following irradiation at low dose rates detected using specific locus test is most probably attributed to the saturation of DNA-repair capacity following high-dose acute exposure (Dubrova *et al.*, 2000b; Russell *et al.*, 1958). However, these data gives further credence to the idea that the ESTRs are mutated via non-targeted mechanism rather than by direct damage (Dubrova *et al.*, 2000b).

It should be noted that according to the results of some publications, ESTR the mutation rate does not always show a linear increase with dose of exposure. For example, the 1.9 fold increase in the ESTR mutation frequency observed in the offspring derived from irradiated premeiotic spermatogonia at 0.5 Gy of γ-rays declined to 1.6 fold increase when the dose increased to 1.0 Gy (Dubrova *et al.*, 1993). Similar findings on the

effects of acute exposure to γ-rays in out-bred Swiss-Webster male mice were reported by Somers *et al.* (2004). According to the results of this study, ESTR mutation rate showed 2.8-fold and 3-fold increases in male mice exposed to 0.5 and 1 Gy, respectively.

The effects of high-LET irradiation on ESTR mutation induction in male mice have been described in two studies. For example, Niwa and co-authors (Niwa *et al.*, 1996) reported roughly similar increases in ESTR mutation rates in the germline of male mice following post-meiotic exposure to fission neutrons with doses of 0.35, 0.7 and 1.02 Gy. In contrast, Dubrova *et al.* (2000) observed practically linear dose-response for ESTR mutation induction in the germline of male mice exposed to fission neutrons on the pre-meiotic stages. The results of this study were later confirmed by Barber *et al.* (2002). The authors showed the very high efficiency of pre-meiotic exposure to 0.4 Gy of fission neutrons on ESTR mutation induction in CBA/H male mice.

A parameter related to the dose response and important in the estimation of radiation sensitivity as well as radiation risk, is the doubling dose. The doubling dose is defined as the amount of radiation that is able to double the spontaneous mutation frequency. In 2005, Dubrova reviewed the doubling dose estimates of ESTR mutation in five mouse strains studied by his Group. In all of these studies the ESTR estimates were made in the germline of male mice exposed to acute X-rays at pre-meiotic diploid spermatogonia. Among the five strains studied, the highest values of doubling doses were those in the BALB/c and C.B17 strains (mean value 0.98 Gy), which are known to be genetically related. The other three strains, CBA/H, C57BL/6 3 CBA/H F1 and 129SVJ 3 C57BL/6 have shown lower mean values of doubling dose at 0.44 Gy.

1.2.3 Chemical mutagens

The early study of Hedenskog *et al.* (1997) provided the first evidence that chemical mutagens can induce ESTR mutations in the germline of treated male mice. The authors exposed male mice to polychlorinated biphenyls (PCB), diesel exhaust, or a mixture of both chemicals and observed an elevated ESTR mutation rate only following treatment with a combination of both chemicals. However, this study was not comprehensive as the authors pooled data obtained from the exposure of premeiotic germ cell stage with that obtained from exposure of postmeiotic stage. Moreover, an elevated mutation rate was detected at one of the two loci used this study.

Barber and co-authors analysed the mutagenic effects of the anticancer drug cisplatin on ESTR mutation rate in male mice, treated either at pre-meiotic or post-meiotic stages of spermatogenesis (Barber *et al.*, 2000). The authors reported no significant difference from that observed in controls.

In 2003, Vilarino-Guell *et al.* analysed the male germline mutagenicity of two alkylating agents, ethylnitrosourea (ENU) and isopropyl methanesulfate (iPMS) and a topoisomerase II inhibitor, etoposide, at different stages of spermatogenesis. They observed significantly higher ESTR mutation rates (2.2-3.0 fold increase) in the germline of male mice following ENU and iPMS exposure at the premeiotic stage of spermatogenesis but a lack of measurable changes following the post-meiotic exposure. In contrast, exposure to etoposide resulted in ESTR mutation induction at meiotic stages only and did not affect post- or pre-meiotic cells. Pre-meiotic exposure to the alkylating agents resulted in a linear dose-response within an interval of doses from 12.5 mg/kg to 25 mg/kg and reached a plateau at higher concentrations.

More recently, Glen *et al.* (2008) have used more sensitive SM-PCR technique to establish ESTR mutation rates in the germline of male mice treated with the alkylating agent ethylnitrosourea and four widely used anticancer drugs – bleomycin, cyclophosphamide, mitomycin C, and procarbazine. They recorded highly significant increases in ESTR mutation rate following pre-meiotic exposure to all studied mutagens. Interestingly, the dose-response of ESTR mutation induced by ethylnitrosourea established by SM-PCR was very close to that obtained using pedigree analysis reported by Vilarino-Guell *et al* (2003).

The abovementioned studies clearly show that ESTR loci provide an efficient system for the detection of germline mutation induced by treatment with chemical mutagens and anticancer drugs.

1.2.4 Mechanisms of spontaneous and induced ESTR mutation

The mechanisms of spontaneous and induced ESTR mutations still remain poorly understood. However, there is a consistent body of evidence that ESTRs mutate in a non-targeted manner rather than being induced via direct DNA damage (Sadamoto *et al.*, 1994; Fan *et al.*, 1995; Dubrova *et al.*, 1998a; Barber *et al.*, 2002; Vilarino-Guell *et al.*, 2003, Glen *et al.*, 2008). For instance, the 4.0 fold increase at ESTR mutation rate observed by Dubrova *et al.* (1998) in the germline of males following premeiotic irradiation would require exceptionally high numbers of damage points (~45,000) to be manifested by direct DNA damage, i.e. the observed increase in the ESTR mutation rate is too high to be induced by direct effects of radiation at loci with such a small size. Also, the increase in the ESTR paternal mutation rates demonstrated after exposure to fission neutrons could not be expected following fewer than the 6 traversals, the number

of traversals expected with 0.5 Gy of fission neutrons, especially when the target is of a small size such as ESTR loci (Dubrova *et al.*, 2000a).

In 2000, Barber *et al.* provided evidence that ESTRs did not mutate via genome wide increases in meiotic recombination events like human minisatellites. When DNA double strand breaks accumulate they can potentially enhance crossovers in cells undergoing meiosis. Barber *et al.* analysed the incidence of crossovers in the germline of male mice exposed to 1 Gy of X-rays or 10 mg/kg of cisplatin. They observed a significant elevation of ESTR mutation only following X-ray exposure with no change in the rate of cross over which indicates that crossing over has no role in induction of ESTR mutation in the germ cells.

Dubrova's group have hypothesized that most likely ESTRs mutate by replication slippage, the same as microsatellites (Yauk *et al.*, 2002; Barber *et al.*, 2004; Dubrova *et al.*, 2005). Indeed, there are many similarities between ESTRs and microsatellites. For example, both groups of loci consist of arrays of short repeats that could be misaligned in the replicating DNA strands, resulting in expansions or contractions in the length of the whole array in the replicating DNA strand. In addition, the similarity in the size of repeats for both types of loci (4–6 bp and 1–6 bp for ESTRs and microsatellites, respectively) and ESTR loci can therefore be regarded as a class of highly-expanded microsatellites.

Dubrova (2005) has shown similarities in the spectra ESTR and microsatellite mutation. The first similarity is the high tendency towards gains of repeats , detected at many microsatellite loci (Ellegren, 2004), also shown in ESTR mutation (Fan *et al.*, 1995; Yauk *et al.*, 2002). Furthermore, both ESTR loci and microsatellites show the same

positive correlation between allele size and rate of spontaneous mutation (Ellegren, 2004; Bois *et al.*, 2001). This correlation is consistent with the hypothesised mechanism of mutation for both types of repeat loci as the longer arrays can promote the polymerase slippage further (Dubrova, 2005).

Further evidence was provided by the work of Yauk *et al.* (2002). The authors analysed the rate and spectra of spontaneous ESTR mutation at the *Ms6-hm* locus in both somatic (brain and spleen) and germline (sperm) cells and showed that the ESTR mutation rate was positively correlated with the rate of proliferation of a given tissue. DNA extracted from tissues with a low mitotic index had lower ESTR mutation rates. The order of ESTR mutation rates was sperm >spleen >brain. This finding indicates that ESTR instability occurs in the actively dividing cells during mitosis or meiosis. A further confirmation for this finding comes from previous data demonstrating the induction of mutation at ESTR loci observed following exposure of dividing, diploid, premeiotic spermatogonia (Dubrova *et al.*, 1998; Barber *et al.*, 2000; Barber *et al.*, 2002; Dubrova *et al.*, 2000). The conclusion from this work is that ESTR mutation occurs in all dividing cells whether somatic or germline.

The most comprehensive study regarding this issue was recently published by Hardwick *et al.* (2009). The authors compared the pattern of ESTR mutation accumulation in tissues with different proliferation capabilities in male mice of various ages (12, 26, 48, and 96 weeks old). No detectable age-related ESTR mutation accumulation was observed in the non-proliferating brain. However, substantially elevated ESTR mutation rates were observed in both sperm and bone marrow taken from old mice, with no change in the spectra of the detected ESTR mutations between old and young mice. These findings are clearly indicating that spontaneous ESTR mutation occurs almost

completely in actively dividing cells via a replication dependant mechanism likely by replication slippage. Based on the findings of this study and previously mentioned work, it can be said that ESTR loci may be considered as a class of expanded microsatellites that mutate by replication slippage in replication proficient cells.

1.2.5 Summary

From all the above mentioned data it is clear that acute and chronic exposure to either high or low-LET radiation are able to induce ESTRs mutations in the germline of exposed male mice. Also, treatment of male mice with chemical mutagens can also induce germline mutations. However, both linear and saturated dose responses have been established for ESTR mutation. ESTR mutation induction occurs in the germline of irradiated male mice via non-targeted mechanisms rather than direct DNA damage that is likely to be induced in dividing pre-meiotic diploid spermatogonia via replication slippage. In conclusion, ESTR loci represent an efficient and robust system for monitoring germline mutation induced by a variety of mutagens. ESTR system has also been used for studying the transgenerational effects following paternal exposure (will be discussed later on).

1.3 Non-targeted effects of ionizing radiation

The old paradigm of radiation biology assumes that the genetic effects of exposure to ionizing radiation are solely attributed to DNA damage induced at the sites affected by the energy deposited in the nucleus. Accordingly, radiation-induced DNA damage takes place during or very near to irradiation of nuclei in targeted cells, and the potential biological consequence will be expressed within one or two rounds of cell division (Kadhim *et al.,* 2013). This paradigm has been challenged by observations that cells that are themselves not irradiated exhibit responses typically associated with direct radiation exposure. Such effects that could be observed in the non-exposed progeny of irradiated cells are known as radiation-induced genomic instability. When these effects occur in non- irradiated cells but presumed to receive damage signals from the directly irradiated cells this effect is termed radiation induced bystander effects (Lorimore *et al.,* 2003).

Many publications describe experimental evidence for the events challenging the prevailing dogma of radiobiology. For example, Weissenborn and Streffer (1988b) showed that the manifestation of ongoing chromosomal damage (structural chromosomal aberrations) many cell cycles following the irradiation of mouse embryos. Besides, this work demonstrated that the frequency of micronuclei was elevated between 24 to 48 hours after irradiation and was termed by authors as 'chromosomal instability'. In addition, an elevated frequency of dominant lethal mutations was found in non-exposed offspring of male mice treated by the α-particle emitter Plutonium-238 (Luning *et al.,* 1976). Furthermore, similar effects were described in non-exposed Chinese hamster ovary cells neighbouring cells traversed by α-particles (Nagasawa and Little, 1992).

In summary, the term ‘non-targeted effects’ describes the adverse effects of irradiation observed in cells whose nuclei were not subjected to direct radiation. These effects include genomic instability in the progeny of irradiated cells, bystander effect, and heritable effects of parental irradiation that can manifest across generations (UNSCEAR, 2006).

1.3.1 Bystander effects

The term ‘bystander effect’ describes the ability of cells affected by a mutagenic agent to convey their manifestations of induced damage to other cells not directly targeted. Different bystander responses are observed in different cell types depending on the type of cells producing the bystander signal after irradiation and the type of cells receiving this signal. Cells which exhibit a bystander effect can be in the immediate vicinity or distantly separated in relation to the target cells. The reported responses were observed both *in vitro* and *in vivo* and include damage-inducible stress responses, sister chromatid exchanges, micronucleus formation, apoptosis, gene mutation, chromosomal instability and transformation after exposure to either high-LET or low-LET radiation (Wright, 2010; Morgan, 2003a,2003b; Morgan and Sowa, 2007; Prise and O'Sullivan, 2009; Kadhim *et al.*, 2013). Both *in vitro* and *in vivo* manifestations of the bystander effect of radiation exposure will be discussed.

1.3.1.1 *In vitro* manifestation of bystander effects

In all experiments studying the bystander effects *in vitro*, it is crucial for any experimental design to be capable of targeting a few cells, one cell or even a subcellular structure rather than overall irradiation. This prerequisite has been met by using a

variety of experimental facilities such as using low fluency particulate radiation, co-culture, microbeam facilities, and media transfer (Nagasawa and Little, 1992; Lehnert *et al.*, 1997; Gerashchenko and Howell, 2003; Prise *et al.*, 2009; Lyng *et al.*, 2002).

1.3.1.1.1 Targeting small proportion of nuclei as traversed by α-particles

One characteristic of the bystander effect of ionizing radiation is that the induced response usually unexpectedly exceeds the possible effect of given radiation dose. Nagasawa and Little described a non-targeted bystander effect for the first time in their paper published in 1992 when they demonstrated sister chromatid exchanges (SCE) in about 30% of Chinese hamster ovary cells exposed to 0.31 mGy of α- particles when only 1% of cells were actually traversed by the α-particles. To explain this, Lehnert *et al.* (1997) demonstrated the release of short-lived factors to the culture medium of α-irradiated normal human lung fibroblasts that were able to induce SCEs in unexposed normal cells at a frequency comparable to that of directly exposed cultures. The activities of these SCEs-inducing factors were efficiently deactivated by superoxide dismutase implicating reactive oxygen species (ROS) in this mechanism (Narayanan *et al.*, 1997).

Alterations in the expression of cell cycle regulators and other protein coding genes have also been reported as radiation-induced bystander response. For example, Nagasawa and Little (1999) reported an unexpectedly high incidence of mutation at the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus in CHO cells at a very low dose of α-particles (below 5cGy). However, exposure to this low dose means that less than one α-particle (0.05-0.3) traverse per nucleus (Nagasawa and Little, 1999). Also, the levels of TP53 and CDKN1A significantly exceeded the amount expected to

be produced by the 2% of human fibroblasts whose nuclei actually traversed by low fluence α-particles (Azzam *et al.*, 1998). Interestingly, this alteration in the expression pattern was greatly reduced by interrupting the functioning of gap junctions by the addition of lindane (Azzam *et al.*, 1998). In 2001, Azzam *et al.* did further work to understand how gap junctions could mediate bystander signalling. Their finding was that the induction of $P21^{Waf1}$protein expression, as well as chromosomal damage, was abolished in cells compromised in gap junction intercellular communication via inactivation of connexion 43. Also, an elevated level of the connexion 43 mRNA was detected *in vitro* following a very low fluency α-particle exposure. Furthermore, a number of post-translational modifications of connexion 43 protein, including phosphorylation and hyper-phosphorylation were found in the bystander cells. Similar effects were also observed following exposure to γ-irradiation, hyperthermia, and t-butylhydroperoxide but not established for UV-irradiation (Azzam *et al.*, 1998).

1.3.1.1.2 Irradiated and non-irradiated cell proximity in co-culture

Using a co-culture system whereby irradiated and non-irradiated cells are in proximity, provides important clues regarding the mechanisms of radiation induced bystander effects. The trans-well co-culture system utilises permeable membrane inserts to place the bystander (naïve cells) into six-well plates containing irradiated cells in absence of direct contact. This system facilitates the study of medium mediated bystander responses excluding the interference of gap junction communication (Yang *et al.*, 2005). In one study, Gerashchenko and Howell (2003) analysed the effect of cell proximity on the proliferation rate of bystander cells by co-culturing γ-irradiated rat liver epithelial cells and non-irradiated cells at different densities. They observed that the bystander cells show a proliferation rate similar to controls when co-cultured with irradiated cells

without direct contact. In contrast, the bystander cells that were mixed with irradiated cells in direct contact exhibited substantially elevated proliferation rates especially when the irradiated and un-irradiated cells were more densely plated (Gerashchenko and Howell, 2003).The authors claimed that under these experimental circumstances direct contact between cells in culture was crucial for conveying these radiation induced signals regardless of whether these signals were gap junction intercellular communication (GJIC) or soluble extracellular factors. This finding provided further experimental evidence for a possible role of gap junctions in conveying the radiation induced bystander signal.

In another study, Yang *et al.* (2005) analysed the low-LET radiation-induced bystander response in co-culture, shedding more light on the mechanism of radiation induced bystander effect**.** They co-cultured normal human fibroblasts exposed to 0.1-10 Gy of X-rays with bystander naïve cells. The radiation induced bystander signals manifested as an increase of micronuclei formation, decrease in survival, enhanced γ-H2AX foci, and accumulation of ^{Waf1}p21 in bystander cells, regardless of radiation dose. Interestingly, treatment with Cu–Zn superoxide dismutase (SOD) and catalase, which are known to scavenge the reactive oxygen species, reduced the accumulated γ-H2AX foci as well as ^{Waf1}p21 production in bystander cells. However, the survival of bystander cells was not affected which means that reactive oxygen species are likely not the only released factors to the culture medium.

1.3.1.1.3 Targeting single cells or group of cells using microbeam

The microbeam approach fulfils two very essential prerequisites for studying the bystander response:(i) the dose delivered to the target cell(s) can accurately be measured; (ii) it provides precise targeting of the irradiated cell or even its subcellular

component. This capability of precise localisation of the target cell or subcellular organelle is important to shed light into the role of intra-and intercellular bystander signalling (Prise *et al.*, 2009). Therefore, the microbeam approach is widely used for studying the responses of bystander cells. One of the studies carried out using a charged particle microbeam revealed that cytoplasmic irradiation was enough to elicit bystander response in non-irradiated radio-resistant glioma cells without targeting the cell nucleus (Shao *et al.*, 2004). Interestingly, this bystander response was abolished after treatment with a nitric oxide (NO) scavenger or membrane raft disruptor (Shao *et al.*, 2004). Indeed, targeting the cytoplasm by using a microbeam was enough to trigger formation of 53BP1protein in both insulted and bystander cells regardless of the dose and the number of cells hit. This finding revealed that DNA direct damage is not the only reason for eliciting radiation-induced 53BP1 foci. Furthermore, this radiation induced 53BP1 foci formation is abolished by inhibition of reactive oxygen species (ROS) and reactive nitrogen species (RNS),but not affected by inhibition of membrane-dependant signalling pathways (Tartier *et al.*, 2007).

It has been reported that mitochondria have an essential role in modulation of radiation-related bystander reactions in human skin fibroblasts. Using microbeam α-irradiation, Zhou *et al.* (2008) demonstrated that mitochondria-depleted human skin fibroblasts were much more sensitive to the induction of bystander reaction compared to their parental mitochondria competent cells. The authors explain the difference in bystander response by the reduction in the basal level of reactive radical species, the possible mediators of bystander response in mitochondria depleted cells compared to mitochondria competent ones. Moreover, in mixed cultures of both mitochondrial phenotypes, targeting only one population of cells with a lethal dose of α-particles

resulted in an evenly reduced bystander response in both cell types. This finding indicates that signals from one cell type can modulate expression of bystander response in another cell type (Zhou *et al.*, 2008).

Three dimensional (3D) tissue constructs were developed by Belyakov *et al.* (2005) and have been utilised to assess radiation-induced bystander response. These constructs are *in vitro* systems aiming to mimic the complexity of the *in vivo* systems in the architecture and microenvironment. Belyakov *et al.* studied the bystander response in 3D reconstructed human skin models, which are very similar to normal human skin microarchitecture, using a charged particle microbeam. They detected substantially increased micronuclei (1.7 fold over control) and apoptosis (2.8 fold over control) in the non-hit bystander cells up to 1 mm distance from irradiated cells.

X-ray microbeam hardware has also been developed (Schettino *et al.*, 2000) and used by number of research groups to address the radiation induced bystander response, but using low-LET radiation this time. Using this X-ray microbeam approach and analysing for the clonogenic survival of hamster fibroblasts as an endpoint, Schittino *et al.* (2003) reported a significant response in bystander cells even if only a single cell was irradiated.

1.3.1.1.4 Induction of bystander responses in naïve cells using media of irradiated cultures

Experiments that involve media transfer are based on the hypothesis that irradiated cells secrete factors (radiation induced signals) into their culture media that are received and

manifested in the non-irradiated bystander cells as a variety of phenotypes including genomic instability (Seymour and Mothersill, 1997), changes in cloning efficiency (Seymour and Mothersill, 1997; Lyng *et al.*, 2000), or enhancement of neoplastic transformation (Lewis *et al.*, 2001).

In one study by Lyng *et al.*, media from the progeny of γ-irradiated human keratinocytes were able to induce apoptosis in non-irradiated bystander cells regardless of radiation dose or the number of passages. Enhancement of apoptosis was associated with rapid calcium flux (30 seconds), loss of mitochondrial membrane potential, and substantial increase in reactive oxygen species. These findings indicated that the radiation-induced signal that initiated apoptosis continued to be produced over several generations of cell division (Lyng *et al.*, 2002).

Several studies demonstrated a role of DNA damage response in mediating radiation-induced bystander effects in experiments that involve media transfer. For example, Kanasugi *et al.* (2007) reported a high incidence of chromosomal alterations in normal human fibroblasts incubated with conditioned medium harvested from irradiated cells exposed to either high LET or low LET ionizing radiation. These chromosomal abnormalities were reduced when the donor cells were treated with DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) inhibitor before irradiation and nitric oxide (NO) scavenger after irradiation. In contrast, treatment of recipient cells with DNA-PKcs inhibitor before addition of the conditioned medium from the donor cells resulted in elevation of bystander chromosomal alterations. The authors concluded that the chromosomal aberrations observed in bystander cells were induced via factors

secreted into the culture medium including NO, and DNA-PKcs-mediated repair machinery is involved partially in the repair processes (Kanasugi *et al.*, 2007).

Another study by Hagelstrom *et al.* (2008) aimed to establish the relationship between the DNA damage response and radiation-induced bystander effect. The authors assessed the role of DNA-PKcs and Ataxia Telangiectasia Mutated (ATM) in the generation and/or reception of radiation-induced bystander signal following γ-irradiation. The cell culture transfer approach was employed in the way that the donor (irradiated) cells were co-cultured with recipient (non-irradiated) cells at very low concentration (1:100 or 1:1000). The incidence of sister chromatid exchange (SCE) was used for estimation of the bystander response. Following γ-ray exposure, both DNA-PKcs and ATM were essential for the generation of the bystander signal that resulted in sister chromatid exchange (the bystander response) in normal human fibroblasts. In contrast, neither DNA-PKcs nor ATM was required for the reception the radiation induced bystander signal.

Moreover, a high level of DNA double strand break damage repair response was observed when human dermal fibroblasts (HDF) were incubated with culture medium of irradiated cells which was positively correlated with radiation dose, but much lower than that of directly irradiated cells. Furthermore, the four cytokines: IL6, IL8, MCP-1 and RANTES levels were significantly elevated in the growth medium and both IL6 and MCP-1 affected the size of the γ-H2AX foci indicating a possible role of cytokines in mediating the bystander response (Dieriks *et al.*, 2010).

1.3.1.2 Bystander effects *in vivo*

In 1974, Brooks *et al.* intravenously injected Chinese hamsters with the α-particle emitter plutonium (^{239}Pu), ensuring that 90% of the substance was accumulated in the liver. The cells closest to the particles were exposed to the highest dose and dose rate, which gradually attenuated for the surrounding cells. When chromosomal damage was analysed, the increased levels of chromosomal aberration remained equally elevated across the tissues. These results revealed that although only a small proportion of liver cells were exposed to particulate radiation, all cells in the liver shared the same extent of damage. Further *in vivo* evidence comes from studying the clastogenic effect (the ability to induce chromosomal damage) of serum from Chernobyl accident survivors. It has been observed that serum extracted from Chernobyl accident survivors is able to significantly increase the frequency of micronuclei in immortalized human keratinocytes twenty years after the fallout (Marozik *et al.*, 2007). Also, signals produced *in vivo* in the bone marrow of CBA/Ca mice one day following γ-irradiation are able to induce DNA damage and apoptosis in non-irradiated bone marrow cells. Among the signalling molecules identified were Fas L, TNF-α, nitric oxide (NO) and superoxide and, additionally, activated macrophages could be implicated in generating of damaging signals (Burr *et al.*, 2010).

The *in vivo* bystander response may also observed in organs which are distantly separated from the original site of irradiation. Indeed, the growth of shielded tumours in the midline dorsum of C57BL/6 mice have been reduced in a dose and dose rate dependent fashion following irradiation of the legs of these mice (Camphausen *et al.*, 2003). This is considered as a kind of 'abscobal effect' which is observed following either radiotherapy or chemotherapy when the radiation or drug is able to induce

bystander effect in distantly separated organ (Morgan, 2003b). Blocking of P53 protein by its inhibitor pifithrin-α was found to abolish radiation mediated tumour size reduction, indicating that P53 could be a key player in radiation mediated abscobal effects (Camphausen *et al.,* 2003). Another study by Koturbash *et al.* (2007) investigated epigenetic alterations in lead-shielded rat spleen tissue 24 hours and 7 months following localized cranial X-irradiation to test the hypothesis that localized X-irradiation could trigger persistent bystander response in distant organs. The authors observed an accumulation of DSBs associated with intense epigenetic modifications in the distant tissues. These epigenetic modifications included significant global DNA hypomethylation, methylation changes in long interspersed nucleotide element (LINE-1) retrotransposon, down-expression of DNA methyltransferases and methyl binding protein MeCP2. Furthermore, overexpression of microRNA-194 which has a role in the regulation of both DNA methyltransferase-3a and MeCP2, was also detected. Interestingly, these changes were long-lasting and persisted for up to seven months following exposure. This study, therefore, provides evidence for a possible epigenetic mechanism for the bystander effect caused by ionizing radiation (Koturbash *et al.*, 2007).

1.3.1.3 Summary

The bystander effect of ionizing radiation is irradiated cells passing on their response to other cells that are not irradiated (Morgan, 2003b). The nature of the radiation induced signals that cause the bystander effect has so far remained elusive. However, bystander responses can manifest in the non-irradiated cells through inter-cellular communication via gap junctions (Azzam *et al.*, 2003) or via diffusible factors that could secreted either *in vitro* in the culture medium or *in vivo* into blood or microenvironment. Among these

factors oxidative species (ROS and NOS), cytokines as well as stress-associated cellular proteins such as TGF-β (Ilyntskyy and Kovalchuk, 2011;; Dieriks *et al.*, 2010; Narayanan *et al.*, 1997, 1999; Dickey *et al.*, 2009). In addition, epigenetic alterations have also proposed as possible mediator of radiation induced bystander effect (Koturbash *et al.*, 2007). Bystander effects have been established both *in vitro* and *in vivo* in a variety of cell and tissue types. In some cases, the *in vitro* bystander response varies depending on the cell type, whereas the bystander response *in vivo* can be affected by the tissue type and genetic background. The manifestation of bystander response has been demonstrated following exposure from high- and low-LET sources and at very low doses/fluency; and in most cases the dose-response is highly non-linear (Kadhim *et al.*, 2013). It is worth mentioning that not all types of cells are capable of generating a bystander signal or responsive to one (Mothersill and Seymour, 1997; Nagar *et al.*, 2003; Mothersill *et al.*, 2001) and these discrepancies possibly occur even within the same population of cells.

1.3.2 Radiation Induced Genomic Instability

Radiation induced genomic instability (RIGI) is defined as the increased rate of acquisition of genetic abnormalities that manifests in the genome of progeny of irradiated cells many generations after initial irradiation (Morgan, 2003a; Wright, 2010). RIGI manifestation can be delayed after irradiation up to 4 years post-radiation or even later in some cases (Morgan, 2003a; Morgan, 2003b). Genomic instability has been studied both in *in vitro* and *in vivo* system using different end-points including chromosomal alterations, changes in ploidy, micronucleus formation, gene mutations and amplifications, and mini- and microsatellite (short tandem repeat) instabilities. All

of these endpoints show significant elevation in non-exposed progeny of irradiated cells (Kronenberg, 1994; Little, 2000; Morgan, 2003a, 2003b).

1.3.2.1 *In vitro* studies

One of the well characterised genomic instability end-points is chromosomal alterations. For instance, Kadhim and colleagues (1992) expanded the clonal descendants from surviving α-particle-irradiated murine haematopoietic cells and analysed the yield of chromosome aberrations. They reported a high frequency of non-clonal chromosomal aberrations in the clonal descendants as a result of exposure to alpha-particles. In another experiment, human-hamster hybrid GM10115 cells were exposed to X-rays and the analysis of the non-exposed progeny showed a significant increase in the frequency of chromosomal aberrations (Limoli *et al.* in 1999; Limoli *et al.*, 2000).

Delayed reproductive death represents another manifestation of radiation induced genomic instability. For instance, progeny of surviving Chinese hamster ovary (CHO) cells isolated after 12-34 population doublings following X-irradiation, showed reduced cloning efficiency as well as a lower attachment ability to culture dishes (Chang and Little, 1991). In addition, the authors reported a significantly elevated fraction of abortive and non-homogeneous colonies 12-23 generations following irradiation (Chang and Little, 1991). This report is in line with earlier studies on plating efficiency of cells post-radiation Mendonca and colleagues (1989). Moreover, a persistent decrease in cloning efficiency was observed over 40 generations in clones derived from normal human embryonic cells that survived 6 Gy of X-rays compared to control clones (Suzuki *et al.*, 1998). While in a recent study, exposure of human osteosarcoma (HOS) cells to depleted uranium (DU) or heavy metals (Ni) resulted in delayed reproductive

death for several generations (36 days) following exposure (Miller *et al.* 2003). Interestingly, while DU stimulated delayed production of micronuclei up to 36 days after exposure, the levels in cells exposed to γ-radiation or Ni returned to normal after 12 days (Miller *et al.*, 2003).

1.3.2.2 *In vivo* studies

Genomic instability following *in vivo* irradiation may represent a crucial step in the initiation of radiation-induced cancers (Little, 2000; Sankaranarayanan and Chakraborty, 1995; Ullrich and Ponnaiya, 1998). Several studies have been performed to analyse the effects of ionizing radiation on genomic stability in mice and humans (reviewed in Morgan, 2003b). However, data from *in vivo* studies are not as conclusive as that describing the *in vitro* effects and sometimes show contradictory findings. Most of what is known from the *in vivo* studies comes from animal experimental data particularly from mice studies.

The effects of ionizing radiation on genomic stability have been extensively studied in different strains of mice using several end-points. For example, an elevated frequency of structural and numerical chromosomal abnormalities, as well as micronuclei, was detected in mouse embryos following exposure to either X-rays or neutrons (Weissenborn and Streffer, 1988a; Weissenborn and Streffer, 1989b). Further evidence for radiation induced genomic instability comes from the *in vivo* study on the long-term effects of irradiation on the yield of chromosome aberrations in C3H mice. Tanaka *et al.* (2008) reported significant increase in the frequency of structural and numerical chromosomal aberrations in the spleenocytes of C3H female mice. This increase in genomic instability was observed over a long period of time (400 days) after exposure.

However, no genetic damage was induced in erythroid stem cells of prenatally γ-irradiated CBA/Ca mice at either 44, 99 or 265 mGy/day (to a total dose of 0.7, 1.6 or 4.2 Gy) after 35 days post-irradiation (Abramsson-Zetterberg *et al.*, 2000). Also no evidence of transmissible chromosomal instability was detected in bone marrow cells 50 or 100 days after *in vivo* exposure of CBA/H mice either to α-particles from the radionuclide ^{224}Ra or to acute X-rays (Bouffler *et al.*, 2001). Furthermore, no chromosomal instability was detected in peripheral blood lymphocytes up to 30 days following whole-body γ-irradiation of C57BL/6 mice (Spruill *et al.*, 1996). The same authors also failed to observe chromosomal instability up to 21 months (Spruill *et al.*, 2000).These results are supported by *in vitro* work by Ponnaiya *et al.* (1997) who also did not observe instability in irradiated cells derived from C57BL/6 mice.

The data obtained from various mice studies highly depend on the experimental strain as well as the sex of the animal. For example, Watson *et al.* (1997) demonstrated that the frequency of α-particle-induced chromosomal instability in haemopoeitic cells of three different strains of mice (CBA/H, DAB/2 and C57BL/6) was dependent on the genotype. According to the results of this study, the irradiated haemopoeitic cells from both CBA/H and DAB/2 strains were 'sensitive' and showed highly significant increase in the frequency of chromosomal instability compared to controls. In contrast, C57BL/6 was considered as 'resistant' compared to the other two strains. The environment in which the mice are bred has also been shown to influence the data obtained from *in vivo* studies as results from the same strain but bred from different colonies and laboratories vary significantly (Morgan, 2003b). Therefore, if extrapolating data from animal studies to human situation, all the delimiting factors should be considered in great detail and with caution.

The non-targeted effects of radiation on the stability of the human genome have also been reported (reviewed in Morgan 2003b; UNSCEAR, 2006). Human data were mostly derived from analysing human cell lines, studies carried on radiotherapy patients as well as occupational or accidental exposure to ionizing radiation. For example, chromosomal instability was observed in both long-term human lymphocyte cultures following *in vitro* irradiation (Holmberg *et al.*,1998) and also in peripheral blood lymphocytes taken from people accidentally exposed to radiation in Estonia in 1994 (Salomaa *et al.*, 1998). However, no evidence of persistent or delayed genomic instability was detected by cytogenetic analysis in 18 individuals receiving fractionated radiation therapy for the treatment of different cancers (Tawn, *et al.*, 2000).

1.3.2.3 Summary of radiation-induced genomic instability

Molecular, biochemical, and cellular mechanisms that initiate and maintain radiation-induced genomic instability are still inadequately defined. One important feature of RIGI is that it is characterized by a significantly higher incidence of chromosomal alterations and mutations, usually about 10–20% more than mutations from targeted effects (Kadhim *et al.*, 2013). Therefore, it is difficult to claim that these unstable phenotypes arise by an induced genetic mutation(s) in one gene or even a family of genes (Morgan, 2003a). Instead, these instability events could be arise via an epigenetic mechanism (Kovalchuk and Baulch, 2008), causing defects in genome maintenance pathways including DNA damage and repair (Cui *et al.*, 1999; Huang *et al.*, 2003; Yu *et al.*, 2001), and/or alterations in cellular homeostasis (Barcellos-Hoff and Brooks, 2001; Baverstock, 2000; Mothersill *et al.*, 2000). Another feature of RIGI is the non-clonal heterogeneity in their expression, whatever the end-point used for detection, and that their biologically damaging responses cannot be anticipated by conventional target

theory (Kadhim *et al.*, 2013; Kadhim *et al.*, 1992). In addition, at low radiation doses, induced genomic instability shows two deviations from what conventionally is expected according to the classical paradigm of radiation biology. First, when the dose-response relationship is considered, the level of response is elevated that expected from higher dose responses. Second, the consequences of the delayed manifestation of radiation-induced damage are always unexpected (Kadhim *et al.*, 2013). The possible mechanisms underlying RIGI will be discussed at section 1.3.4.

1.3.3 Transgenerational instability

Given the evidence for RIGI in somatic cells there is a need to assess the ability of this radiation-induced signal to persist through meiosis, pass through the germline, and manifest in the non-exposed offspring, destabilising their genomes. The phenomenon of radiation-induced transgenerational instability can be defined as an enhanced rate of *de novo* mutation that can be observed in the non-exposed offspring of irradiated parents (Dubrova, 2003). The first experiment to show transgenerational instability as elevated rates of germline mutations was obtained by Luning *et al.* (1976). The authors observed an elevated rate of dominant lethal mutations in the non-exposed F_1 offspring of male mice injected with Plutonium. Transgenerational instability (TI) has since been reported by several researchers using a variety of endpoints, in human (Suskov *et al.*, 2008; Aghajanyan and Suskov, 2009; Aghajanyan *et al.*, 2011) or mouse somatic cells (Dubrova *et al.*, 2000; Barber *et al.*, 2002; Barber *et al.*, 2006; Hatch *et al.*, 2007; Barber *et al.*, 2009; Vorobtsova, 2000; Shiraishi *et al.*, 2002; Niwa and Kominami, 2001).

TI is characterised by two unique features: (i) the instability phenotype cannot be attributed to Mendelian segregation of *de novo* mutations induced in the germline of irradiated parents as it equally manifests in their first (F_1) and second (F_2) generation offspring (Dubrova *et al.,* 2000; Barber *et al.,* 2002; Barber *et al.,* 2006; Barber *et al.,* 2009); (ii) the instability signal acts in *trans* affecting the non-irradiated maternally-derived allele following paternal exposure (Barber *et al.,* 2009; Shiraishi *et al.,* 2002; Niwa and Kominami, 2001).

1.3.3.1 Developmental abnormalities as an end point

In a number of early studies, TI was detected as developmental malformations induced in the developing embryo. For example, Müller and associates (1999) detected increased lethality and malformations in 19-day-old foetuses following paternal radiation exposure of 2.8 Gy of γ- radiation from ^{137}Cs source. This elevated lethality occurred after exposure of all stages of spermatogenesis, with the exception of early spermatogonia.

In addition, studies using the pre-implantation embryo chimera assay also reported transgenerational effects induced by paternal irradiation (Wiley *et al.,* 1997; Baulch *et al.,* 2001). This assay utilizes mouse embryo aggregation chimeras consisting of one irradiated embryo paired with an un-irradiated embryo containing blastomeres labelled with fluorescein isothioxyanate (Obasaju *et al.,* 1988; Obasaju *et al.,* 1989). Male mice were irradiated 6 to 7 weeks before mating to assess changes in the proliferation of F_1 and F_2 embryonic cells using the pre-implantation chimera assay. The authors measured the competitive cell proliferation in chimeric embryos and found that the F_1 embryos conceived 6 to 7 weeks after paternal irradiation showed a proliferation disadvantage

that persisted without degradation in the F_2 generation of embryos when F_0 males received 1.0 Gy of γ-irradiation (Wiley *et al.*, 1997). Using the same experimental design, Baulch *et al.* (2001) evaluated the effects of paternal irradiation on the pattern of gene expression in F_3 offspring of irradiated males. The authors analysed the activity of receptor tyrosine kinase, protein kinase C and MAP kinases, as well as the levels of nuclear proteins TP^{53} and $p21^{waf1}$. The activity of all three protein kinases was altered, and nuclear levels of TP^{53} and $p21^{waf1}$ protein were higher in F_3 offspring with a paternal F_0 radiation history compared to non-irradiated litter-mates.

1.3.3.2 Increased cancer incidence

1.3.3.2.1 Mouse studies

Increased cancer incidence and enhanced disease progression have been studied to address the heritable effects of radiation exposure. These studies aimed to test the hypothesis that radiation-induced genetic alteration in paternal germ cells can lead to carcinogenesis in their non-exposed offspring. Shoji *et al.* (1998) conducted experiments to determine whether genetic damage induced in paternal germ cells by exposure to fission neutrons could lead to tumourgenesis in their offspring. C3H/HeNCrj male mice were irradiated with fission neutrons, at doses of 0 and 12.5 cGy and mated with C57BL/6NCrj females two weeks after exposure. Higher proportions of abnormal sperm and embryo lethality were observed among the F_1 offspring in the irradiated group compared to the non-irradiated group. Moreover, the incidence of liver tumours among the F_1 offspring increased in males which showed that radiation exposure may have caused genetic transmission of liver tumour-associated traits. Also, a high incidence of heritable tumours, especially in the lungs, was observed

in the offspring of ICR male mice exposed to X-rays. This increase in frequency of induced heritable tumours was dose dependent between 0.36-5.04 Gy of X-rays (Nomura, 1982). However, this high frequency of heritable tumours was not confirmed by Cattanach *et al.* (1995 and 1998).

Other studies aimed to test the hypothesis that exposure of male mice to radiation can increase the vulnerability of their offspring to develop tumours following their exposure to carcinogens. Vorobtsova *et al.* (1993) observed a substantial increase in the incidence of skin cancer in the offspring of high-dose, acutely X-irradiated males treated with 12-O-tetradecanoylphorbol-13-acetate compared to treated progeny of non-irradiated parents. Similar effects were observed for the incidence of leukaemia and lymphoma on the offspring of exposed (injected with ^{239}P) male mice treated with methylnitrosurea (Lord *et al.*, 1998a; b). Further, Nomura (1983) reported a significant increase in the incidence of lung tumours in the offspring of irradiated ICR males after postnatal treatment with urethene (Nomura, 1982). In contrast to this finding, the urethane promoting treatment of the offspring of X-irradiated CBA/J males did not enhance the incidence of lung tumours (Mohr *et al.*, 1999).

It can be speculated that inter-strain differences, particularly differences in predispositions to cancer, may alter their cancer risk. Also, it seems that germline irradiation *per se* is not sufficient to induce cancer in the future generation. However, the induced genomic instability that can pass on and accumulate in the progeny of irradiated parents renders them more susceptible to the subsequent exposure or tumour promoting agents.

1.3.3.2.2 Human studies

Whether cancer incidence increases in the offspring of occupationally or accidentally exposed fathers remains controversial. The contradictions, in part, could be attributed to temporal and geographical variations (Dickinson and Parker, 2002) or population mixing (Gilham *et al.*, 2005). Gardner *et al.* in their case control study in 1990 correlated the increased incidence of leukaemia and non-Hodgkin's lymphoma among children living near the Sellafield power plant in the UK with their fathers' occupation in the power plant, and the external dose they were exposed to during their work prior to conception. A lot of controversy follows this conclusion especially after re-examination. For example, Draper *et al.* (1997) failed to establish the same correlation with an extensive study based on 35,949 diagnoses of childhood cancer with matched controls, and the cohort study by Parker *et al.* (1993) on childhood leukaemia near Seascale did not support Gardner's hypothesis. However in the results of their cohort study, Dickinson and Parker (2002) established a statistical correlation between paternal preconceptional irradiation and a high incidence of childhood leukaemia and lymphoma when they widened the temporal and geographical boundaries. It is worth mentioning that all studies reviewed so far have offered some evidence for the transgenerational effect of radiation. However, the genetic basis of such effect is nearly absent making explanation of these results very complicated.

1.3.3.3 Chromosomal instability among the children of irradiated parents

Using chromosomal aberrations as an endpoint, a number of recent studies have reported the manifestation of chromosomal instability in children of irradiated nuclear power plant workers (Pilins'ka *et al.*, 2005; Aghajanyan and Suskov, 2009). In one

study, elevated frequencies of chromosomal aberration, gene mutation, and apoptotic markers were observed in lymphocytes taken from liquidators of the Chernobyl nuclear power plant accident and their non-exposed children (Suskov *et al.*, 2008). In addition, more recently, Aghajanyan *et al.* (2011) reported significantly elevated frequencies of chromosomal aberration and chromatid breaks in lymphocytes of fathers who worked as liquidators in the Chernobyl power plant and their non-exposed children. Also, a substantial increase in aberrant genome frequency was found in blood samples taken from the children of irradiated fathers following the *in vitro* exposure to ^{137}Cs γ-irradiation. This result may indicate that the radiosensitivity of human genomes can be enhanced following low dose irradiation (Aghajanyan and Suskov, 2009).

1.3.3.4 Mouse mutation assays

The mutated version *pink-eyed dilution* p^{un} gene possesses a 70 kb tandem duplication that results in loss of eye colour as well as diluting the coat colour in mice. Deletion of one copy of the duplicate via intra-chromosomal homologous recombination reverts the gene mutation and restores the colour to eye and fur. This somatic reversion makes this locus a good candidate for studying the transgenerational effects of environmental carcinogens by either scoring black spots on the light grey fur using fur spot assay or black cells on the epithelium of the eye of the offspring using eye spot assay following exposure (Reliene and Schiestl, 2003).

This assay has been used to study the transgenerational effect of ionizing radiation. For example, Shiraishi *et al.* (2002) analysed offspring of irradiated males (6 Gy of X-rays) that derived from either irradiated spermatogonia or sperm using the somatic reversion of mutated p^{un} using p^{un} eye spot assay, searching for the effect of ionising radiation that

could pass transgenerationally via male germline. Two reciprocal crosses: ♀C3H/HeJp^j/p^j x ♂C57BL/6Jp^{un}/p^{un} and ♂C3H/HeJp^j/p^j x ♀C57BL/6Jp^{un}/p^{un} were carried out such that each one of the F_1 offspring will inherit one p^{un} allele that derived from irradiated father and the other from unirradiated mother. They observed a two-fold increase in the number of spots in the retinal epithelium of the offspring derived from irradiated spermatozoa. On the other hand, no significant increase was recorded in the offspring conceived with irradiated spermatogonia. Interestingly, the unirradiated maternally derived p^{un} allele shows nearly the same reversion frequency as the paternally derived irradiated allele which clearly indicates that the radiation induced signal that is delivered via sperm can act in *trans,* affecting the unexposed maternal allele.

1.3.3.5 Tandem repeat sequences

Tandem repeat DNA loci (reviewed in section 1.3) with high rates of spontaneous germline mutation have been employed for studying population genetics, individual identification, and germline mutation induction and transgenerational instability (Burke *et al.*, 1991; Jeffreys *et al.*, 1997; Jeffreys *et al.*, 1999; Dubrova *et al.*, 1993; Dubrova *et al.*, 1998; Barber *et al.*, 2002; Barber *et al.*, 2006; Barber *et al.*, 2009; Mughal *et al.*, 2012)

1.3.3.5.1 Human studies

Many factors complicate the study of TI in exposed human populations. Among these is the inconsistency in nature of irradiation that the studied population has been exposed to, as well as uncertainties regarding estimates of doses. In addition the availability of representative control groups that well match the exposed group in ethnicity and life

style (see section 1.1.3.4.1). However, Dubrova *et al.* (2002) presented evidence for the potential transgenerational effect of radiation in the human germline using an efficient and sensitive human-minisatellite system. Blood samples were taken from parents and offspring of 40 three-generation families living in the Beskaragai district, near the nuclear weapon test site that is known to have elevated levels of ionizing radiation, with effective doses more than 1Sv. At the same time blood collected from 28 three generation non-irradiated families that share the same ethnic origin, year of birth and matched paternal age were selected as a control group. A substantially elevated germline mutation rate (1.7-fold increase) was observed in the F_1 offspring. This rate was 1.5 fold higher in the second generation. In contrast, no significant changes in germline mutation rate were detected in the families that survived the Hiroshima and Nagasaki atomic bombings using human minisatellites (Kodaria *et al.*, 1995; Kodaria *et al.*, 2004).

1.3.3.5.2 Mouse expanded simple tandem repeats

The first study to analyse the possibility that paternal irradiation could lead to transgenerational increases in mutation rates at ESTR loci in the mouse germline was performed by Dubrova *et al.* (2000a). This study showed elevated ESTR mutation rates in the germline of both male and female F_1 offspring of male mice directly exposed to high linear energy transfer (high-LET) fission neutrons. This transgenerational effect was further analysed by Barber *et al.* (2002). They assessed the ESTR mutation rate in the germline of first and second generation offspring of three strains of inbred male mice, CBA/H, C57BL/6 and BALB/c exposed to either 0.4 Gy of high-LET fission neutrons or 1-2 Gy of low-LET X-rays. Their findings were that the ESTR mutation rate was elevated in the germ line of both F_1 and F_2 generation offspring derived from

fathers exposed to either high-LET or low-LET radiation. They also provide evidence that the transgenerational effect is not strain specific as it manifested consistently in F_1 and F_2 offspring across the three strains of mice analysed.

This radiation-induced transgenerational effect has been consistently reported by Dubrova's group utilising single molecule PCR analysis of ESTR loci in different experimental designs. These studies demonstrated substantially elevated ESTR mutation frequencies in the germline and somatic tissues in the offspring of male mice exposed to X-rays regardless of the stage of spermatogenesis (Barber *et al*., 2006; 2009; Hatch *et al.*, 2008).

A study by Barber *et al.* (2006) provided evidence that radiation-induced TI manifested in the non-exposed offspring of irradiated male mice widely destabilising their genomes. The authors analysed mutation frequencies at the X-linked hypoxanthine guanine phosphoribosil transferase (*hprt*) locus as well as the *Ms6-hm* ESTR locus in the non-exposed F_1 offspring of irradiated male mice. In parallel to the significant increase in ESTR mutation frequencies observed both in somatic and germline, the *hprt* locus showed more than a three-fold increase in the offspring of irradiated males compared to controls. By considering the location of *hprt* on X-chromosome which represents the non-exposed maternal allele, it appears that radiation induced signal has the potential to widely destabilise the genome of the offspring of exposed fathers.

This well documented transgenerational effect of radiation exposure raised a very important question regarding the presence of a threshold dose below which no transgenerational effect could be triggered. Indeed, a recent publication by Mughal *et al.* (2012) failed to detect the transgenerational effects in the germline and somatic tissues

of F_1 offspring of male mice exposed either to 10–25 cGy of acute or 100 cGy of chronic γ-rays. In contrast, the F_1 ESTR mutation rates were significantly elevated following acute paternal exposure to 50 and 100 cGy of γ-rays. The results of this study imply that the manifestation of TI in the offspring of exposed male mice is triggered by a threshold dose of acute low-LET irradiation.

1.3.3.6 Transgenerational effect following exposure to chemical mutagens and anticancer drugs

According to the results of some recent studies, paternal exposure to chemical mutagens and anticancer drugs can also destabilise the F_1 genomes. Substantially elevated ESTR mutation rates have been demonstrated in the germline of CBA/Ca and BALB/c that were treated with the alkylating agent ethylnitrosourea either in premeiotic spermatogonia or mature sperm. This elevation was equally manifested in the germline of the non-exposed F_1 offspring (Dubrova *et al.*, 2008). Similarly, highly elevated ESTR mutation rates were detected in both somatic (bone marrow) and germline (sperm), in the non-exposed progeny of male mice injected with equivalent doses of cyclophosphamide (CPP), mitomycin C (MMC), or procarbazine (PCH) ; commonly used as chemotherapeutic drugs. These data draw attention to the possible genetic risks for the children born to cancer chemotherapy survivors (Glen and Dubrova, 2012). The abovementioned studies also provide insight to the features of the phenomenon of transgenerational instability. Indeed, considering the profound differences in the spectrum of damage induced by irradiation and chemical mutagens, it would appear that TI cannot attributed to a specific sub-set of DNA lesions, such as radiation-induced double-strand breaks (DSB), and may therefore be attributed to a stress-like response to a global DNA damage (Dubrova *et al.*, 2008).

1.4 Mechanisms of radiation induced genomic instability

The phenomenon of radiation induced genomic instability (RIGI) is well defined regarding its manifestation and cellular phenotypes. However the underlying mechanism is as yet unknown. RIGI is known to manifest in either somatic cells (genomic instability) or germline (transgenerational instability). The non-exposed progeny of irradiated cells display a high frequency of nonclonal genetic mutations such that it is no longer explainable with a hypothesis based on direct DNA damage. It was therefore suggested that RIGI can be underlined by epigenetic mechanisms (Kovalchuk and Baulch, 2008), defects in genome maintenance pathways including DNA damage and repair (Cui *et al.*, 1999; Huang *et al.* , 2003; Yu *et al.*, 2001), or alterations in cellular homeostasis (Barcellos-Hoff and Brooks, 2001; Baverstock, 2000; Mothersill *et al.*, 2000). In this section the possible mechanisms for the phenomenon of RIGI will be discussed.

1.4.1 Can double strand breaks be implicated?

Despite being the principal cytotoxic lesion induced by ionizing radiation, the induction of DNA DSBs by itself does not appear to be responsible for the initiation and perpetuation of RIGI (Suzuki *et al.*, 2003; Wright, 2010). Instead, DSBs could have a role in the initiation of RIGI. This notion has been supported by evidence that DNA damaging agents that lead to DNA breakage induce delayed chromosomal instability. For instance, the study of Kaplan and Morgan (1998) when they observed delayed chromosomal instability after incorporation of ^{125}I-iododeoxyuridine, which causes DNA damage at the site of their decay. Another study reported that chromosomal

instability could be induced by radiomimetic drugs such as bleomycin and neocarzinostatin (Limoli *et al.*, 1997).

Double strand breaks can be induced via variety of endogenous and exogenous agents, including ionizing radiation and some chemical mutagens. The endogenous agents include free radicals or factors produced during regular metabolic reaction (van Gent *et al.*, 2001). In addition, DNA double strand breaks can arise during DNA replication (Figure 1.1). During DNA synthesis, and under normal circumstances, the replication machinery usually overcome any impediment such as DNA adducts secondary structures or tightly bound proteins that can cause replication fork stalling and consequently compromise genome integrity. This is usually achieved via checkpoints where DNA integrity could be constantly checked, as well as DNA replication being coordinated with repair, chromosome segregation and cell cycle progression (Aguilera and Gomez-Gonzalez, 2008). However, under circumstances of replication stress when replication inhibited and/or S-phase checkpoint inactivated, the replication fork can collapse causing disassembly of replisome (replication machinery) leaving single-strand DNA gaps and DSBs (Cobb *et al.*, 2005; Sogo *et al.*, 2002).

Genomic instability can also manifest during the DSB repair process itself. It is known that cells have two mechanisms for the repair of DNA double strand breaks: the homologous recombination (HR) and non-homologous end-joining (NHEJ) (Haber, 2000). The faster NHEJ can work throughout the entire cell cycle. However, it is very error prone as its mechanism is based on just modifying the broken DNA ends and ligates them together regardless of homology (Lieber, 2008). In contrast, HR is slower and restricted to G2 or S-phase as it requires the use of the undamaged sister chromatid

as a template for the reconstitution of the original sequence and therefore, it is error free (Thompson and Schild, 2001). Repair of double strand breaks by joining of the DNA strands is crucial for cell survival, however; it can compromise genome integrity (Aguilera and Gomez-Gonzalez, 2008; Burma *et al.*, 2006; Sonoda *et al.*, 2006), particularly error prone NHEJ which possibly causes losses or rearrangements of genetic information via mis-rejoining of the two strands (Hartlerode and Scully, 2009, Lieber, 2010) or alters DNA sequences during processing of the broken DNA ends by exonucleases or endonucleases. Therefore, despite the cell escaping lethality via DSB repair system, it may survive with a loss of heterozygosity and/or gross genome rearrangements (Suzuki *et al.*, 2011).

The DSB repair process may induce a permanent epigenetic mark on chromatin architecture that could mediate the epigenetic memory of the initial insult (Orlowski *et al.*, 2011). It is well known that chromatin architecture partitions the genome into less compact active domains (euchromatin) or highly compacted inactive domain (heterochromatin), making chromatin an important regulator of gene expression by controlling the accessibility of transcription machinery depending upon the degree of packing of chromatin. Therefore, each cell has an epigenetic identity formed by these epigenetic marks which must transmit faithfully during mitosis for proper function of the cell (Li and Reinberg, 2011). By considering the crucial role of chromatin architecture in maintaining the expression pattern of the cell, it is probable that severe disruption of chromatin architecture such as DSB can lead to alterations or modifications in the epigenetic marks that could lead to disorder in the epigenetic regulatory pathways, if not repaired properly (Orlowski *et al.*, 2011). One source of this epigenetic disruption could be attributed to repair process itself which, if done

faithfully, should lead to restoration of chromatin organisation and hence, normal cellular functions. For example, NHEJ, the rapid and frequent way to respond and repair the IR-induced DSB, is error prone and causes disturbances in chromatin structure as well as sequence due to the absence of homologous template (Lieber *et al.,* 2003; Natarajan *et al.*, 2008).

Indeed, chromatin in the immediate vicinity to the site of DSB undergoes modulations in response to DNA damage to enable the accessibility of the repair machinery. As a direct response to DNA DSB, mammalian histone variant H2AX is phosphorylated on Ser139 to generate γH2AX which appear in the form of subnuclear foci in the vicinity of the site of DSB, which spread broadly around the DSB (Pilch *et al.,* 2003; Rogakou *et al.,* 1998). According to Fernandez-Capetillo *et al.* (2004) the phosphorylation of γH2AX facilities chromatin re-modelling in the close vicinity of DSB to form facility for recruitment of the components involved in the DSB-repair machinery (NHEJ and HR). Thus, a variety of repair factors such as the MRN complex, MDC1, BRCA1, 53BP1 and Rad51 were detected in γH2AX foci.

The abovementioned results imply that DNA DSB repair can lead to permanent chromatin modifications which could persist during mitosis and passed to newly formed cells. In other words, if the repair process caused any chromatin remodelling, this in turn could lead to alteration in the expression status of a certain genes, then this newly generated expression phenotype could persist the long term or forever based upon the stability of *de novo* epigenetic mark created (Orlowski *et al.*, 2011). One possible scenario has been proposed by Cuozzo *et al.* (2007) after they induced a site-specific DNA DSB in HeLa or mouse embryonic stem cells (ES) with the restriction

endonuclease *I-SceI*, whose repair is carried out via HR. The authors observed that DNA repair was associated with newly generated methylation which inactivated the recombined gene (green fluorescent protein in their case). As detected by chromatin immunoprecipitation and RNA analysis, the DNA methyl transferase 1 was linked to chromatin at the recombination site. The methylated phenotype disappeared from recombinants produced in stem cell deficient in Dnmt1 which indicates a role of Dnmt1in the *de novo* methylation and subsequent inactivation of the recombined gene. Another support for this scenario comes from the work of O'Hagan *et al.* (2008) who established an experimental model in which DSB could be induced within exogenous promoter construct of E-cadherin CpG-island. The results of this study showed that although DNA repair machinery resumed the promoter activity in the most of the cells, a small batch of cells acquired promoter inactivation phenotype and passed it on to their progeny. This silencing was found to be associated with promoter hypermethylation (O'Hagan *et al.*, 2008; Orlowski *et al.*, 2011).

Suzuki *et al.* (2011) suggested that as the induction of radiation-induced DSBs can be delayed after the initial insult, the presence of such delayed DNA damage may potentially explain the manifestation of RIGI in the progeny of exposed cells. The authors analysed the distribution of P53 binding protein 1 (53BP1) foci, a key component of the cellular responses to DNA double-strand breaks (Schultz, *et al.*, 2000) in the progeny of CHO and xr5 cells exposed to 10 Gy of acute X-rays. They observed a higher incidence of 53BP1 foci in the descendants of both CHO and xrs-5 that survived X-irradiation compared to non-irradiated cells which was explained by the authors as delayed induction of DSB. The finding of Suzuki *et al.* supported the earlier work of Barber *et al.* (2006) who reported DNA lesions several generations after the initial

exposure using phosphorylated histone H2AX foci as a marker for DNA double strand breaks.

1.4.1.1 Summary of DNA double strand breaks

DNA double strand break, the principal lesion induced by irradiation, cannot be ignored when talking about mechanisms underlying the phenomenon of RIGI. However, it is likely that DSBs *per se* cannot be responsible for initiation and perpetuation of RIGI. Instead, DNA DSBs may indirectly mediate initiation and/or perpetuation of RIGI most likely during the process of DSBs repair via promoting an epigenetic alteration. One possible source of epigenetic disruption may arise via the very error-prone NHEJ repair that may cause disturbances in chromatin structure as well as sequence alterations. Another source is chromatin modulation in the site of DSBs, including formation of γH2AX foci as well as 53BP1 foci. DNA damage response and DSB repair could be the main player in mediating the epigenetic memory of insult when induced chromatin modification could persist during mitosis and passed on to the newly formed cells, i.e. when the repair process induces a chromatin modification that leads to alteration in the expression status of certain gene, and the newly generated expression phenotype could persist for long term (Orlowski *et al.*, 2011).

1.4.2 Oxidative stress and mitochondrial dysfunction

One of the possible causes for RIGI is oxidative stress and the corresponding elevation of reactive oxygen (ROS), and nitrogen species (RNS) as well as inflammation induced by ionizing radiation in the target tissues. It is well established that the presence of elevated levels of free radicals and oxidative stress products such as oxidative base damage is associated with radiation induced chromosomal instability *in vitro* (Clutton *et*

al., 1996; Limoli *et al.*, 1998). Persistent increases in reactive oxygen species (ROS) were observed in cultures of cells showing radiation induced genomic instability suggesting a role for enhanced oxidative stress in maintaining the unstable phenotype (Limoli *et al.*, 2003). Limoli and colleagues (2003) induced chromosomal instability in the human-hamster hybrid line GM10115 cell line chronically exposed to hydrogen peroxide (H_2O_2). Irrefutable evidence that oxidative stress is implicated in radiation induced genomic instability was reported by (Limoli *et al.*, 2001) when they irradiated cells in the presence of free radical scavengers such as DMSO, glycerol, or cysteamine and they recorded reduction in the incidence of chromosomal instability. Furthermore, Roy *et al.* (2000) studied the effects of hypoxia on X-ray-induced delayed effects and found that hypoxia (2% oxygen) significantly reduced the induced delayed effects of X-ray irradiation compared to cells cultured under normal oxygen conditions (20%).

Mitochondria are considered the major natural cellular source of ROS, producing them during their normal metabolic activities. It has also been thought that mitochondrial dysfunction following mutagen exposure may have a role in the maintenance of elevated ROS in genetically unstable cells. Also decreases in mitochondrial membrane potential, the activity of manganese superoxide dismutase (MnSOD), respiration rates and mutations in a mitochondrial electron transport chain proteins, and succinate dehydrogenase level, have all been associated with genomic instability (Limoli *et al.*, 2003; Samper *et al.*, 2003; Kim *et al.*, 2006; Slane *et al.*, 2006).

A more recent study by Mukherjee *et al.* (2012) has focused on inflammatory response as a possible cause for RIGI *in vivo*. The authors irradiated mice which were kept on a diet containing anti-inflammatory drugs and found in these animals a substantial

reduction of chromosomal instability associated with a decrease in the level of inflammatory markers in the bone marrow cells. The authors concluded that radiation induced chromosomal instability is not an intrinsic property of the cell and most likely attributed to a subsequent inflammatory reaction following the secondary damage that manifests as a non-targeted delayed effect.

According to the results of the abovementioned experiments, the secretion of inflammatory factors from the irradiated tissue may provide a plausible explanation for the phenomenon of non-targeted effects, including radiation-induced genomic instability, bystander effects or delayed cell death. However, it cannot explain the phenomenon of transgenerational instability considering that the genomic instability is the same phenomenon in both somatic and germ cells (Morgan, 2003b). Indeed, there is no possible explanation of how secreted inflammatory factors or ROS could mediate the transmission of genomic instability across the male germline especially with the negligible cytoplasmic part of the sperm (Karotki and Baverstock, 2012).

1.4.3 Epigenetic modifications and radiation induced genomic instability

Direct gene mutation, radiation induced lesions such as DSBs, or changes in level of mRNA cannot explain the unexpected non-clonal manifestation of RIGI (Limoli *et al.,* 1997, Morgan *et al.,* 1998, Snyder and Morgan, 2005). Instead, epigenetic deregulation including alterations of pattern of expression, DNA methylation and histone modifications are likely to be the mechanisms accounting for RIGI.

Methylation of cytosine residues of CpG dinucleotides is a crucial epigenetic mechanism for transcriptional inactivation and chromatin condensation (Bird and Wolffe, 1999; Orlowski *et al.*, 2011). These epigenetic marks can be maintained across multiple cell divisions. Promoter methylation can repress transcription of some genes by preventing transcription factors from reaching their target sequences (Bird, 2002).However, it is not always the case as in some cases histone modifications are required alongside DNA methylation to produce a transcriptionally repressive phenotype (Kass *et al.*, 1997). This interaction mechanism is mediated via a family of proteins that specifically bind to methylated DNA and modulate histone modification which includes MBD1, MBD2, MBD3 and MeCP2 (Boeke *et al.*, 2000; Jones *et al.*, 1998; Nan *et al.*, 1998; Orlowski *et al.*, 2011). DNA methylation also has a role in silencing of transposable elements. In mice, transcription of the intracisternal A particle (IAP) elements is controlled via promoter methylation which is massively unregulated in the embryos lacking Dnmt-1 (Walsh *et al*,. 1998). Also, the long interspersed nuclear elements 1 (LINE-1) and Alu elements are inactivated by hypermethylation (Bestor, 2005). Taken together, the abovementioned results indicate that DNA methylation can be regarded as a mediator of RIGI.

Indeed, in many reports ionizing radiation exposure has been associated with changes in methylation profiles and is suggested as an initiator of RIGI (Tawa *et al.*, 1998, Kovalchuk and Baulch, 2008). For example, Kaup *et al.* (2006) demonstrated that CpG methylation alteration can persist up to 20 population doublings post-irradiation of human keratinocyte cell line. Furthermore, using an arbitrarily primed methylation sensitive PCR, the authors demonstrated that irradiation causes reproducible alterations in the methylation profiles of this cell line and one of these altered sequences was a

retrotransposon element (Kaup *et al.*, 2006). Profound epigenetic changes have also been observed following exposure of GM10115 cells to low-LET X-rays and high-LET iron ions (Aypar, *et al.*, 2011a). Alterations in DNA methylation either in specific genes or repeat elements as well as alterations in the level of miRNA have been recorded. However, these epigenetic changes were not accompanied by chromosomal instability at delayed time and the authors claimed that acquisition of epigenetic changes by irradiated cells does not necessarily initiates chromosomal instability (Aypar, *et al.*, 2011a).

Strong evidence that DNA methylation could be implicated in the mechanism of radiation-induced genomic instability has been presented by the *in vitro* work of Rugo *et al.* (2011). They found that γ-irradiated mouse embryonic stem cells could 'remember' the genome-destabilising effect for weeks after the initial insult which could spread into neighbouring cells. The most striking finding was that the manifestation of this destabilization signal through mitosis is mediated by methyltransferases (Dnmt1 and Dnmt3a) and their disruption ceases the signal transmission through mitosis (Rugo *et al.*, 2011).

It has been reported that the promoter of p^{16} (INKa) was differentially hypermethylated in a tissue and sex specific fashion following whole body low dose X-irradiation (Kovalchuk *et al.*, 2004) and that was correlated with genomic instability. Interestingly, these alterations were dose-dependent, persistent and linked with radiation induced lesions (DSBs) (Pogribny *et al.*, 2004). The reason behind these sex and tissue specific alterations could be the radiation induced alterations in DNA methyltransferases (Raiche *et al.*, 2004). Indeed, acute X-irradiation of rat leads to global DNA

hypomethylation that is accompanied by declines in the levels of maintenance (Dnmt1) and novel (Dnmt3a and 3b) methyltransferases as well as the methyl-binding protein MeCP2 in rat mammary gland. These alterations were also found to be linked with DNA repair as reported by Loree *et al.* (2006). Similar observations have been recorded by Koturbash *et al.* (2006) in the thymus of the non-exposed offspring of irradiated male C57Bl/6 mice mated 7 days post-irradiation. They recorded significant reduction in the level CpG methylation which was accompanied by a substantial decrease in DNA methyltransferases as well as methyl-CpG-binding protein MeCP2.

However, in one recent study by Armstrong *et al.* (2012), it was reported that global DNA hypomethylation has no role in radiation induced genomic instability or radiosensitivity, at least in mouse embryonic stem cells. That said, functional DNA methyltransferases are possibly required for radiation induced genomic instability.

Another epigenetic alteration that profoundly affects cellular activities and maintenance and that is known to be altered post irradiation is histone modifications. Histone post-translational modifications (PTMs) are important controllers of active or inactive chromatin states which have profound effects on the accessibility for chromatin to replication, transcription or repair (Orlowski *et al.*, 2011; Jenuwein and Allis, 2001). These modifications include acetylation, phosphorylation, ubiquitination, and methylation and each type of modification has a different regulatory role. For example, histone acetylation is associated with a relaxed, transcriptionally active chromatin state whereas deacetylation leads to a compacted repressive state (Jenuwein and Allis, 2001).

Histones can be methylated at certain residues and modulate the chromatin state. For instance, methylation of lysine 9 of histone H3 is correlated with condensed chromatin transcriptional silencing, whereas lysines 4 and 27 of histone H3 methylation results in transcriptionally active chromatin (Jaenisch and Bird , 2003; Ilnytskyy and Kovalchuk, 2011). One of the most important histone modifications is phosphorylation of H2AX at serine 139 (γ-H2AX) which represents an early mark for response to DSBs forming γ-H2AX foci (Rogakou *et al.*, 1998; Sedelnikova *et al.*, 2003).

Using a mouse model Pogribny *et al.*, (2005) have reported a decrease in histone H4-lysine 20 trimethylation in the thymus following fractionated whole body exposure to 0.5Gy of X-rays which could result in a less compacted chromatin state. Also, the more compact transcriptionally inactive chromatin domains were less susceptible for induction of DSBs by γ-irradiation compared with the relaxed regions. Interestingly, immediately after γ-irradiation, chromatin in the immediate vicinity of DSBs appeared to be less compact. However, this relaxed state became more compact following enhancement of H3K9 methylation, within 40 minutes (Falk *et al.*, 2008).

In conclusion, the association between epigenetic modification and RIGI is well documented and provides a good explanation for the phenomenon. However, it is not known how these epigenetic modifications could drive the initiation or propagation of sequential events that lead to the instability phenotype (Karotki and Baverstock, 2012).

1.4.4 Possible role of extracellular matrix and cell signalling, telomere dysfunction, and centrosome damage

It is well established that the extracellular matrix (ECM) and cell signalling plays a major role in maintenance and regulation of cell and tissue functions. For instance, they alter patterns of gene expression (Bissell *et al.*, 1982; Bissell and Barcellos-Hoff, 1987). The living cell is greatly affected by their surrounding cells and there is always cross talk between neighbouring cells via signals through the ECM. These signals can affect cell proliferation and/or differentiation. It has been demonstrated that cells growing in tissues always have lower rates of proliferation than those of the single or cultured cells which is attributed to inhibitory signals that decrease cellular proliferation (Soto and Sonnenschein, 2004). Therefore, any stress factor such as ionizing radiation can alter these signalling pathways and cross talks between cells by affecting the ECM environment. One piece of evidence that supports this hypothesis comes from an experiment conducted by Barcellos-Hoff (1993) who γ-irradiated female BALB/c mice and analysed the ECM changes and activity of transforming growth factor β (TGFβ) in the mammary glands post-irradiation. The authors detected alterations in the ECM as well as activation of TGFβ. In addition to its role in regulation of cellular functions such as apoptosis, cell growth, chemotaxis, and differentiation (Massague *et al.*, 2000), TGFβ could have a role in mediating radiation exposure of the ECM (Ehrhart *et al.*, 1997) as well as abnormal extracellular signalling result from irradiation.

Telomere dysfunction has also been proposed as a mechanism of RIGI. Telomeres are terminal tandem repeat nucleotide arrays that known to protect the chromosome end from abnormal fusions and/or DNA rearrangements (Greider, 1991; Greider, 1996). Their direct role in maintenance of genome integrity makes telomeres likely candidates

for studying their potential role in RIGI. For example, a significantly increased telomere loss as well as duplication associated with chromosomal instability has been observed in normal human fibroblasts surviving X-irradiation compared to the control level (Ojima *et al.*, 2004). More recently, Berardinelli *et al.* (2013) have demonstrated a correlation between telomere length, dysfunction and chromosome mis-segregation when human primary fibroblasts were irradiated with either X-rays or low energy protons. Interestingly, according to the results of this study, a significant telomere shortening was observed in cells 96 hours following exposure to X-rays. On the other hand, high-LET exposure to neutrons significantly increases the telomere length 24 and 96 hours after irradiation. However, as far as telomere aberrations are concerned it can provide an explanation for only chromosomal instability as a manifestation of RIGI. On the other hand, other manifestations of RIGI such as an increased rate of point mutations cannot be explained by telomere dysfunction (Karotki and Baverstock, 2012).

Due to its important role in cell division, centrosome damage has been suggested as possible cause for RIGI. Indeed, the centrosome is the main centre for organizing microtubules in mammalian cell which is necessary for normal behaviour of chromosomes in cell division. During mitosis the centrosomes nucleate the formation of astral microtubules and through them the centrosomes can determine the spindle polarity (Sluder and Nordberg, 2004). Maxwell *et al.* (2008) provided evidence that centrosome damage could initiate genomic instability when they demonstrated centrosome aberration in normal human mammary epithelial cells as an early event in the first cell cycle following irradiation. This centrosome damage occurred and accumulated in a dose dependant manner and the viable progeny of these cells show

genomic instability manifested as aneuploidy, tetraploidy and spontaneous DNA damage.

1.4.5 Possible mechanisms of transgenerational instability

In the last section (1.3.3.4), the potential mechanisms for the radiation-induced genomic instability were discussed with some reference to transgenerational instability, considering them the same phenomenon in somatic and germline cells. Herein the focus will be on the unique features of the phenomenon of transgenerational instability that could mediate its manifestation across generations. In all experiments that attempted to address the transgenerational effect induced by genotoxic agents such as ionizing radiation (Barber *et al.*, 2002; 2006; 2009), chemical mutagen (Dubrova *et al.*, 2008), or anticancer drugs (Glen and Dubrova, 2012) in the germline of the non-exposed offspring of exposed fathers, the stress induced destabilization signal is likely to be epigenetic. This epigenetic signal is transmitted via sperm and persists the epigenetic reprogramming in early embryogenesis. The reason why this signal is thought to be epigenetic is that the instability phenotype is out of Mendelian expectations as it inherited by all offspring and equally manifested in both F_1 and F_2 generations (Dubrova *et al.*, 2000; Dubrova *et al.*, 2008; Barber *et al.*, 2002; Barber *et al.*, 2006; Barber *et al.*, 2009; Glen and Dubrova, 2012). In addition, the instability signal acts in *trans*, affecting the non-irradiated maternally-derived allele following paternal exposure (Dubrova *et al.*, 2000; Barber *et al.*, 2009; Shiraishi *et al.*, 2002; Niwa and Kominami, 2001). However, the nature of this epigenetic signal is as yet unknown.

1.4.5.1 DNA damage-response and DNA double strand repair as memory mediators

There is some evidence that the epigenetic memory that mediate passage of the destabilization signal via the paternal germline may be settle in the link between replication and DNA repair (de Boer *et al.*, 2010). Indeed, DNA damage responses can trigger an epigenetic alteration that is maintained across cell division. The work of Cuozzo *et al.* (2007) provided such evidence. The authors analysed DNA methylation changes occurred during homologous recombination DSB repair in HeLa or mouse embryonic stem cells and showed that some alterations were transmissible to progeny of these cells via DNA methyltransferase-1. Another study by Barber *et al.* (2006) reported that radiation-induced transgenerational destabilisation observed in offspring of irradiated male mice can be attributed to the presence of persistent endogenous DNA lesions (double and single-strand break) that passed the male germline and manifested across generations.

Hatch *et al.* (2008) recorded substantially elevated ESTR mutation rates in the germline (sperm) and somatic (bone marrow) cells of the offspring derived from irradiated spermatozoa of either normal BALB/c or non-homologous end-joining pathway deficient (*scid*) male mice. According to the results of this study, ESTR mutation frequencies were highly elevated in the germline and somatic tissues of offspring of irradiated *scid* males mated with non-exposed BALB/c females. In contrast, ESTR mutation rates in the offspring of irradiated BALB/c males mated with non-exposed *scid* females did not significantly differ from those in controls, thus implying potential contribution of DSB repair in the manifestation of transgenerational instability.

The *in vitro* data published by O'Hagan *et al.* (2008) provide further evidence for the manifestation of DSB repair-induced epigenetic alterations. According to the results of this study, following the repair of DSBs induced within exogenous promoter of the E-cadherin CpG-island of most cells resumed the transcription of this gene. However, a small batch of cells acquired this promoter inactivation phenotype and passed it to their progeny. This silencing was found to be associated with promoter hypermethylation. These observations indicates that DNA damage response and DSB repair are potential mediators for the epigenetic memory of genotoxic insult that is required for the manifestation of radiation induced signal across the male germline.

1.4.5.2 Possible role of DNA methylation and histone modifications

It is well documented that DNA methylation is highly dynamic during germ-cell development (reviewed in Smallwood and Kelsey, 2012) and known to be asymmetrical between male and female germ cell development. Also, the presence of some methylated loci that can escape being cleared during epigenetic reprogramming makes DNA methylation a potential candidate for epigenetic transmission (Daxinger and Whitelaw, 2012). For instance, some retrotransposons such as intracisternal A-type particles (IAPs) keep their methylation in mouse mature gametes as well as early pre-implantation (Lane *et al.*, 2003). Another example from the work of Borgel *et al.* (2010) who analysed DNA collected from different developmental stages of the mouse including mature gametes, morula, and blastocyst. They found no change in methylation in promoters of ~100 non-imprinted, non-repetitive genes which means that they could escape being erased during post-fertilization methylation reprogramming.

The reviewed work so far suggests that DNA methylation and post-translational histone modifications can play an important role in the transmission of the radiation induced instability signal across male germline. However, they give no explanation for how these epigenetic marks could escape the challenge of being erased during spermatogenesis. During the last stage of spermatogenesis, profound morphological changes take place in the post-meiotic spermatids to form mature sperm. These morphological changes are accomplished by the replacement of histones with more basic and highly compacted protamines which are expected to erase any histone mark induced by irradiation. The DNA-protamine complex is also known to be transcriptionally inert and inaccessible to DNA repair machinery (Marchetti and Wyrobek, 2008; Wouters-Tyrou *et al.*, 1998; Meistrich *et al.*, 2003; Boissonneault, 2002). One question that needs to be asked is how these epigenetic marks, especially histone marks, can resist these massive changes during spermiogenesis. One answer is that not all histones are replaced with protamines during spermiogenesis. Instead, about 1-2% of histones in the genome of mature sperm in mouse and 4% of human sperm genome are retained in nucleosomes (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010). From these histone marks that resisted clearing were extensive histone H3 lysine 27 trimethylation (H3K27me3) that is associated with gene silencing and marked promoters of some genes that are known to repress during gametogenesis and/or early development (Hammoud *et al.*, 2009; Brykczynska *et al.*, 2010). The authors revealed that these retained marks could carry the epigenetic instructions from one generation to another via sperm. However, it is worth mentioning that none of these studies explained how these epigenetic marks can escape erasure in epigenetic reprogramming during early development (Burton and Torres-Padilla, 2010) if they pass through the germline.

1.4.5.3 Non-coding RNA

Most, if not all studies, addressing the transgenerational transmission have focused on the nucleus. However, non-coding RNAs (RNAi) have recently emerged as possible modulators of epigenetic inheritance (Daxinger and Whitelaw, 2012). It is well known that mature sperm nucleus is highly condensed, transcriptionally and translationally inactive. However, all RNA species were detected in mature sperm (Krawetz, 2005) for example, long non-coding RNAs (LncRNAs) and a variety of small RNAs such as microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and PIWI-interacting RNAs (piRNAs), which are known to have a role in gene silencing (Zhao *et al.*, 2006; Watanabe *et al.*, 2006).

It has been reported that small RNAs can travel between cells in worms and plants (Melnyk, *et al.*, 2011). Also, silencing of transposable elements is mediated by siRNA in some animals, plants and fungi (Soltkin *et al.*, 2009) and even in mouse oocytes siRNA is essential for silencing of retrotransposons (Watanabe *et al.*, 2008; Tam *et al.*, 2008; Murchison *et al.*, 2007).

PIWI-interacting RNAs or piRNA, are of a size ranging from 24-31bp.Their name is derived from the PIWI clade of proteins of the Argonaute family they known to bind to. The piRNAs are involved in silencing of transposable and repetitive elements in the germline of mice, fruit flies, and zebra fish and hence they play role in genome stability in their germline (Daxinger and Whitelaw, 2012). piRNA was found to be expressed at a high level in spermatocytes (Saito and Siomi, 2010; Gan *et al.*, 2011) and it could have a role in parental imprinting (Watanabe *et al.*, 2011).

Another class of small RNA is the one including micro RNAs (miRNAs) which are known to repress protein coding genes by hybridising their mRNAs post-transcription. Rassoulzadegan *et al.* (2006) have reported that miRNAs have a role in the epigenetic inheritance of the *kit* locus across generations in mice. Breeding heterozygous mice which carry the mutant Kit^{tm1Alf} allele along with the wild type *kit* allele produces the wild type genotype mice in the expected proportion. However, the phenotype was skewed to heterozygous parental phenotype which revealed the involvement of transgenerational epigenetic inheritance. Interestingly, the level of *kit* mRNA in the wild type offspring that carry the mutant phenotype was substantially reduced to levels similar to the heterozygous. Also, injection of the two miRNAs miR-221 and miR-222 known to potentially target *Kit* mRNA into wild-type zygotes resulted in mice with the white-tail phenotype characteristic for the heterozygous (Rassoulzadegan *et al.*, 2006).

Radiation-induced changes in the spectra of microRNAs have recently been described. For example, alterations in the level of expression of miRNA following low (0.1Gy) and high (2.0Gy) doses of low LET X-rays were observed in human fibroblasts. These altered miRNAs were found to target regulatory genes of cell cycle checkpoints and apoptosis (Maes *et al.*, 2008). Alterations in the miRNA spectrum were also demonstrated following exposure either to high LET (Fe ions) or low LET (X-rays) in GM10115 cell lines (Aypar *et al.*, 2011a).

In conclusion, the abovementioned results imply that non-coding RNA may be regarded as possible mediators for the germline transmission of transgenerational effects. However, none of these studies has attempted to establish whether this may be the case for the offspring conceived from transcriptionally inactive stages of spermatogenesis, including late spermatids or mature sperm.

1.5 Heritable effects of maternal irradiation

1.5.1 Female germ cell development

Mammalian oogenesis is a complex and prolonged process which begins in the early stages of embryogenesis and continues during adulthood. Oogenesis starts with germ cell specialisation, followed by migration into genital ridge, colonisation of the developing gonad, differentiation into oogonia that undergo several rounds of mitosis, launching to meiosis and finally resting at late meiotic prophase until ovulation at puberty. In mice, differentiation of primordial germ cells (PGCs) starts at embryonic day 5.5 to 6.0 in the proximal epiblast induced via bone morphogenetic protein-4 (BMP-4) and BMP-8b signalling from the extraembryonic ectoderm. The BMP-4 triggers key transcriptional mediators that are essential for acquiring the germ cell features (Lawson *et al.*, 1999; Ying *et al.*, 2001; Ohinata *et al.*, 2009). The PGCs multiply and migrate to the genital ridge between the 9-12 days of gestation (Borum 1961; Mtango *et al.*, 2008). The migration of PGCs to the genital ridge is achieved by a combination of morphogenetic movements and self-propulsion (Picton *et al.*, 1998) and mediated by Kit ligand (KL or Stem cell factor) which is expressed in the somatic cells through the migratory way and received by c-kit receptor expressed on the surface of PGCs (Fleischman, 1993). In the genital ridges, PGCs differentiate according to the available pair of sex chromosomes (XX or XY) to either oogonia or spermatogonia which are both mitotically and transcriptionally active (Picton *et al.*, 1998). The groups of oogonia connect together via intercellular cytoplasmic bridges and form what called germ cell clusters or cortical cords by surrounding themselves by mesonephros-derived somatic cells (van den Hurk and Zhao, 2005). In the mouse, between days 14-16 of gestation the oogonia undergo about 4 cycles of mitosis before launching to meiosis

whereas in humans continue dividing mitotically over a period of several months (Picton, 2001). Within the cords, oogonia begin pre-meiotic DNA synthesis (days 12-13) which completed for the majority of oogonia by day sixteen (Lima-de-Faria and Borum, 1962; Peters *et al.,* 1962). Oogonia that fail to proceed into meiosis soon degenerate (Beaumont and Mandl, 1962). From this point onwards only primary oocytes remain, which means there is no possibility of additional germ cell renewal. On embryonic days 14-16 most oocytes are in the leptotene and zygotene phases of meiosis, reaching pachytene on days 17-18 (Lima-de-Faria and Borum, 1962). The early diplotene stages start to appear on about the eighteenth day and late diplotene (dictyate) stage is reached on the day of birth. Four to five days after birth all surviving oocytes are in the dictyate or so-called 'resting stage' (Borum, 1961). The process of follicle formation starts and continues parallel to meiosis. During folliculogenesis, the dictyate oocytes lose the intercellular bridges and become surrounded with single layer of flattened cells called pregranulosa cells forming the early stage primordial follicle (Picton, 2001; Gougeon, 1996). Mouse oocytes arrested at this stage (dictyate stage), that surrounded by a single layer of flattened granulosa cells which forms 'primordial follicle' and remain at this stage until the individual reaches sexual maturity (~5-6 weeks of age). After the onset of sexual maturity follicles begin to mature into 'growing follicles' which contain initially two and then many layers of follicular cells. Oocyte development then proceeds over a 6 week period to become fully mature and for an oocyte to reach ovulation (Oakberg, 1979).

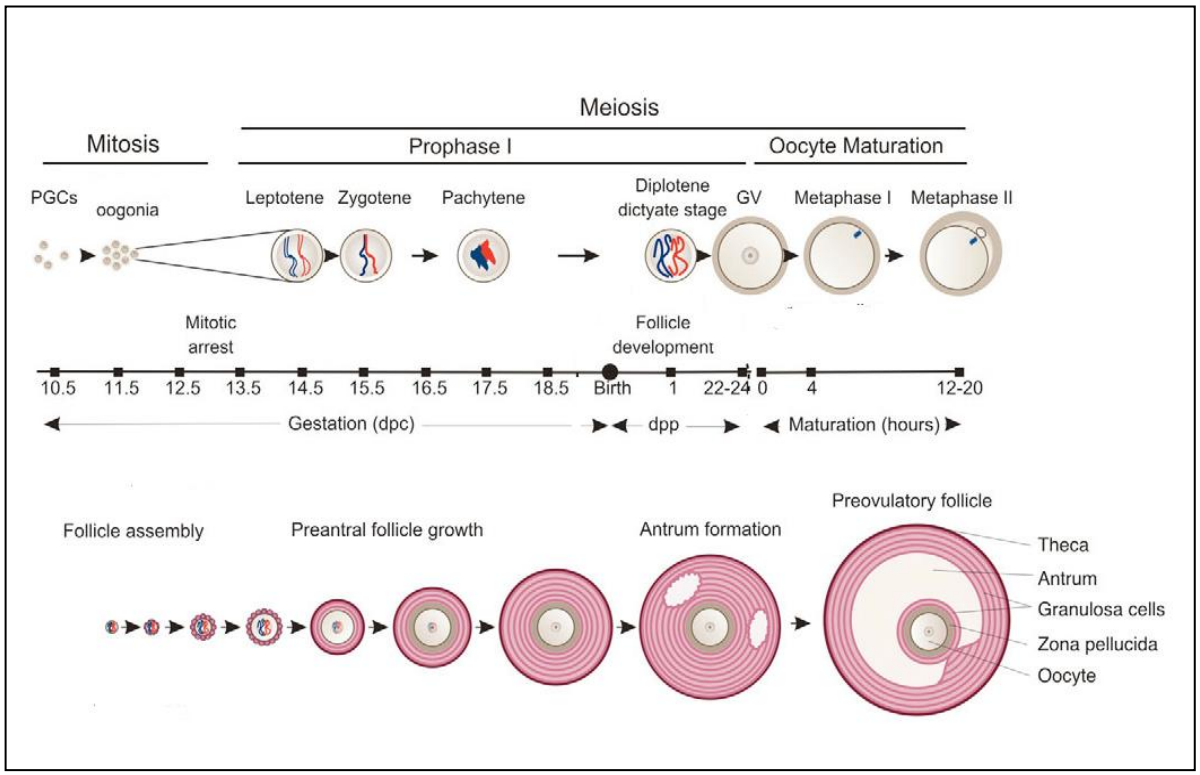


Figure1.1 : Stages of oogenesis (adapted from Racki and Richter, 2006).

The upper panel summarises the process of oogenesis and the lower panel describes the folliculogenesis.

PGCs: primordial germ cells, dpc: day post-coitum, dpp: day post-partum, GV: germinal vesicle.

1.5.2 DNA repair in oocytes

The first direct evidence for DNA repair in oocytes was presented in the publication by Masui and Pedersen (1975). Using the unscheduled DNA synthesis technique, the authors showed that fully matured mouse oocytes exposed to ultraviolet (UV) irradiation were capable of repairing DNA damage. According to the results of this publication, UV dose-dependent unscheduled DNA synthesis was also observed in resting oocytes taken from 2- to 3-day-old mice and growing oocytes taken from 12–13-days old mice. Interestingly, the uptake of tritiated thymidine was six times greater in growing oocytes compared with resting oocytes (Pedersen and Mangia, 1978). Similar results were also obtained on the isolated mouse dictyate oocytes taken from both young (8-14 weeks) and old (12 – 15 months) mice. The authors concluded that the mouse dictyate oocyte has a capacity for DNA repair that was independent of the maternal age (Ashwood-Smith and Edwards, 1996; Guli and Smyth, 1989).

Further evidence supporting the results of these studies was obtained by Matsuda and Tobari (1988). The authors fertilised the mouse oocyte with UV-irradiated sperm and analysed the yield of chromosomal alterations at the metaphase of the first cleavage. According to the results of this study, the observed frequency of chromosome aberrations was highly enhanced in the oocytes pre-treated by the two DNA-repair inhibitors – caffeine and ara-C, thus indicating that UV-induced DNA damage in sperm can be effectively repaired in the oocytes.

Fritz-Niggli and Schaeppi-Buechi (1991) provided the first evidence for repair of DNA damage in *Drosophila melanogaster* oocytes. The authors analysed the effects of X-ray exposure to a priming dose of 0.02 Gy on the yield of chromosome aberrations in the

oocytes later exposed to a substantially higher dose of 2 Gy and observed a significant reduction in chromosome damage (adaptive response). These results were later confirmed in mice by Jacquet *et al.* (2008). Interestingly, the authors failed to observe the manifestation of adaptive response in the less sensitive earlier oocytes (two weeks before ovulation).

More recently, microarray analysis of the global gene expression in mouse oocytes at different maturity stages has revealed that there is an over-expression of DNA repair genes and DNA damage response genes throughout oocyte development (Adriaens *et al*., 2009; Pan *et al.*, 2005; Hamatani *et al.*, 2008). In a comprehensive study using primate oocyte (Rhesus monkey), Zheng *et al.* (2005) analysed the expression patterns of 48 mRNAs covering the pathways involved in DNA repair in germinal vesicle (GV) and MII stage oocytes. They observed the presence of mRNAs encoding for all repair pathways including mismatch repair (MMR), base excision repair (BER) proteins related to single strand break (SSB) repair, and double strand break repair (DSB) with some variation in the level of expression between stages of oogenesis.

In conclusion, reviewed work clearly shows that the mammalian oocyte is DNA repair proficient that is able to repair either spontaneous or mutagen-induced DNA damage. However, the efficiency of repair shows some stage variation.

1.5.3 Mutation induction in the maternal germline

Over the past decades, several attempts have been made to address the potential genetic effect of maternal exposure to ionising radiation. These experiments (discussed in section 1.1) were designed to measure the induced mutation using traditional phenotypic assays such as dominant lethality (DL), specific-locus test, and cytological analysis of chromosomal aberrations.

Using the specific locus test, Searle and Phillips (1971) compared the mutational yields from irradiation of mitotically dividing, premeiotic primordial spermatogonia and their precursors with that of oogonia and their precursors. They irradiated the pregnant female mice with 1.085 Gy of fission neutrons before the twelfth day of gestation and mated their offspring with members of the seven-locus tester stock. Similarly elevated mutation frequencies were recorded for both irradiated males and females. This observation is consistent with the finding of a recent study using ESTR mutation as an end point as a similar elevation was established in the germline of both males and females irradiated *in utero* at the twelfth day of gestation (Barber *et al.*, 2009). Obviously, in both studies (Searle and Phillips, 1971; Barber *et al.*, 2009), male and female germ cells were irradiated at the mitotically active stage before launching to meiosis.

The sensitivity of the meiotically arrested dictyate oocyte to ionising radiation at different stages of maturity has also been addressed using the specific locus test. The period of time elapsed between radiation exposure and conception greatly affects the mutational yield. For example, the data from early studies (Table 1.1) assessing the irradiation of dictyate oocytes revealed that when the interval between exposure of the

female and conception of the litter was less than 7 weeks, high frequencies of specific locus mutations were measured, *i.e.* when dictyate oocytes in maturing follicles were irradiated. However, the yield of mutations decreases dramatically when younger oocytes in immature follicles are irradiated (offspring conceived more than 7 weeks after maternal irradiation) (Russell, 1965a and b). The very low mutation frequencies obtained with irradiation of less mature oocytes (Table 1.1) can be explained by the presence of very efficient repair system, selection against mutated oocytes, or that dictyate oocytes at this stage of development are very resistant to radiation induced genetic damage (Searle, 1974; Russell, 1968).

These findings are supported by the work of Brewen *et al.* (1976). The authors analysed the yield of chromosome aberrations in X-irradiated dictyate oocytes at different stages of maturity and observed that mature oocytes are more radiation sensitive than immature as detected by the extent of chromosomal aberration in each stage. In another study, X-irradiation of NMRI female mice with various doses resulted in structural chromosomal alterations in MII oocytes as well as 2-cell embryo following exposure to 2 Gy. However, almost all chromosomal alterations failed to be detected in surviving embryos after 13.5 days of pregnancy (Reichert *et al.*, 1984).

Table 1.3: Frequencies of specific locus mutations after X-ray acute exposure to arrested oocytes

Irradiation-conception interval	Dose, Gy	Regime	Frequency per locus
Control	-	-	0.49×10^{-5}
< 7 weeks	0.5	Unfractionated	1.03×10^{-5}
	2	Unfractionated	9.04×10^{-5}
	4	Unfractionated	22.14×10^{-5}
	4	Fractionated	21.12×10^{-5}
	4	Fractionated	9.73×10^{-5}
> 7 weeks	0.5	Unfractionated	0

Data taken from Searle (1974).

Tease and Fisher (1996) compared radiation sensitivity of the pre-ovulatory stage oocyte to the dictyate oocytes using both chromosomal aberration and dominant visible mutations. Again, the sensitivity of pre-ovulatory stage was ~6 times more than that of dictyate oocytes as detected by the extent of chromosomal aberration and the number of visible mutations. In contrast to the finding of Reichert *et al.* (1984), chromosomal rearrangements were detected in 5 of 9 individuals carrying the dominant visible mutation. This finding indicates that radiation induced chromosome alterations in female germ cells are not completely eliminated during pre-natal development, instead a proportion has the potential to manifest in the next generation.

The effect of irradiation of pregnant female mice very early post fertilisation at zygote stage has also been addressed. Pils *et al.* (1999) reported a significantly increased incidence of sterility as well as prenatal mortality following exposure of female Heiligenberger mice to 1.0 Gy of X-rays at the zygote stage. In addition, the authors

found slightly increased incidence of congenital malformations in the F_1offspring of the irradiated females. However, Jacquet and colleagues (2010) did not observe any significant increases in the incidence of developmental defects, or the frequency of chromosome aberrations in the F_1 offspring of irradiated females belonging to the radiosensitive strains CF1 and ICR. The authors explain this discrepancy by different radiosensitivity of mouse strains.

Fractionation of radiation dose has a profound effect on the efficiency of mutation induction in late oocytes. The fractionation of a 4 Gy exposure into eight separate exposures (of 0.5 Gy) over a 75 minute period significantly reduced the mutation frequency (Russell, 1968). However, two fractions of 2 Gy separated by 24 hours gave results very similar to that of a single exposure. This discrepancy may be explained by the fact that lower doses of exposure a proportion of radiation-induced DNA damage can properly be repaired (Russell, 1968).The dose rate also affects mutation frequencies after irradiation. Chronic exposure to ionizing radiation results in a very low yields of mutations (Russell, 1972). This may be due to the effects of both dose rate and of oocyte maturation which is sometimes difficult to distinguish with such prolonged exposures. For example, the delivery of 4 Gy exposures at $0.9x10^{-3}$ Gy/min takes over 5 weeks. So litters conceived within 7 weeks following the end of the irradiation period will have received part of the dose at greater than 7 weeks before conception, *i.e.* during the earlier apparently less sensitive phase of oocyte development.

The reviewed work regarding female exposure indicates that mature oocytes are more sensitive to radiation than immature oocytes dependent on the stage of folliculogenesis. By considering that follicles work as functional suncytium, oocyte sensitivity to

radiation may be affected by the way they respond to radiation-induced DNA damage that can be changed according the follicle stage of maturity (Ruiz-Herrera *et al.*, 2012). In addition, there is a growing body of evidence that oocytes maintain their capacity to repair DNA damage during their maturation process (Ashwood-Smith and Edwards, 1996; Pan *et al.*, 2005; Zheng *et al.*, 2005; Menezo *et al.*, 2007; Hamatani *et al.*, 2008). It was also proposed that mature oocytes may repair genetic damage by error prone DNA repair mechanisms which may explain the presence of chromosome alterations in MII oocytes (Reichert *et al.*, 1975; Tease and Fisher, 1996; Griffin *et al.*, 1990; Ruiz-Herrera *et al.*, 2012). On the other hand, immature oocytes cannot efficiently repair radiation-induced genetic damage, which thereby result in cell death and not contribute to the offspring (Ruiz-Herrera *et al.*, 2012).

1.6 Aims and objectives

The existing experimental data regarding the genetic effects of maternal exposure to ionising radiation do not allow a comprehensive assessment of the genetic risk for humans (UNSCEAR, 1993). Given the profound differences in the biology of male and female germ cells, the numerous data on the mutagenic and transgenerational effects of paternal irradiation cannot be used to infer whether the same processes also occur following maternal exposure. This study was therefore specifically designed to evaluate the direct and transgenerational genetic effects of maternal irradiation in mice and compare them with those following paternal exposure.

The specific aims of this project are:

- To analyse the pattern of ESTR mutation induction in the germline of female mice exposed to 1 Gy of acute X-rays.

- To investigate whether maternal irradiation can result in transgenerational changes affecting mutation rates in the germline and somatic tissues of their non-exposed first-generation offspring.

- To analyse and compare the genome-wide pattern of gene expression in the non-exposed first-generation offspring of irradiated male and female mice.

2 MATERIALS AND METHODS

Materials

2.1 Chemicals

Chemical reagents were supplied by Biowittaker Molecular Applications (BMA) (Rockland, USA), Fisher Scientific (Loughborough, UK), Flowgen (Ashby de la Zouch, UK), and Sigma-Aldrich Company (Poole, UK).

2.2 Molecular reagents

Molecular reagents were obtained from ABgene (Epsom, UK), Ambion, Inc. (Warrington, UK), Biowittaker Molecular Applications, BMA (Rockland, USA), Ivitrogen UK (Paisley, UK), New England Nuclear, NEN, Life Sciences (Division of Perkin-Elmer Life Sciences Ltd, Zaventem, Belgium), New England Biolabs, NEB (Hitchin, UK), Promega (Southampton, UK), Qiagen Ltd. (Crawley, UK), Roche Diagnostics, Roche Applied Science (East Sussex, UK), Sigma-Aldrich Company (Poole, UK), Stratagene (Amsterdam, The Netherlands)

2.3 Radiochemical Reagents

The radiochemical reagent α-^{32}P-dCTP was purchased from PerkinElmer (Austria).

2.4 Enzymes

Protease K (used for DNA extraction) was obtained from Sigma-Aldrich (Poole, UK), restriction endonucleases *AluI, MseI* and DNA polymerase large (Klenow) fragment were purchased from New England Biolabs (NEB) (Hitchin, UK) and High Fidelity PCR system were purchased from Roche Diagnostics (Mannheim, Germany).

2.5 Oligonucleotides

DNA oligonucleotide probes where purchased from Sigma-Aldrich (Poole, UK).

2.6 Molecular Weight Markers

The 1kb DNA ladder purchased from Invitrogen UK (Paisley, UK), the 100bp DNA ladder acquired from Promega (Southampton, UK) and λ DNA ladder digested with *Hin*d III was supplied by ABgene (Epsom, UK).

2.7 Kits

High Fidelity PCR system was purchased from Roche Diagnostics (Mannheim, Germany). All these kits were used by study for the expression profile analysis (microarray): SuperScript Double-Stranded cDNA synthesis kit (Invitrogen, Paisley, UK), Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany), NimbleGen One Colour DNA labeling Kit, NimbleGen Hybridization Kit and NimbleGen Wash Buffer Kit (Roche Diagnostics Ltd., Burgess Hill, UK).

2.8 Equipment

All equipment used for the purposes of the laboratory work were purchased from Agilent Technologies (Waldbronn, Germany), Bio-Rad (Hemel Hempstead, UK), Eppendorf (Hamburg, Germany), Fisher Scientific (Loughborough, UK), Genetic Research Instrumentation (Braintree, UK), Heraeus Instruments (Hanau, Germany), NanoDrop Technologies (Ringmer UK), Purite Ltd (Oxon, UK), Qiagen (Crawley, UK), Roche Diagnostics (Mannheim, Germany), Thermo Scientific (Ashford, UK), and Ultra Violet Products Life Sciences (Cambridge, UK).

2.9 Solutions

Table 2.1 summarises all solutions used during study. All buffers were prepared according standard methods (Sambrook and Russell, 2001).

Table 2.1: List of solutions

Solution	**Composition**
Lysis Buffer A (100ml)	5M NaCl 2mls (0.1M) 0.5M EDTA 5mls (0.025M) 1M TrisHCl pH 8 2mls (0.02M) dH_20 91mls
Lysis Buffer B (100ml)	10% SDS 10mls (1%) 0.5M EDTA 2.5mls (0.0125M) 1M TrisHCl pH 8 1ml (0.01M) dH_20 86.5mls
Phenol-Chloroform	Phenol: chloroform: isoamyl alcohol 25:24:1 (v/v) saturated with 10 mM Tris, pH 8.0
Proteinase K	25 mg/ml (dissolved in distilled water)
Ethidium Bromide	10 mg/ml (dissolved in distilled water)
DNA loading dye (stop dye)	5xTAE, 12.48g Ficoll 400, 0.1g bromophenol blue.
5X TAE Buffer	0.2M Tris Acetate, 0.1M Sodium Acetate, 1mM EDTA (adjust pH to 8.3 using glacial acetic acid)
10X TBE	89mM Tris, 89mM Boric Acid, 2 mM EDTA (pH 8.0)
Southern Depurinating Solution	0.25 M HCl
Southern Neutralising Solution	0.5 M Tris-HCl (pH 7.5), 3 M NaCl
Southern Denaturing Solution	0.5 M NaOH, 1 M NaCl

Table 2.1(continued)

Oligo Labelling Buffer (OLB)	**Solution A:** 625µl 2M TrisHCl pH8 25µl 5M MgCl2 350µl distilled water 18µl 2-mercaptoethanol 5µl dATP 5µl dGTP 5µl dTTP **Solution B:** 2M Hepes (free acid) titrated to pH 6.6 with NaOH. **Solution C:** Hexadeoxyribonucleotides or Pd6 random hexamers (Pharmacia) dissolved in 550µl of 1 x TE **OLB is:** 20µl of Soln. A + 50µl of Soln. B + 30µl of solution C Mix solutions, dispense into small aliquots and store at -20°C.
10X TE Buffer	100mM Tris-HCl [pH 8.0], 10mM EDTA
1 M Sodium Phosphate Solution (pH 7.2)	**For 1L:** 121.7 g $Na_2HPO_4.2H_2O$ (MW 177.96) i.e. 0.68 M 49.3 g $NaH_2PO_4.2H_2O$ (MW 156.01) i.e. 0.32 M or 97.1 g Na_2HPO_4 (MW 141.96) i.e. 0.68 M 49.3 g $NaH_2PO_4.2H_2O$ (MW 156.01) i.e. 0.32 M
Church Buffer	0.5 M Na Phosphate, 7% SDS, 1mM EDTA
Phosphate wash solution	0.04M Na Phosphate, 0.5% SDS
20X SSC Solution	3M NaCl, 0.3M Tri-Sodium Citrate, adjust pH to 7.0 with NaOH
High stringency wash (HSW)	0.1X SSC, 0.01 % SDS
Low stringency wash (LSW)	1X SSC, 0.1 % SDS
Oligo Stop Solution (OSS)	20 mM NaCl, 20 mM Tris, 2 mM EDTA, 0.25% SDS, 1µM dCTP
4M Betaine	FW=117.15 g 4M = 468.6 g in 1000ml dH_2O Then 25 ml needed 11.72gm
DEPC water	5µl Diethyl Pyrocarbonate in 100ml sterile water (Autoclaved)
Dilution buffer	5mM Tris HCl pH of 7.5 and 5µg/ml Salmon Sperm DNA

2.10 Mice

BALB/c and CBA/Ca mice, 45-52 days old were obtained from Harlan (Bicester, UK) and housed at the Division of Biomedical Services, University of Leicester. All animal procedures were carried out under the Home Office project licence No. PPL 80/2267.

2.10.1 Control group

This group consisted of the offspring of the non-irradiated parents. The offspring of two reciprocal crosses between BALB/c and CBA/Ca mice were used in this study. Each F_0 male was mated with two females from another strain. F_0 males were subsequently culled and both tails and spleens were taken. After weaning (at 3 to 4 weeks old) most offspring were culled and tissues (tails and spleens) taken. F_0 females were culled also after weaning and tails and spleens were taken. Eight males were taken from each cross, weaned and kept until they reached 8 weeks of age, before being culled and multiple tissues taken (tail, spleen, caudal epididymis, bone marrow, kidney and brain) and kept at -80°C until being analysed.

2.10.2 Maternally irradiated group

One week after arrival BALB/c and CBA/Ca female mice were given 1 Gy of acute X-rays delivered at 0.5Gy min^{-1}, (250 kV constant potential, HLV 1.5 mm Cu, Pantak industrial X-ray machine, Connecticut, USA). Irradiated females were mated to non-irradiated males from another strain within 1 week after exposure. This group consisted of the offspring resulting from two reciprocal crosses between 32 CBA/Ca females and 16 BALB/c males (104 individual), as well as 32 BALB/c females and 16 CBA/Ca males (135 individual). At weaning (3-4 weeks old), most offspring were culled and tails and spleens were taken. F_0 females were also killed at this point and tails and spleens taken. In addition, eight F_1 males from each reciprocal cross were kept until

they were 8 weeks of age. These mice were then culled and multiple tissues taken (tail, spleen, caudal epididymis, bone marrow, kidney and brain) and kept at -80°C until the analysis.

2.10.3 Paternally irradiated group

One week after arrival BALB/c and CBA/Ca male mice were given 1 Gy of acute X-rays delivered at 0.5Gy min^{-1}, (250 kV constant potential, HLV 1.5 mm Cu, Pantak industrial X-ray machine, Connecticut, USA). Irradiated males were mated to non-irradiated females from another strain within 1 week after exposure. This group consisted of the offspring resulting from two reciprocal crosses between 5 CBA/Ca males and 10 BALB/c females (49 mice), as well as 5 BALB/c males and 10 CBA/Ca females (32 mice). At weaning (3-4 weeks old), most offspring were culled and tails and spleens were taken. F_0 females were also killed at this point and tails and spleens taken. In addition, eight F_1 males from each reciprocal cross were kept until they were 8 weeks of age. These mice were then culled and multiple tissues taken (tail, spleen, caudal epididymis, bone marrow, kidney and brain) and kept at -80°C until the analysis.

Methods

2.11 Methods of DNA extraction

2.11.1 DNA extraction for pedigree analysis

2.11.1.1 Proteinase K digestion

One third of the tail from each F_1 mouse and about a tenth of spleen from F_0 (parents) were chopped in a Petri dish. After chopping, the tissues were transferred into 15 ml MaXtract Phase Lock tubes (Qiagen) containing equal 1ml of lysis solution A and 1 ml of lysis solution B (table 2.1). Following this, 30 µl of 25 mg/ml Proteinase K was added, the tubes capped tightly mixed very well and incubated overnight at 55^{o}C in water bath.

2.11.1.2 Phenol chloroform extraction

To clean up the DNA, half of the volume from digestion step (1ml) of phenol/chloroform/isoamyl Alcohol (25:24:1 ratio) was added and the tube capped tightly and inverted several times. Tubes were spun in a swing rotor Eppendorf Centrifuge 5804 at 3,100 rpm for 5 minutes. The supernatant was transferred to a clean tube, to which 1 ml of chloroform was added and spun again at 3,100 rpm for 5 minutes in the swing rotor Eppendorf Centrifuge.

2.11.1.3 Ethanol precipitation

DNA was precipitated in 2-3 volumes of 100% ethanol with one tenth of 3 M sodium acetate (pH5.2) (usually 6 ml ethanol and 200 µl sodium acetate). The DNA pellet was removed using pipette and transferred into a labelled eppendorf tube containing 500 µl

80% (v/v) ethanol and spun for 2 minutes at 13,000 rpm in a 5415- D centrifuge. The supernatant was removed and DNA pellets were then air dried and re-suspended in appropriate volume of ultrapure water (usually 300 – 500µl) and kept at -20^{o}C.

2.11.2 DNA extraction from for Single Molecule PCR analysis

During the process of DNA extraction for single molecule PCR analyses, all solutions and equipment must be single molecule clean (UV- sterilised) and all extraction procedures have been done in a laminar flow hood to minimize the risk of contamination.

2.11.2.1 Proteinase K digestion

DNA extraction from the brain and bone marrow tissues for SM-PCR follows the same procedure which start by spinning number heavy 15ml MaXtract Phase Lock tube (Qiagen) correspond to the number of samples to be extracted for 5 minutes at 3,100 rpm in Eppendorf Centrifuge 5804. After centrifugation, equal volumes of SM-PCR clean lysis buffer A and B were added (1ml lysis buffer A and 1ml lysis buffer B). Tissue samples were placed in a sterile Petri dish and then finely chopped. One ml of SM-clean PBS was added to the brains samples and they were transferred to the Phase lock tube containing lysis buffer. The bone marrow samples were placed in 500 µl PBS, frozen at -80^{o}C and after complete thawing transferred directly into the Phase Lock tube containing lysis buffer. Following this, 30 µl of 25 mg/ml Proteinase K was added, the tubes capped tightly mixed very well and incubated overnight at 55^{o}C in water bath.

2.11.2.2 Phenol chloroform extraction

To clean up the DNA, 1ml of phenol and 1ml chloroform added to the digested tissue from the previous step and tube capped tightly and mixed by inversion. Tubes were spun in a swing out rotor (Eppendorf Centrifuge 5804) at 3,100 rpm for 5 minutes. The supernatant was transferred to a clean tube, to which 1 ml of chloroform was added and spun at 3,100 rpm for 5 minutes in the Eppendorf Centrifuge 5804 for 5 minutes.

2.11.2.3 Ethanol precipitation

DNA was then precipitated in 2-3volumes of 100% ethanol with one tenth of 3 M sodium acetate (pH5.2) (6 ml ethanol and 250 µl sodium acetate pH 5.2). The DNA pellet was removed using pipette and transferred into a labelled eppendorf tube containing 500 µl 80% (v/v) ethanol and spun briefly at 13,000 rpm in a 5415- D centrifuge for 1 minute. After removal of the supernatant, the DNA pellets were then air dried and re-suspended in appropriate volume of 5mM Tris HCl (usually 100 – 200µl) and kept in -20^{o}C.

2.11.3 DNA extraction from sperm for Single Molecule PCR analysis

Sperm DNA prepared from caudal epididymis according to the method described by Yauk *et al.* (2002). This method depends on the fact that sperm is resistant to the processes that lyse the somatic cells (epithelial or white blood cells) which therefore can be lysed and filtered out. The sperm/somatic cell ratio was monitored microscopically during the process until complete lysis of somatic cells. To minimize the risk of contamination, all manipulations were carried out in laminar flow hood.

2.11.3.1 Differential lysis of somatic and sperm cells

This method uses the both caudal epididymi of each individual which were placed in a sterile Petri dish, excess fats were removed and finely chopped using a sterile scalpel blade. After chopping, 1ml of PBS was added to the tissue and mixed. A metallic mesh (S-3770 metallic meshes, Sigma) was made into a cone shape and placed into the top of a screw cap eppendorf and the tissue in PBS transferred into the metallic mesh; pipetted up and down until complete filtration. Following filtration, the mesh discarded and 5µl of filtrate was placed on haemocytometer and examined under microscope in order to visualize the sperm/somatic cell ratio. The tube containing filtrate (PBS-tissue suspension) was then spun for 2 minutes at 13,000 rpm in a 5415-D centrifuge. The supernatant was then removed completely and 1ml of 1x SCC added and mixed thoroughly (vortex) until the tissue become completely suspended. For somatic cell lysis, 10µl of 10% SDS added, tubes capped and mixed by inversion several times and spun at 13,000 rpm in a 5415- D centrifuge for 2 minutes. The supernatant was discarded and 960µl of 0.2xSCC was added and mixed well by vortex until the pellet becomes fully suspended. At this step, 5µl of the suspended pellet was placed on the counting chamber and visualized under microscope (no somatic cells should be present this time).

2.11.3.2 Proteinase K digestion

To the suspension from the previous step, 70µl of β-mercaptoethanol, 100µl of 10% and 20µl of 25 mg/ml proteinase K were added. Following this step, the tubes capped tightly, mixed thoroughly by repeated inversion and incubated for 3 hours at 37^{o}C in water bath.

2.11.3.3 Phenol/Chloroform extraction

After Proteinase K digestion, the contents of each tube were transferred into labelled 15ml MaXtract Phase Lock tube (Qiagen) which previously spun for 5 minutes in a swing rotor Eppendorf Centrifuge 5804 at 3,100 rpm. The process of phenol extraction proceeded according to the 2.11.2.2 step

2.11.3.4 Ethanol precipitation

DNA precipitated according to section 2.11.2.3.

2.12 Total RNA extraction from kidney for microarray analysis

RNA samples were extracted from the kidney samples taken from the offspring of control and irradiated parents using TRI Reagent (Sigma) method. This method is an improvement of work done by (Chomczynski and Sacchi, 1987) for total RNA extraction. The TRI Reagent is a mixture of guanidine thiocyanate and phenol in a monophase solution which dissolves DNA, RNA and protein on homogenization or lysis of tissue sample. After addition of 1-bromo-3-chloropropane and centrifugation, the mixture separated into 3 phases: an aqueous phase containing the RNA, the interphase containing DNA, and an organic phase containing proteins. Each component can then be isolated after the phase separation.

2.12.1 Tissue homogenization

Suitable RNaseZap, DEPC-treated sterilised homogenizer probe was unwrapped, assembled into homogenizer (Multi-Gen 7, PRO Scientific Inc.) and cooled in dry ice for 30-40 seconds prior to use. One frozen kidney (previously kept in -80^{o}C) was then transferred into sterile 15 ml centrifuge tube containing 7ml of TRI Reagent (Sigma),

homogenized for 20 seconds, and left for 5 minutes at room temperature. After incubation, the content of the tube was aliquoted into 8 sterile labelled eppendorf tubes.

2.12.2 Phase separation

Two hundred microliters of 1-bromo-3-chloropropane (Sigma) was added to each tube containing app. 1 ml of tissue homogenate. The mix was vigorously shaken for 25 seconds and left for 2.5 minutes at room temperature. Following incubation, the tubes were spun at 12,000 rpm in Eppendorf 5415 R Centrifuge for 10 minutes at 4^{o}C. The upper aqueous layer was then transferred into a new labelled sterile eppendorf tube.

2.12.3 Isopropanol precipitation and washing

To each tube, 560 µl of isopropanol (1 µl per 1 µl aqueous phase) was added, tubes capped, mixed by repeated inversion, and incubated for 10 minutes at room temperature. After incubation, tubes were spun at 12,000 rpm in Eppendorf 5415-R Centrifuge for 10 minutes at 4^{o}C, the supernatant discarded, and the pellet kept on ice until next step. The RNA pellet was then washed twice by addition of 1ml of 75 % ethanol (v/v; prepared with DEPC-treated water) followed by repeated inversion until the pellet was completely taken away from the bottom of the tube and centrifugation for 5 minutes on 12,000 rpm in Eppendorf 5415 R Centrifuge at 4^{o}C. After complete removal of the alcohol, RNA air dried for 4 minutes and then re-suspended in 80-150 µl depending on the amount of pellet. After became completely dissolved, RNA was temporarily pooled into a single solution for purposes of concentration measurement. After estimation of RNA concentration (see 2.23.1), RNA was stored at -80^{o}C.

2.13 ESTR mutation detection

2.13.1 Estimation of DNA concentration

DNA samples were mixed briefly and spun. The NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) was blanked with a proper blank (distilled water in case of pedigree analysis and 5mM Tris buffer in case of SM-PCR) and the DNA concentration of 1.5 µl of sample was then measured and the results have been recorded.

2.13.2 Digestion of genomic DNA for pedigree analysis

For digestion of genomic DNA, 20µl reaction containing 10µg of each DNA sample, 2 µl NE Buffer 2, and 2 µl *Alu* I enzyme (NEB10U/µl) was incubated at 37^{o}C overnight. The reaction was stopped by adding 5 µl of stop loading dye (Table 2.1).

2.13.3 Digestion of genomic DNA for single molecule PCR analysis

Prior SM-PCR amplification, genomic DNA was digested with *MseI* restriction enzyme. This restriction enzyme does not cleave the sites outside the *Ms6-hm* locus complementary to the PCR primers (Barber *et al.*, 2009). DNA was digested in 20µl reaction mix containing approximately 6µg of genomic DNA, 1x NEB buffer 2, 20 ng of DNase free-BSA, and 25 U of *MseI* enzyme mixed at 37 °C for 2 hours. DNA was precipitated in 2-3 volumes of 100 % ethanol and 10 % of 3 M NaAc (pH 5.2) at -80 °C overnight (for at least 2 hours). The tubes were centrifuged for 20 minutes at 12,000 rpm in a 5415- D centrifuge, following another centrifugation for 20 minutes. The supernatant was discarded and the pellet washed in 80% ethanol, dried, and re-suspended in 50 µl of 5 mM Tris HCl. Finally, DNA concentration was measured and diluted into concentration of 10ng/µl with 5mM Tris HCl and kept at -20°C.

2.13.4 PCR amplification of the BALB/c derived *Hm-2* allele for pedigree analysis

During analysis of germline mutation induction in pedigrees, it was clear that the *Hm-2* allele derived from BALB/c mice could not be detected by southern blotting analysis of *AluI* digested DNA samples. This was attributed to the very small size of the BALB/c derived allele (~1.6 kb). ESTR mutation detection at the BALB/c derived allele was therefore carried out using PCR. PCR was performed using High Fidelity PCR system Kit (Roche Diagnostics, Germany) in the presence of a PCR enhancing agent; betaine which enhances PCR specificity by reducing the secondary structure in GC-rich regions facilitating strand separation (Henke *et al.*, 1997). Genomic DNA was amplified in 10 µl containing 1x buffer (with $MgCl_2$, 1.5 mM), 0.2 mM dNTPs, 0.4 µM primers (Hm-2 C and Hm-2 D, Table 2.2), 1 M betaine, 1 Unit polymerase enzyme (Expand High Fidelity Enzyme mix) and 250 pg of template DNA. PCR was performed in thin-walled 0.2 PCR tubes (ABgene). The PCR conditions were: 5 min at 96°C for denaturing followed by 21 cycles of 96°C for 30 seconds, 58°C for 30 seconds and 68°C for 6 minutes ending with final extension for 10 minutes at 68°C. PCR reactions were carried out on a PCT-225 DNA Engine Tetrad™2 Thermal Cycler (MJ Research, USA).

Table 2.2: Primer sequence

Primer	Primer sequence
Hm2C	GATGACTGTCAGAGCAGGGA
Hm2D	CCCTCTGCTTTGTGCTTGTG

Table 2.3: PCR Protocol for *Hm-2* amplification

Component	**1x reaction**
10X Buffer	1µl
2mM dNTPs	1µl
10 µM primer C	0.4 µl
10 µM primer D	0.4 µl
4M Betaine	2.5 µl
High Fidelity Enzyme mix (3.5 U/ µl)	0.3 µl
DNA (250 Pg/ µl)	1 µl
H_2O	3.4 µl
Total	10 µl

2.13.5 Single molecule optimization for *Ms6-hm* locus

Using dilution buffer (Table 2.1), the 10ng/µl stock DNA was diluted into the following concentrations: 100pg/µl, 50 pg/µl, 20 pg/µl, 10pg/µl, 5pg/µl and 2pg/µl. A master-mix, enough for the optimization of two different DNA samples, was prepared using the High Fidelity PCR system with the components listed in the table below.

Table 2.4: components of the master mix for SM-PCR optimisation

Component	**1 x reaction**	**Final concentration**
10 x PCR buffer (High Fidelity PCR kit, Roche)	1	1x PCR buffer
2mM dNTPs (High Fidelity PCR kit, Roche)	1	200µM
10mM Forward primer (Sigma)	0.4	0.4 mM
10mM Reverse primer (Sigma)	0.4	0.4 mM
4M Betaine (table 2.1)	2.5	1M
Water	3.4	---
3.5 U/µl Enzyme mix (High Fidelity PCR kit, Roche)	0.3	1.054 mM
DNA (at required concentration)	1	Variable

Table 2.5: Primer sequence for *Ms6-hm* SM-PCR amplification

Primer name	Primer sequence
Hm1.1 F	5′ - AGA GTT TCT AGT TGC TGT GA- 3′
Hm1.1 R	5′ - GAG AGT CAG TTC TAA GGC AT- 3′

Eight PCR reactions containing 1 µl of serially diluted DNA samples were amplified in 0.2 ml tube strips of 8 on a PTC-225 DNA Engine Tetrad (MJ Research, Waltham, MA, USA). The PCR conditions were the following: 3 minutes at 96°C, followed by 29 cycles of 96°C for 20 seconds, 58°C for 30 seconds and 68°C for 3 minutes. The program terminated with additional incubation of 10 minutes at 68°C.

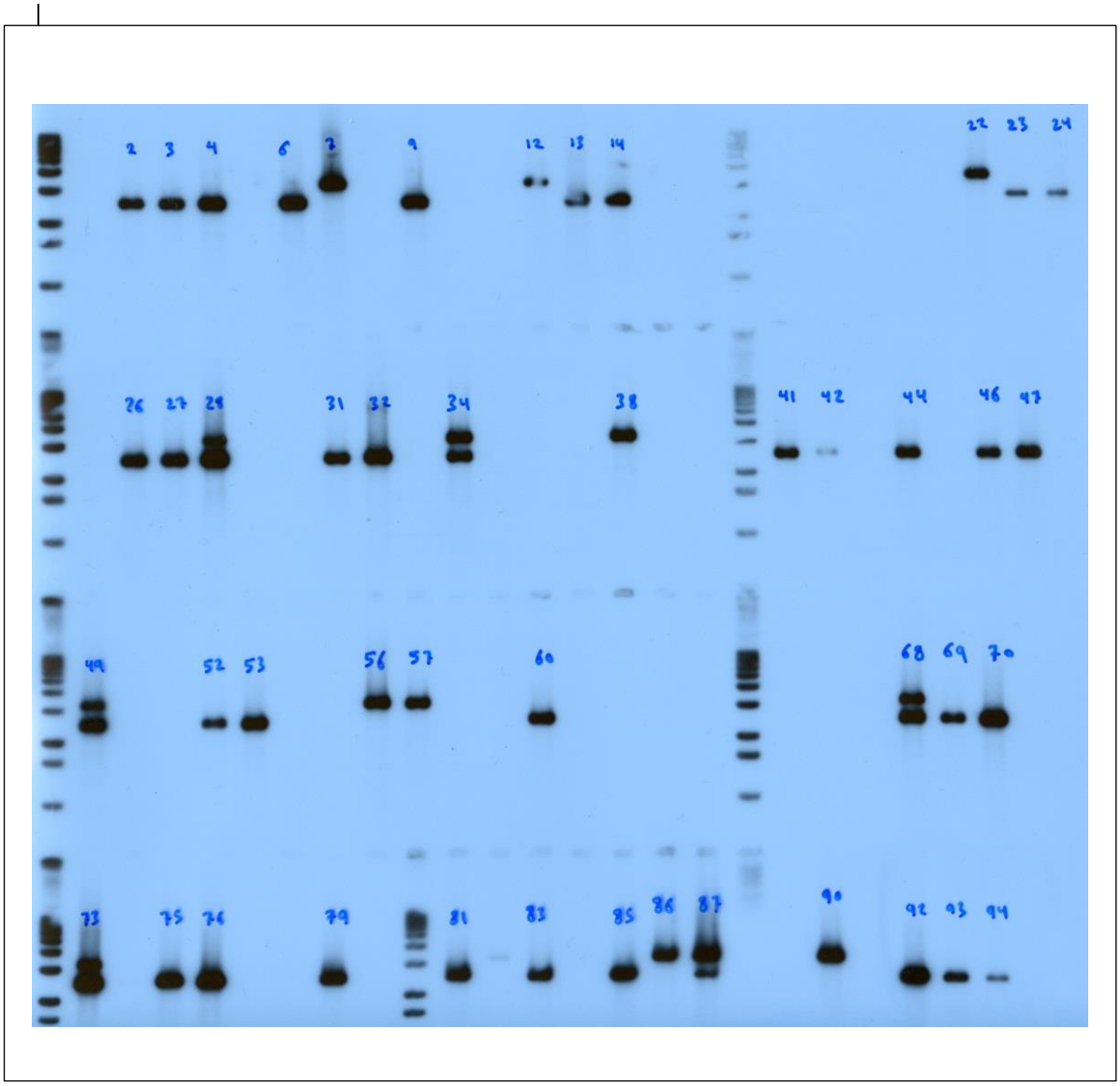
2.13.5.1 Estimating single molecule concentration

PCR products were resolved on agarose gel and detected by Southern blotting (see 2.15) and the autoradiographs examined to establish the concentration of DNA concentration at which approximately 50% of PCR reactions were negative.

2.13.6 SM-PCR for Poisson analysis

The single molecule concentration established as described in section 2.13.5.1 was used for another set of PCR reaction. This PCR reaction was performed on 1µl of DNA samples at single molecule concentration in 96-well plates following the same PCR protocol discussed previously in section 2.13.5. PCR product was resolved on agarose gel and detected using southern blotting and hybridization. On average 100-140 positive reactions from each DNA sample were used for ESTR mutation detection (Figure 2.1).

Figure 2.1: An autoradiograph representing the Poisson analysis.



Autoradiograph showing Southern blot containing 96 SM-PCR products prepared using the estimated single molecule concentration and hybridised with the Ms6-hm probe. The SM-PCR products were detected in 46 reactions (positives), and 50 reactions yielded no products (negatives). Hand-written numbers indicate positive reactions.

2.14 Agarose gel electrophoresis

2.14.1 Electrophoresis of *AluI*-digested genomic DNA (pedigree analysis)

Forty centimetre long 0.8 % (w/v) agarose gels were prepared by mixing 4.8g of LE agarose (SeaKem^{TM}, BMA) and 600ml of 1xTBE buffer containing 0.5µg/ml ethidium bromide. Two stepladders were loaded next to DNA samples; 2 µg of λ Hind III (ABgene, UK) as a visible ladder for monitoring the process of electrophoresis and 200 ng of 1 Kb ladder (Invitrogen, UK) for band size estimation in the autoradiographs. The first lane (far right one) loaded with both ladders (10 µl of 200 ng/µl λ stock + 2 µl of 1 Kb 100 ng/µl stock + 2 µl loading dye and 6 µl of dH_2O). One lane near the middle of the gel and the last lane (the far left one) loaded with the 1 Kb ladder only (2 µl of 1 Kb 100 ng/µl stock + 2 µl loading dye and 16 µl of dH_2O). Eight microgram of *AluI* digested DNA samples (20µl) were loaded on the gel and electrophoresed for approximately 48 hours at 120 V using Bio-Rad or Fisher Scientific power packs until the 1.6kb band had reached the gel's end. Electrophoresis tanks were made in-house (Bio/Medical Joint Workshop, University of Leicester).

2.14.2 Electrophoresis of BALB/c-derived *Hm-2* PCR product (Pedigree analysis)

1% (w/v) agarose gels were prepared by mixing 6g of LE agarose (SeaKem^{TM}, BMA) and 600ml of 1xTBE buffer containing 0.5µg/ml ethidium bromide following the same procedure described in section 2.14.1. Prior to loading and to make it visible and easy to load, 5µl of loading dye (5xTAE, 12.48g Ficoll 400, 0.1g Bromophenol blue) was added to each PCR reaction. Each well of the 40 cm long gel was then loaded with 5µl of each PCR reaction and electrophoresed alongside with two step ladders; 100bp DNA

ladder (200ng, Promega) for band size estimation in the autoradiographs and the 1kb ladder (2µg, Invitrogen) as a visible ladder for tracing the electrophoresis process. The electrophoresis process was continued for 24 hours at 150 volt until the 2027bp band had migrated up to five centimeters to the gel's end.

2.14.3 Agarose gel electrophoresis for single molecule optimisation and Poisson analysis

1% (w/v), short agarose gels were prepared by mixing 2g LE-agarose (SeaKem^{TM}, BMA) and 200ml of 0.5xTBE buffer containing no ethidium bromide. The hot agarose was then poured into 25 x 24cm gel trays with 4 rows of 26 teeth gel combs. The prepared gels were then moved to the running tanks containing 0.5x TBE as running buffer. To facilitate process of loading, 5µl of loading dye (5xTAE, 12.48g Ficoll 400, and 0.1g Bromophenol blue) was added to each PCR reaction prior to loading and 5µl of this mix was then loaded. PCR products were electrophoresed at 180 volt for 2 hours until the Bromophenol blue had migrated to the next row of wells.

2.14.4 Long gel electrophoresis of SM-PCR product for mutation scoring

Long gels were prepared as 1% (w/v) LE-agarose (SeaKem^{TM}, BMA) in 1xTBE buffer containing 0.5µg/ml ethidium bromide cast on 40 x 20cm gel trays. Five microliters of PCR reaction was loaded on the gel and electrophoresed at 180 volt for approximately 28 hours until the 2027bp band of the 1kb ladder had reached the end of the gel.

2.15 Southern blotting

Blotting of DNA to the nylon membrane for mutation detection was based on the work of (Southern, 1975).

After the process of electrophoresis has completed, the gel was placed in a tray and depurinated in 0.25 M HCl for 2x10 min. This depurination causes cleavage of the glycosidic linkage between purine bases (A or G) which finally lead to breakage of the polynucleotide chain converting DNA molecules into smaller DNA fragments within the gel which transfer more quickly. Following depurination, the gel was alkali-denatured for 2x20 min in denaturing solution (0.5 M NaOH, 1M NaCl) that converts the double-stranded DNA molecules to single-stranded by breakage of their hydrogen bonds. Denaturation facilitates DNA molecules transfer and their subsequent binding to nylon membranes as well as making nucleotide sequences of interest available for probe hybridization (Brown, 2001). The gel was then neutralized in 0.5 M Tris-HC1/3 M NaCl, pH 7.5 solution for 2 x 10 min. After neutralization, the gel was transferred into the blotting apparatus and MAGNA nylon membrane soaked in 2xSSC was placed on top of the gel, followed by 3 pieces of Whatman 3MM blotting paper and a stack of paper towels, covered with a glass plate and weights. DNA was transferred to nylon membrane by the capillary transfer method using 20xSSC as the transfer buffer (Southern, 1975) for at least for 5 hours. The membrane was rinsed in 2xSSC, placed in a 3MM folded paper and dried for fifteen minutes in an 80˚C oven; DNA was cross linked to the membrane by exposure to 7x104 J/cm2 of UV light in the RPN 2500 ultraviolet cross-linker (Amersham Biosciences). The procedure for blotting gels containing PCR products slightly differed. The he gels were depurinated for 2x5 minutes, denatured for 2x10 minutes and 1x15mins and neutralized for 2x5 minutes and 1x20 minutes.

2.16 Preparation of synthetic repeat probes

2.16.1 Probe synthesis

All synthetic repeat probes for detection of ESTR loci *Ms6-hm* and *Hm-2* were synthesized by PCR amplification of their synthetic primers (Table 2.7) using 11.1x PCR buffer (Jeffreys *el al*, 1990).

The Ms6-hm probe was synthesised in 20µl reaction containing 2µl 11.1x PCR buffer (Table 2.7), 1 µM of each primer (act also as a template) and 2.5 Units *Taq* polymerase. The Hm-2 probe synthesis was carried out in 7 µl reactions containing 0.63 µl 11.1x PCR buffer, 0.4 µM of each primer (act also as a template) and 0.07 units *Taq* polymerase. The PCR amplification was carried out in PTC-225 DNA Engine Tetrad (MJ Research, Waltham, MA, USA) with these conditions: 20 cycles of at 96°C for 20 sec and 70°C for 20 sec, followed by 40 cycle of at 96°C for 30 sec plus 70°C for 1 min 30 sec. Synthesized repeat probes were purified using a QIAquick PCR Purification kit (Qiagen) according to the manufacturers' instructions.

Table 2.6: Components of 11.1x PCR buffer

Component	**Concentration of stock solutions**	**Final concentration in PCR reaction**
Tris-HCL (pH 8.8)	1 M	45 mM
Ammonium Sulphate	1 M	11 mM
MgCl2	1 M	4.5 mM
2-mercaptoethanol	100%	0.045%
EDTA (pH 8.0)	10 mM	4.4μM
dATP	100 mM	1 mM
dCTP	100 mM	1 mM
dGTP	100 mM	1 mM
dTTP	100 mM	1 mM
BSA	50 mg/ml	13μg/ml

Table 2.7: Primers for probe synthesis

Sequence similarities
Ms6-hm G|GGCA|
Hm2 |GGCA|

Loci	Primer	Primer sequence
Ms6-hm	HMA	GGGCAGGGCAGGGCAGGGCA
	HMB	CCGTCCCGTCCCGTCCCGTC
Hm2	HM2FOR	GGCAGGCAGGCAGGCAGGCA
	HM2REV	GTCCGTCCGTCCGTCCGTC

2.17 Probe labelling and recovery

The Ms6-hm and Hm-2 probes were labelled according to the random primed labelling reaction described by (Feinberg and Vogelstein, 1984). This method is based on incorporation of α-^{32}P-dCTP into newly synthesized DNA using randomly generated hexamers by the *E. coli* DNA polymerase Klenow fragment. The labelling was performed in 40μl reaction containing 10ng of probe (boiled for 6 minutes and chilled in ice for denaturation), 12 μg of DNase-free BSA, 6μl of OLB, 5 U of Klenow enzyme (5,000U/ml, NEB) and 1.5 of ^{32}P α–dCTP (1000Ci/mmol). The labelling mix was

incubated overnight at room temperature. After incubation, the probe was then recovered by ethanol precipitation using 100µg salmon sperm DNA (sigma-Aldrich) as a carrier, followed by washing with 80% (w/v) ethanol. The probes were then re-suspended in 400 µl of ultrapure water, and denatured for 6 minutes at 98ºC in hot plate prior to hybridization.

2.18 Hybridization

Membranes were pre-hybridized for 30 minutes by soaking in a Church buffer (modified from Church and Gilbert, 1984; Table 2.1) in a tube that is constantly rotated in 65°C hybridization oven (ThermoHybaid, ThermoScientific, UK). The pre-hybridization solution was then replaced with ~15ml of Church buffer (65°C), contained the previously denatured appropriate radioactive labelled probe. The blots were hybridized overnight in hybridization oven at 65°C.

2.19 Post-hybridization washing

Hybridization solution was poured off and the blots washed once in 30ml of Phosphate Wash Solution (40mM $NaHPO_4$ and 0.5% SDS) at 65°C. The phosphate wash followed by 2-5 times in high stringency wash solution (0.1% (w/v) SSC, 0.01% (w/v) SDS) each for 10 minutes at 65°C until the counts/sec of the wash solutions where about 5-10.

2.20 Autoradiography

The membranes were wrapped in Saran Wrap and placed in an appropriate size autoradiographic cassette containing an intensifying screen. An appropriate size piece of Fuji Rx100 X-ray film (Fiji Film, USA) was placed directly onto the membrane. The

cassette was sealed and put into the -80˚C freezer where it was left for a period of 24 hours to one week depending on the strength of signal.

2.21 Stripping and re-hybridization

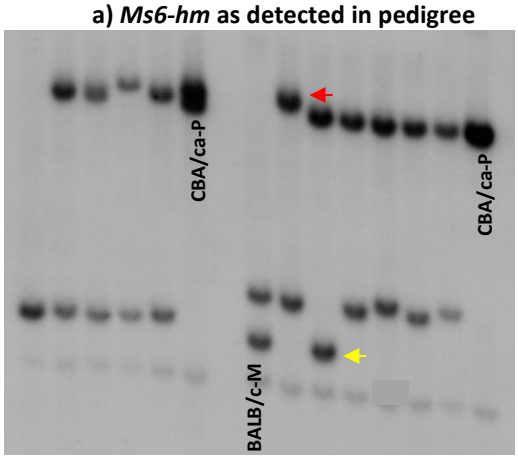
To detect DNA bands derived from another ESTR locus, nylon membrane were stripped by placing in a tray contains boiling 0.1% SDS (w/v). The tray was shacked for 1 min and repeated until the total radioactive count was below 5counts/sec. The stripped membranes were re-hybridized as described above.

2.22 Mutation scoring

In this study ESTR mutations were detected either at the *Ms6-hm* and *Hm-2* loci (pedigree analysis) or at the *Ms6-hm* locus alone (SM-PCR analysis). For germline mutation detection in pedigrees, the autoradiographs were analysed over the well-resolved region between 2.5 and 22kb and between 2-5 kb in case of SM-PCR. Similar to previous studies (Dubrova, 2005; Barber *et al*., 2009), DNA bands that show a shift of at least 1 mm relative to the progenitor allele were scored as mutants (Fig 2-4). During scoring, the autoradiographs were coded and scored blindly to prevent any bias. Moreover, each autoradiograph was scored by one individual and checked by a second individual.

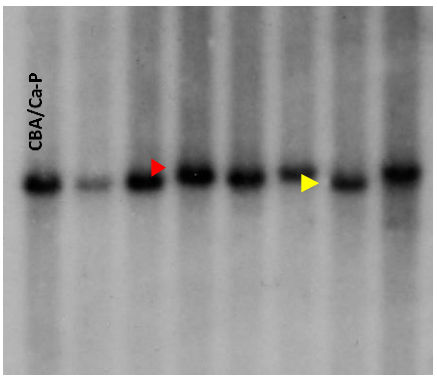
Figure 2.3: Examples of ESTR mutations

a) *Ms6-hm* as detected in pedigree



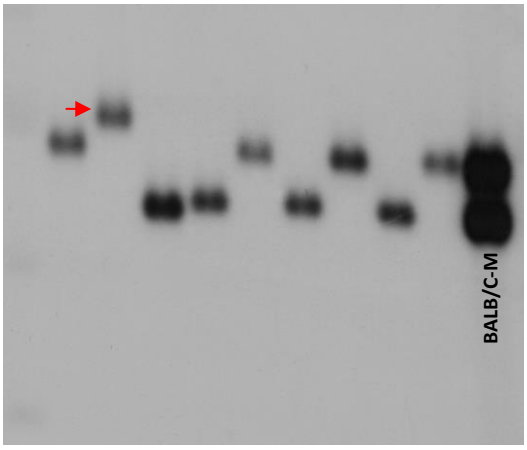
Paternal allele is represented by P (CBA strain) and maternal allele is represented by M (BALB/c). Red arrows refer to gain repeat-gain mutation and yellow arrows refer to repeat-loss mutation.

b) *Hm-2* (CBA-derived allele) as detected in pedigree



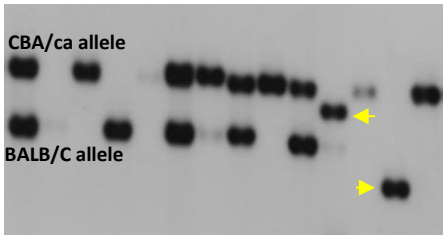
P represents the paternal allele derived from CBA/Ca strain. Red arrows refer to repeat-gain mutation and yellow arrows refer to repeat-loss mutation.

c) PCR amplified *Hm2* alleles (BALB/c allele)

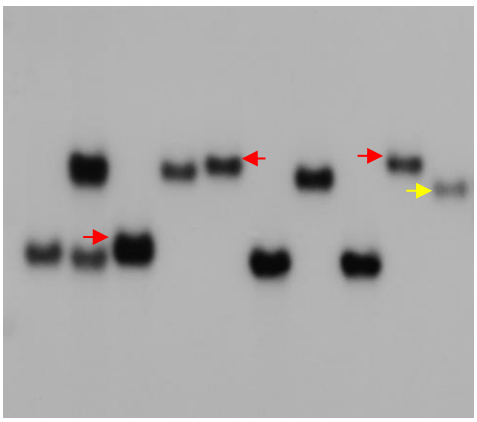


BALB/C-M refers to the ESTR allele derived from BALB/C mother. Red arrow refer to gain of some repeats

d) Examples of *Ms6-hm* mutations as detected by SM-PCR



Yellow arrows refer to repeat-loss mutation.



Red arrows refer to gain repeat-gain mutation and yellow arrows refer to repeat-loss mutation.

2.23 Methods for RNA analysis (microarray analysis)

2.23.1 Estimation of RNA concentration

After extraction, RNA samples were immediately frozen and kept at -80°C except for 5µl of each sample which used for concentration measurement purpose. Concentration of RNA samples was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The concentration of RNA samples used in this work ranged from 1000 to 2100 ng/µl, with A_{260}/A_{280} ratio ≥ 1.8 and A_{260}/A_{230} ratio ≥ 1.8.

2.23.2 Checking RNA quality

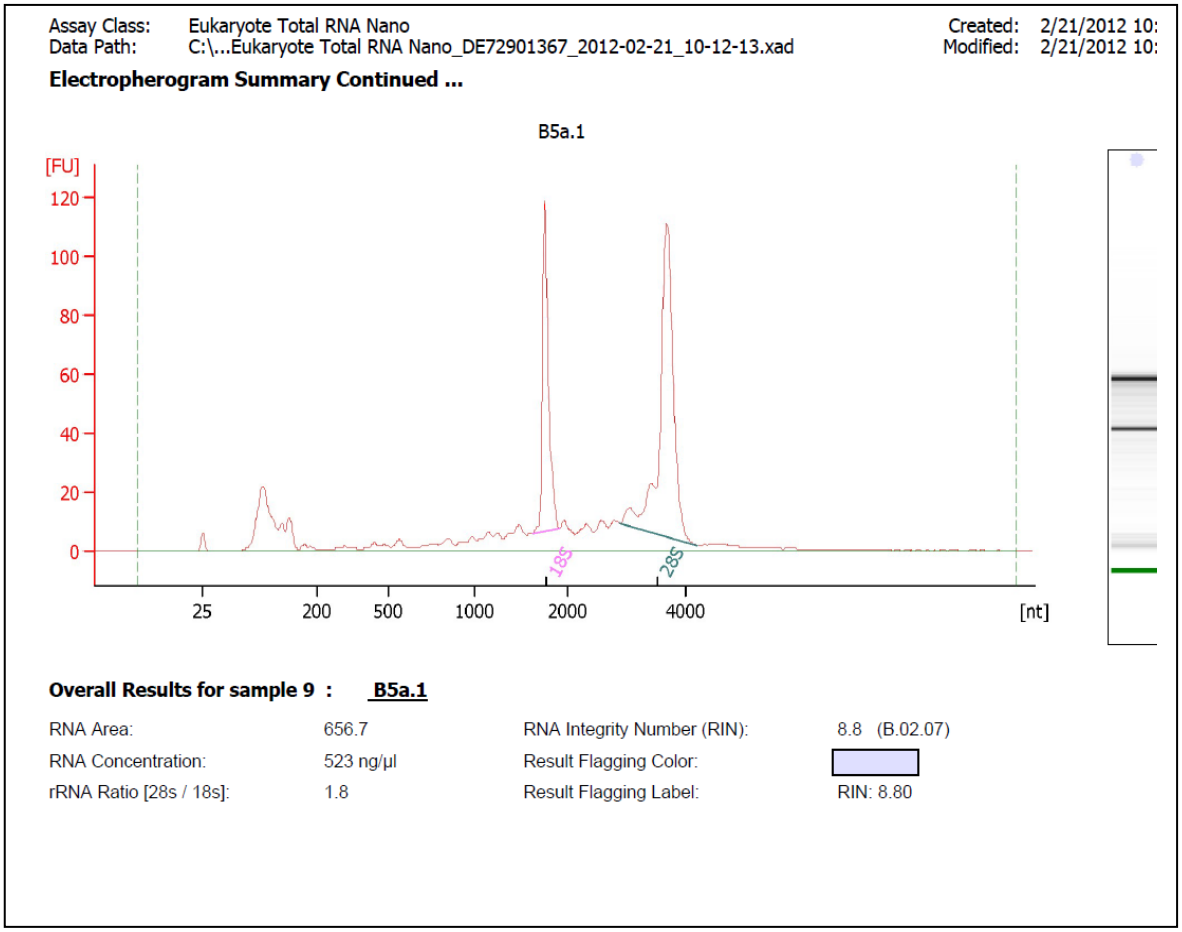
RNA quality check was established on the Agilent 2100 Bioanalyzer using Agilent RNA 6000 Nano Kit (Agilent, Waldbronn, Germany). The principle of RNA quality evaluation is based on the fact that these kits contain chips equipped with a system of interconnected set of microchannels that is used for a size-dependant nucleic acid fragment separation via electrophoresis.

550 µl of RNA 6000 of Nano gel matrix reagent was pipetted into a spin filter, centrifuged at 1500 g for 10 minutes at room temperature. The filtered gel was then aliquoted into 56 µl aliquots in 0.5 ml RNase-free microfuge tubes which are stable for up to 4 weeks. The RNA 6000 Nano dye mix equilibrated at room temperature for 30 minutes was vortexed for 10 seconds, spun briefly, collected and 1 µl of it was then added to 65 µl aliquot of filtered gel from the previous step. After addition of the dye, the solution vortexed well and the tube has spun at 13000 g for 10 minutes at room temperature. This prepared gel-dye mix stable for only one day.

Nine microliters of the gel-dye mix has added to a RNA 6000 Nano chip. To all 12 sample wells, including the ladder well, 5 µl of RNA 6000 Nano marker was added. One microliter of RNA ladder was added to the ladder well and 12 RNA samples containing 250 ng of total RNA were loaded on the chip. The chip was placed horizontally in the adapter of the IKA vortex and vortexed for 1 minute at 2400 rpm and run in the Agilent 2100 bioanalyzer within 5 minutes.

Figure (2.4) presents an electropherogram showing the RNA peaks of successful sample run (total RNA). The Agilent Bioanalyzer software was used to establish the integrity of RNA samples. The RIN index introduced by Agilent technologies was estimated to assess the RNA quality. The RIN software classifies the eukaryotic total RNA by using numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact (Schroeder *et al*., 2006). The RIN index for the sample shown on Figure 2.4 is 8.8 and the minimum RIN number used for the microarray analysis in this study was 8.0.

Figure 2.3: Electropherogram of an RNA sample



Electropherogram showing the RNA peaks of successful sample run (total RNA). The RIN software classifies RNA sample by using numbering system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact. The RIN number for the sample shown is 8.8

2.23.3 cDNA synthesis

cDNA synthesis was performed using SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen, UK).

2.23.3.1 First strand synthesis

First strand synthesis was carried out in 0.2 ml tube (RNase free) containing 10µg of total RNA, 1µl of oligo dT primer (100 pmol/µl) in total volume of 11µl. The samples were heated to 70ºC for 10 minutes in a thermocycler, spun briefly and placed on ice for 5 minutes. After incubation, 4µl of 5x first strand buffer, 2 µl of 0.1M dTT, 1µl of 10mM dNTPs mix and 2µl of SuperScript II RT were added. The tubes were then mixed gently and incubated for 60 minutes at 42ºC. After incubation, the samples spun briefly and kept on ice until the second strand synthesis.

2.23.3.2 Second strand synthesis

To the first strand reaction all of the following have been added in the indicated order:

Table 2.8: components of the second strand synthesis reaction

Component	**Volume**
First strand synthesis reaction	20
DEPC Water	91
5X Second Strand Buffer	30
10 mM dNTP Mix	3
10 U/µl DNA Ligase	1
10 U/µl DNA Polymerase I	4
2 U/µl RNase H	1
Total	150

The tubes were gently mixed, spun briefly and incubated for 2 hours at 16ºC. After incubation, 2 µl of 5U/µl T4 DNA polymerase was added and tubes were incubated at 16°C for 5 minutes. The tubes were then placed in a PCR chiller rack and 10 µl of 0.M EDTA was added, mixed stored at -20°C.

2.23.3.3 RNase A clean up

To the tubes from second strand synthesis reaction, 1 µl of 4 mg/ml RNase A solution was added, mixed, briefly spun and incubated at 37°C for 30 minutes. After incubation, the contents of the sample tubes transferred into labelled Phase Lock tubes (centrifuged for 2 minutes at 12,000 g) containing 170µl of phenol : chloroform, mixed and spun at 12,000 g for 5 minutes. The aqueous phase was then collected into clean labelled 1.5ml tubes.

2.23.3.4 cDNA precipitation and quantification

cDNA samples were precipitated by adding 16 µl 7.5 M ammonium acetate, 7 µl of 5 mg/ml glycogen and 326 µl of ice-cold absolute ethanol. The tubes were then mixed by repeated inversion and centrifuged at 12,000 g for 20 minutes. The supernatant was discarded and the pellet washed twice with 80 % (v/v) ethanol, dried and rehydrated with 20 µl of nuclease free water. cDNA concentration was measured on the NanoDrop 2000 spectrophotometer. The samples with the concentration of >100ng/ µl and the A_{260}/A_{280} and A_{260}/A_{230} ratios >1.8 were used in this work.

2.23.4 Labelling of cDNA

NimbleGen One-Colour DNA Labeling Kit (Roche, Germany) was used for cDNA labeling.

2.23.4.1 Preparation of Cy3 Random Nanomers

For dilution of the Cy3-Random Nonamers, 1.75 µl of β-mercaptoethanol (Sigma) was added to 998.25 µl of Random Primer Buffer. The Cy3-Random Nonamers was diluted in 924 µl of Random Primer Buffer with β-mercaptoethanol and aliquoted as 40µl

individual reaction volumes in 0.2 ml thin-walled PCR tubes and stored at -20°C, protected from light.

2.23.4.2 Denaturation

One microgram of each cDNA sample and 40µl of diluted Cy3-Random Nonamers was pipetted in 0.2ml thin-walled PCR tube and the volume completed to 80µl with nuclease free water. The tubes were then mixed well, heat denatured in 98°C for 10 minutes and chilled quickly in an ice water-bath for 2 minutes.

2.23.4.3 Synthesis

For each sample prepared from denaturation step, 10 µl of 10mM dNTPs Mix, 8 µl of Nuclease-free water and 2µl of Klenow Fragment (3'→5' exo-) 50U/µl were added. The tubes were then mixed by pipetting, spun briefly and incubated for 2 hours at 37°C in a thermocycler protected from light. After incubation, the reaction has stopped by addition of 21.5µl of stop solution (0.5M EDTA; 5M NaCl), and tubes were vortexed and spun briefly.

2.23.4.4 Precipitation

The content of the tubes described in the previous section was transferred into 1.5ml tubes (RNase free) containing 110 µl of isopropanol, vortexed and incubated at room temperature for 10 minutes protected from light. The tubes were spun for 10 minutes at 12,000 g in 4°C, the supernatant was discarded, and the pellet rinsed with 500µl 80% (v/v) ice cold ethanol. After addition of ethanol, the pellet was dislodged by pipetting few times, spun for 2 minutes at 12,000g, the supernatant pipetted and the pellet has dried in vacuum concentrator for 5 minutes on low heat protected from light. The pellet

was then rehydrated with 25µl nuclease-free water and the concentration was measured on the NanoDrop 2000.

2.23.5 Hybridization and washing

The NimbleGen Hybridization System 4 (Roche) was used in this project according to the manufacturer protocol.

2.23.5.1 Preparation of sample

The Cy-labelled cDNA dried pellet (4µg) was re-suspended in 3.3µl of Sample Tracking Control (STC) solution, which contains Cy3-labelled cDNA samples that hybridizes to a known location on the array. The tubes were vortexed and spun to collect the pellet. Using the components of the NimbleGen Hybridization kit, the following master mix was prepared to hybridize single 12x135K slide.

Table 2.9: Microarray hybridisation master mix

Component	**Volume**
2X Hybridization Buffer	88.5 µl
Hybridization Component A	35.4 µl
Alignment Oligo	3.6 µl
Total	127.5 µl

8.7µl of master mix was added to each sample, which were vortexed and centrifuged. The pellet was denatured at 95°C for 5 min and then incubated at 45°C for 5 minutes.

2.23.5.2 Hybridization

Twelve samples were loaded on the NimbleGen 12x135K Mouse Expression Arrays (MM8 genome build, Roche). The slides were placed into the NimbleGen Hybridization System 4 and were hybridized at 42°C for 19 hours.

2.23.5.3 Washing of hybridized arrays

NimbleGen Wash Buffer Kit and NimbleGen Array Processing Accessories (Roche) were used for washing. The solutions used for washing are listed in Table (2.10). The mixer-slide assembly was removed from the Hybridization System 4 and loaded into the Mixer Disassembly tool immersed in the shallow dish containing pre-wormed Wash I (42°C). The mixer was then peeled off carefully while keeping the mixer-slide assembly submerged and with complete avoidance of horizontal movements and scraping with the mixer across the slide which was gently agitated for 15 seconds. Keeping the bar code in the top, the slide was transferred into a slide rack in the tank containing Wash I and agitated vigorously but constantly for additional 2 minutes. The slide rack was then transferred to wash II tank and washed for 1 minute with constant vigorous agitation followed by 15 seconds washing in the wash III tank. After washing the slide was dried by centrifugation for 2 minutes at low speed and scanned.

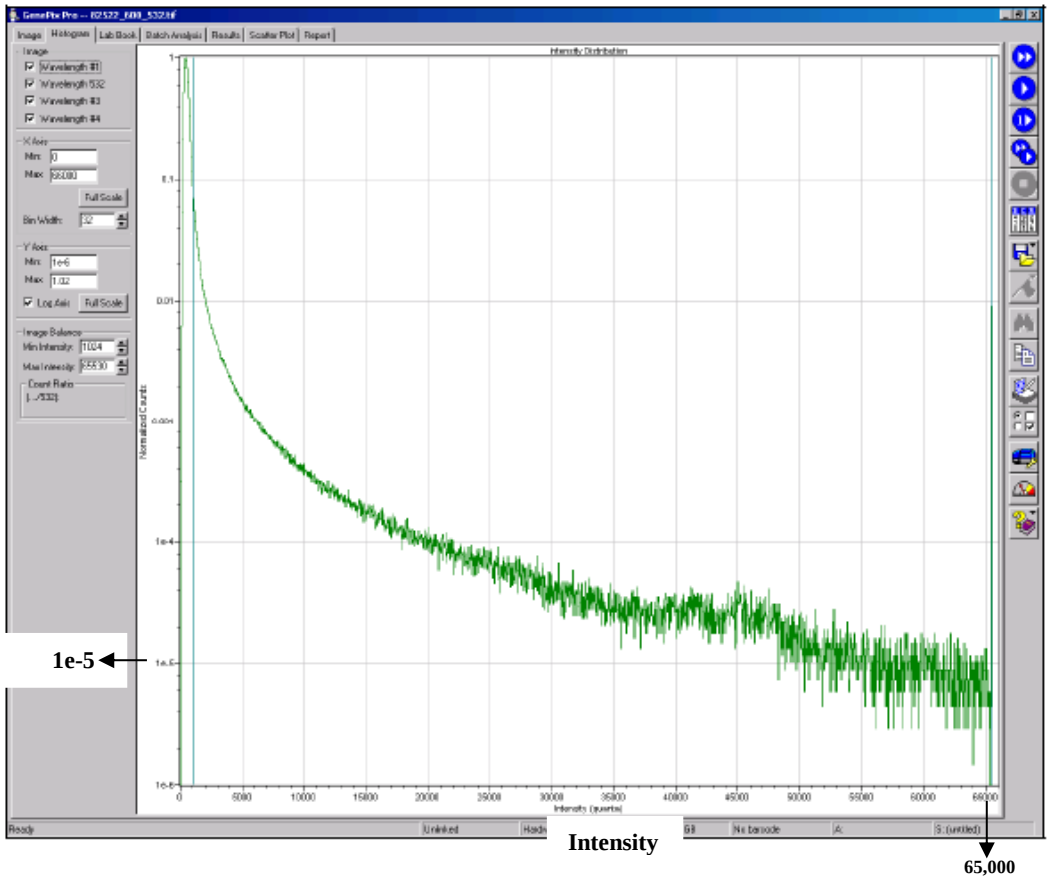
Table 2.10: preparation of microarray washing solutions

Component	**Wash I user-supplied dish**	**Washes I, II, and III tanks**
Ultra-pure water	243 ml	243 ml
10X Wash Buffer I, II, or III	27 ml Wash Buffer I	27 ml from each buffer to the corresponding tank
1M DTT (reconstituted by adding 1.2 ml of water)	27 µl	27 µl
Total	270 ml	270 ml

2.23.6 Array scanning

The slides were scanned on the Axon 4200 AL scanner (Axon Instruments) according to the manufacturer protocol (USA). The images were scanned and processed using GenePix Pro 6.0 program (Molecular Devices). The quality of the scans was validated by generating of the intensity curve with the baseline below 10-5 (Figure 2.4)

Figure 2.4: Example of histogram



The histogram displays the normalized count of pixels of all intensities and the optimum one should have 1e-5 normalized counts at the 65,000-intensity level (saturation). The PMT gain should be increased when the normalized counts at the 65,000-intensity level are less than $1e^{-5}$ and should be decreased if the normalized counts at the 65,000-intensity level are more than $1e^{-5}$.

2.23.7 Data Analysis

The images were processed using the NimbleScan v2.5 program (Roche). Pair reports containing the raw data with the probe intensities were generated for each of twelve sub-arrays. The pair files were then used to generate normalized expression data using the Robust Multistep Average (RMA) algorithm the Robust Multistep Average (RMA) algorithm (Bolstad *et al*., 2003; Irizarry *et al*., 2003; Irizarry *et al*., 2003).

Prior to analysis, the microarray data were databased using Microsoft Excel 2010. The data were analyzed using Statistica v7.1 (StatSoft Ltd., Bedford, UK) and Systat 13 (Systat Software Inc., London, UK). BioProfiling.de analytical software was used for the GO-analysis (Antonov, 2011).

Non-normalized and normalized microarray data were deposited on the Gene Expression Omnibus database (submission number GSE42933).

3 ESTR MUTATION INDUCTION IN THE GERMLINE OF IRRADIATED FEMALE MICE

3.1 **Introduction**

In contrast to paternal exposure, little is known about the effects of maternal exposure on mutation induction in the germline of irradiated parents. The available data from female exposure have been derived from studies utilising classical assays such as specific locus test or dominant lethality. As already mentioned, the sensitivity of these assays for detecting the effects of exposure to low and medium doses of ionising radiation is quite low (Russell, 1965a,b ; Russell, 1972). Given that the most common types of human exposure to ionising radiation are attributed to this very range of doses, the validity of any extrapolation on the abovementioned mouse data to humans therefore remains questionable.

The results of previous studies clearly show that expanded simple tandem repeat (ESTR) loci represent a very sensitive system for monitoring mutation induction in the mouse germline. Indeed, ESTR loci have successfully been used for monitoring mutation induction in the germline of male mice exposed to ionising radiation (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998; Barber *et al.*, 2002; Barber *et al.*, 2009; Somers *et al.*, 2004) and chemical mutagens (Vilarino-Guell *et al.*, 2003; Glen *et al.*, 2008). According to the results of these studies, the sensitivity of this technique substantially exceeds that for the Russell 7-locus test, allowing mutation induction detection at much lower doses of exposure. However, to date, little is known regarding the effects of maternal irradiation on ESTR mutation induction in the mouse germline. So far, the

effects of acute exposure to X-rays on ESTR mutation induction in the maternal germline have been analysed by Barber and co-authors (2009). However, the results of this study describe the effects of *in utero* irradiation on ESTR mutation induction in mice and to date, the mutagenicity of exposure during adulthood remains unknown. This is why the current project was designed to establish the effects of maternal irradiation during adulthood on ESTR mutation induction in the mouse germline.

3.2 **Experimental design**

The offspring of two reciprocal crosses between BALB/c and CBA/Ca inbred mice were analysed in this study. The *Ms6-hm* and *Hm-2* ESTR loci alleles in these strains show clear size differences that allow an unambiguous establishment of the parental origin of mutations. CBA/Ca and BALB/c females were given whole-body acute irradiation of 1 Gy of X-rays delivered at 0.5 $Gymin^{-1}$ and mated with control males from another strain one week following irradiation (Figure 3.1) ensuring that their offspring were derived from irradiated meiotically-arrested dictyate oocytes in maturing follicles (Searle, 1974).

A combination of pedigree-based approach and PCR amplification was employed to detect the *de novo* ESTR mutations that occurred in the germline of control and irradiated parents and passed to their offspring. As already mentioned, the allele size for both ESTR loci substantially differed. Thus, the CBA/Ca derived *Hm-2* allele was detected by genomic southern blotting at ~16kb, whereas the size of BALB/c-derived *Hm-2* allele was only ~1.6kb and therefore mutation scoring at this allele required PCR amplification. On the other hand, mutation detection at the CBA/Ca- and BALB/c-derived alleles of the *Ms6-hm* locus was carried out using Southern Blotting. To

estimate the rate by which ESTR mutation occurred in the parental germline, the total number of mutants found in the offspring was divided into the total number of ESTR bands detected in each group. In control families this estimate corresponds to the rate by which spontaneous ESTR mutations occur in the germline of (F_0) parents, whereas in the irradiated families it also reflects mutation induction in the parental germline.

ESTR mutants were detected in DNA samples extracted from tails of the first-generation offspring of control and irradiated parents. The control group consisted of 218 offspring of the two reciprocal crosses, namely: ♀BALB/c x ♂CBA/Ca (135 offspring) and ♀CBA/Ca x ♂BALB/c (83 offspring). The exposed group included 231 offspring of irradiated females resulted from the same reciprocal crosses: ♀BALB/c x ♂CBA/Ca (127 offspring) and ♀CBA/Ca x ♂BALB/c (104 offspring).

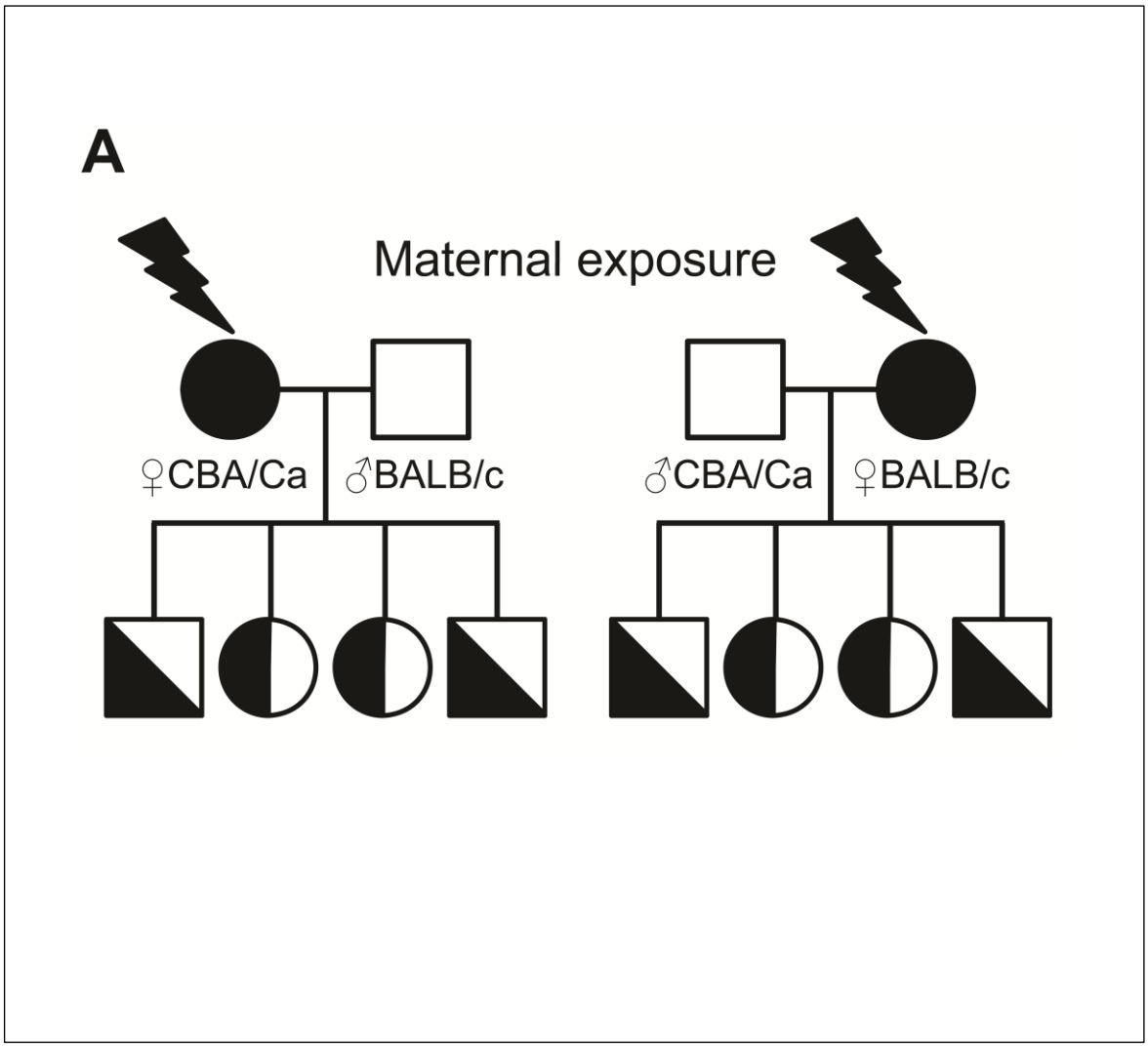


Figure 3.1 Experimental design of ESTR mutation detection in the germline of directly exposed (F_0) females. *De novo* ESTR mutations were detected by profiling DNA samples extracted from tails of all the offspring and their parents that correspond to germline mutation induction.

3.3 **Results**

3.3.1 **The frequency of *de novo* ESTR mutation in the offspring of reciprocal crosses**

The ESTR mutation rates of the two reciprocal crosses CBA/Ca x ♀BALB/c and ♂BALB/c x ♀CBA/Ca were compared. In both control and irradiated groups, the maternal and paternal mutation rates for CBA/Ca x ♀BALB/c and ♂BALB/c x ♀CBA/Ca crosses were estimated and compared using Fisher's exact test (Table 3.1). The comparison revealed that the ESTR mutation frequencies did not significantly differ between two reciprocal crosses within either the control or the irradiated group (Table 3.1). Therefore, the data from the two crosses were aggregated.

Table 3.1: ESTR mutation frequencies in the germline of control and irradiated parents from two reciprocal crosses

Group, cross	No mutations[a]	No bands	Frequency	Prob[b]
Control				
- maternal				
♀BALB x ♂CBA	21 (10+11)	270	0.0778	
♀CBA x ♂BALB	6 (4+2)	159	0.0377	0.1424
- paternal				
♀BALB x ♂CBA	12 (8+4)	261	0.0460	
♀CBA x ♂BALB	10 (5+5)	163	0.0614	0.6312
Irradiated				
- maternal				
♀BALB x ♂CBA	16 (8+8)	251	0.0637	
♀CBA x ♂BALB	16 (11+5)	208	0.0769	0.7102
- paternal				
♀BALB x ♂CBA	17 (9+8)	253	0.0672	
♀CBA x ♂BALB	16 (9+7)	207	0.0773	0.8097

[a]Number of mutations detected at *Ms6-hm* and *Hm-2* loci is given in parenthesis.
[b]Probability of difference between the two reciprocal crosses (Fisher's exact test, two-tailed).

3.3.2 **The effects of irradiation during adulthood on ESTR mutation induction in the maternal germline**

Given that the parental origin of *de novo* ESTR mutants found in the offspring of control and irradiated females can be un-ambiguously established (see above), in each group the separate estimates of paternal and maternal mutations rates were obtained (Table 3.2). The data presented in this table show the number of ESTR mutations detected at the *Ms6-hm* and *Hm-2* ESTR loci and the total number of ESTR bands. In both groups, these two values were used to estimate ESTR mutation rates in the maternal and paternal germline. According to the data presented in Table 3.2, ESTR mutation rates in the germline of non-exposed fathers from the two groups did not significantly differ. The same was also true when ESTR mutation rates were compared in the germline of control fathers and mothers (Fisher's exact test, p=0.55). In addition, ESTR mutation rates in the germline of exposed females couldn't achieve significant difference compared to their non-exposed male partners (p=1). Most importantly, a comparison of ESTR mutation rates in the germline of irradiated and control females failed to detect any significant increases in the exposed group. Taken together, the results presented show that exposure to 1 Gy of acute X-rays during adulthood does not alter ESTR mutation rate in the maternal germline.

Table 3.2: ESTR mutation frequencies in the germline of control and irradiated parents (pedigree data)

Group	No mutations[a]	No bands	Frequency	Ratio to control	Prob[b]
Control					
- maternal	27 (14+13)	429	0.0629	-	-
- paternal	22 (13+9)	424	0.0519	-	-
Irradiated					
- maternal	32 (19+13)	459	0.0697	1.11	0.7881
- paternal	33 (18+15)	460	0.0717	1.38	0.2795

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

3.3.3 **The spectra of ESTR mutation**

The spectra of paternal and maternal ESTR mutations are compared in Table 3.3. For each locus, the incidence of mutations attributed to gain and loss of a certain number of repeats did not significantly differ between control and irradiated families. The data on their mutation spectra were therefore pooled. Among the 59 maternal mutants, 31 (52.5%) were attributed to gains and 28 (47.5%) to losses of repeats. The incidence of gains (58.2%) and loses (41.8%) among the 55 paternal mutants was practically indistinguishable from that for ESTR mutations occurring in the maternal germline (Table 3.3). Therefore, the data from this study imply that the spectra of paternal and maternal ESTR mutations are very similar, if not identical.

Table 3.3: Comparison of ESTR mutation spectra in the maternal and paternal germline

Locus	Gains	Losses	Total
Ms6-hm			
- maternal	16 (48.48%)	17 (51.52%)	33
- paternal	21 (67.74%)	10 (32.26%)	31
χ^2, df=1	2.39	P = 0.1221	64
Hm-2			
- maternal	15 (57.69%)	11 (42.31%)	26
- paternal	11 (45.83%)	13 (54.17%)	24
χ^2, df=1	0.68	P = 0.4096	50
Total			
- maternal	31 (52.54%)	28 (47.46%)	59
- paternal	32 (58.18%)	23 (41.82%)	55
χ^2, df=1	0.36	P = 0.5485	114

3.3.4 **Conclusions**

The findings of this study are summarised below:

- Irradiation of the dictyate oocytes in maturing follicles does not affect ESTR mutation rate in the germline of female mice exposed during adulthood.
- The spectra of ESTR mutations occurring in the paternal and maternal germline do not significantly differ.

Discussion

Due to the complexity of oogenesis, in the majority of the studies conducted so far, the mutagenic effects of exposure to ionising radiation have been established in the paternal germline while data describing female exposure still remains relatively scarce. Previous work has established that the pattern of ESTR mutation induction dramatically differs across spermatogenesis. Indeed, exposure to a mutagen during the pre-meiotic stages of spermatogenesis results in germline mutation induction, whereas post-meiotic exposure (spermatids or sperm) does not affect ESTR mutation rates (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998; Barber *et al.*, 2002; Barber *et al.*, 2009; Somers *et al.*, 2004; Vilarino-Guell *et al.*, 2003; Glen *et al.*, 2008). These findings have also been supported by the findings of one recent study on the effects of *in utero* irradiation on ESTR mutation rates in the germline of adult mice. The authors reported that the *in utero* targeting of the pre-meiotic stages of oogenesis resulted in ESTR mutation induction in the maternal germline (Barber *et al.*, 2009).

The present study has assessed ESTR mutation rate in the germline of female mice exposed during adulthood. As already mentioned, in the irradiated females the non-dividing meiotically arrested dictyate oocytes in maturing follicles were targeted. The data presented here are in agreement with the results of the abovementioned studies on the stage-specificity of ESTR mutation induction in the germline of exposed male mice (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998; Barber *et al.*, 2002; Barber *et al.*, 2009; Somers *et al.*, 2004; Vilarino-Guell *et al.*, 2003; Glen *et al.*, 2008). These data also support the findings showing the mutagenic effects of *in utero* irradiation on ESTR mutation rate in male and female mice (Barber *et al.*, 2009).

According to the results of previous studies, ESTR mutation induction almost exclusively occurs in replication-proficient germ cells. Indeed, targeting the replication-proficient spermatogonia and primordial germ cells substantially enhances the ESTR mutational yield, whereas the exposure of non-dividing post-meiotic spermatids and mature spermatozoa that do not undergo DNA replication does not affect mutation rates in these cells (Dubrova *et al.*, 1993; Dubrova *et al.*, 1998; Barber *et al.*, 2002; Barber *et al.*, 2009; Somers *et al.*, 2004; Vilarino-Guell *et al.*, 2003; Glen *et al.*, 2008).

The same holds true for the pattern of ESTR mutation induction in the maternal germline (Figure 3.1). Irradiation of the mitotically active pre-meiotic foetal stages of oogenesis results in ESTR mutation in the maternal germline (Barber *et al.*, 2009). According to the results of current study, exposure of non-replicating meiotically arrested oocytes does not destabilise ESTR loci in the maternal germline (Figure 3.2). This dependence of ESTR mutation induction upon the proliferative capacity of the cells was observed not only in the germline, but also in somatic tissues. Indeed, the analysis of ESTR mutation frequencies in the somatic tissues of both male and female mice irradiated either *in utero* or during adulthood has revealed radiation-induced increases only following targeting replication-proficient tissues. In addition, the frequency of ESTR mutation in these tissues was associated with the mitotic index (Barber *et al.*, 2009; Yauk *et al.*, 2002).Taken together, these results suggest that similar mechanisms may underlie spontaneous and, possibly, radiation-induced ESTR mutation in the paternal and maternal germline, as well as in their somatic tissues.

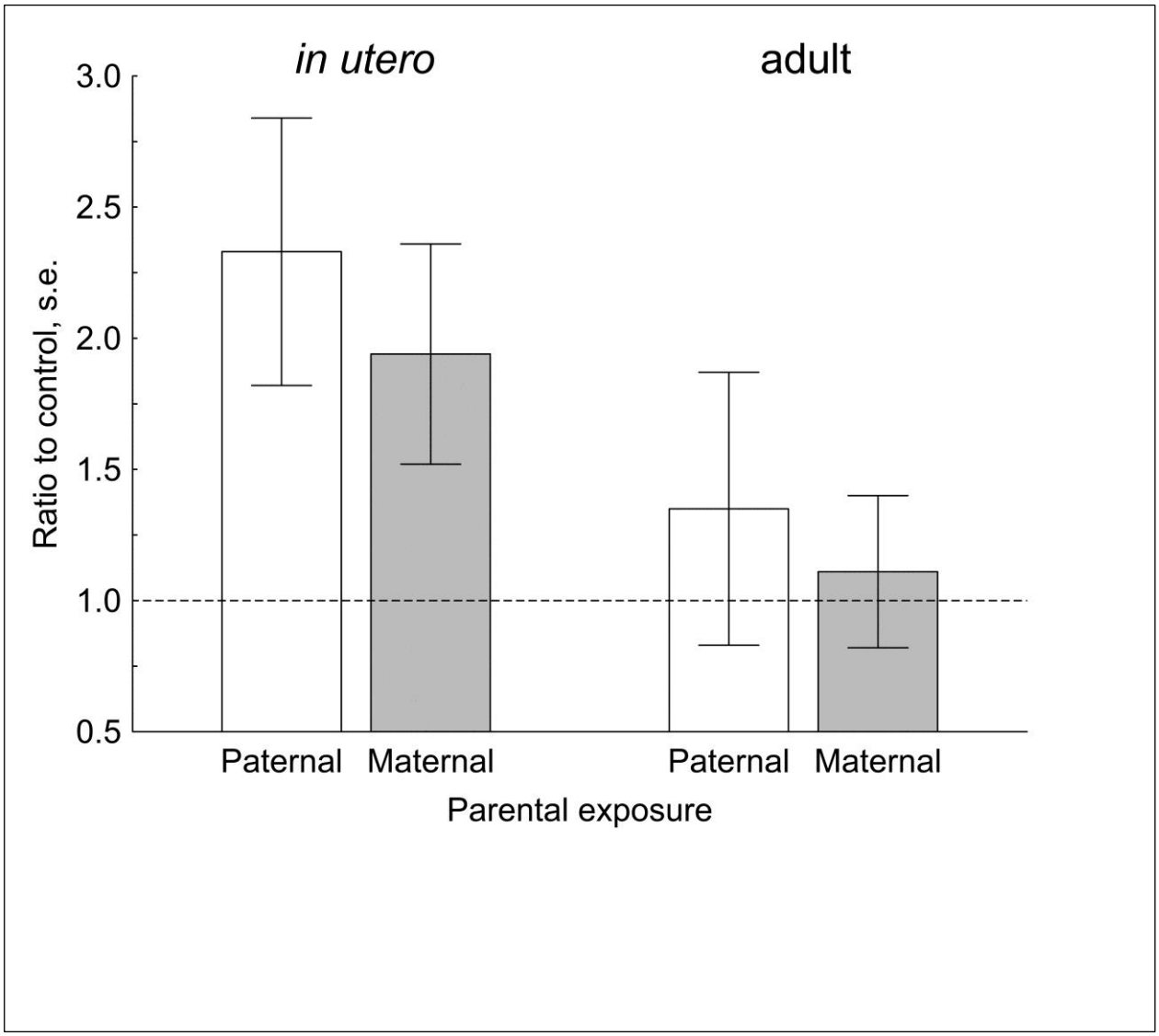


Figure 3.2. ESTR mutation induction in the germline of male and female mice following irradiation either *in utero* or adulthood. Data for the effects of *in utero* maternal irradiation are taken from Barber *et al.* (2009) and that for adult maternal irradiation are taken from this study. Data for the effects of post-meiotic (spermatid stage) irradiation in adult male mice are taken from Barber *et al.* (2000).

The findings of this study also provide further insights into the mechanism of ESTR mutation. They show that the rates and spectra of spontaneous ESTR mutation in the paternal and maternal germline are very similar. This similarity implied that the same processes might be responsible for spontaneous paternal and maternal ESTR mutation. Two recent studies have attempted to analyse the frequencies and spectra of spontaneous ESTR mutation in tissues with different proliferation indexes in male mice of different age (Hardwick *et al.*, 2009), and in different stages of spermatogenesis (Shanks *et al.*, 2008). The results of both studies show that spontaneous ESTR mutation occurs almost completely in actively dividing cells by a replication-dependant mechanism, thus implying that replication slippage can alone explain the very high ESTR mutation rate in the germline and somatic tissues of male mice. Taken together, the data reported here and the results of the previous studies may indicate that the same mechanism might be responsible for spontaneous ESTR mutation in females as well as males. However, as the frequency of ESTR mutation in the germline of male mice significantly increases with age (Hardwick *et al.*, 2009), in the germline of elderly males, it should substantially exceed that in female mice of the same age. This expected difference, which remains to be proven, reflects the profound differences between the male and female germ cell biology. It could be explained by the fact that spermatogenesis in adult mice is an on-going process characterised by high proliferation activity of stem cells that facilitate the accumulation of ESTR mutations during successive rounds of DNA replication. On the other hand, the meiotically arrested oocytes do not undergo any further divisions. The absence of DNA replication at this stage of mouse oogenesis greatly suppresses spontaneous ESTR mutation.

Conclusion

The data presented here show that targeting of non-replicating meiotically-arrested dictyate oocytes does not affect ESTR mutation rate in the germline of irradiated female mice. These are in agreement with the results of previous studies on the stage-specificity of ESTR mutation induction in the germline of male mice exposed to ionising radiation and chemical mutagens. These findings further support the replication-driven model of spontaneous ESTR mutation by replication slippage.

4 THE EFFECTS OF PARENTAL IRRADIATION DURING ADULTHOOD ON THE MANIFESTATION OF TRANSGENERATIONAL INSTABILITY IN THEIR OFFSPRING

4.1 Introduction

It is well established that ionising radiation can not only induce mutations seen in directly exposed somatic cells, but can also result in delayed effects with new mutations arising many cell divisions after the initial insult (Morgan, 2003a; Morgan, 2003b). It has been suggested that the manifestation of radiation-induced genomic instability may contribute to the accumulation of mutations in somatic cells, thus increasing the risk of cancer (UNSCEAR, 2006). Given, it was logical to test the hypothesis that parental irradiation may also destabilise the genomes of non-exposed offspring. Indeed, a number of mouse studies have provided strong evidence for transgenerational destabilisation of the offspring genomes following paternal exposure to either ionising radiation or chemical mutagens (Barber *et al.*, 2002; Barber *et al.*, 2006; Barber *et al.*, 2009, Dubrova *et al.*, 2008; Hatch *et al.*, 2007). By contrast, the transgenerational effects of maternal irradiation remain poorly understood. The results of one recent study showed that maternal *in utero* exposure did not affect mutation rates in the F_1 offspring, whereas the similarly exposed F_1 offspring of prenatally irradiated male mice were genetically unstable (Barber *et al.*, 2009). These data raise the important question whether maternal irradiation during adulthood can destabilise the offspring genomes. This study was therefore designed to establish whether maternal irradiation during adulthood can destabilise the genomes of F_1 offspring.

4.2 Experimental design

The offspring of two reciprocal crosses ♀BALB/c x ♂CBA/Ca and ♀CBA/Ca x ♂BALB/c were used in this study. CBA/Ca and BALB/c females and males were given whole-body acute irradiation of 1 Gy of X-rays delivered at 0.5 Gy/min and mated to their non-exposed partners within 2 to 5 days following exposure (Figure 4.1). This mating scheme ensured that the offspring of irradiated males and females were derived either from exposed post-meiotic spermatozoa or dictyate oocytes in maturing follicles respectively (Searle, 1974).

To evaluate the transgenerational effects of maternal and paternal irradiation, the frequency of ESTR mutation was measured at the *Ms6-hm* ESTR locus using SM-PCR as previously described in section 2.11. If the transgenerational effects manifest in the offspring of irradiated parents, then the frequency of ESTR mutation in the germline and somatic tissues of these animals should significantly exceed that in controls. It should be noted that the abovementioned effects are distinct from the phenomenon of mutation induction in the germline of irradiated parents where extra mutations only occur in the exposed/irradiated germ cell. In this case, mutation induction in the directly targeted genomes should occur following exposure to practically all doses of radiation. On the other hand, the transgenerational effects in the offspring may not manifest following paternal exposure to some doses.

DNA samples were extracted from sperm (taken from caudal epididymis), bone marrow and brain of the 8-week old F_1 male offspring conceived either by control parents or irradiated males and females. DNA samples were analysed from 4 animals in the control group such that 2 offspring were taken from different litters for each of the

reciprocal crosses. Among the offspring of irradiated males and females, DNA was similarly prepared from 4 males from each group (2 offspring from different litters for each of the reciprocal crosses). Mutations were scored (Section 2.22) at both the BALB/c- and CBA/Ca-derived alleles (Figure 2.4)

To increase the statistical power, on average 170 amplifiable molecules were analysed for each sample. The statistical power of this study was estimated using the Poisson approximation, whereby 1.5 fold increase in ESTR mutation frequency in the offspring can be detected with α=0.05 level of significance.

The frequency of ESTR mutation, μ in each tissue was estimated by dividing the number of mutants, m by the total number of amplifiable DNA molecules, λ. The standard error of mutation frequency, seμ was estimated according to the following equation:-

$$se\mu = \mu\sqrt{\left(\frac{se\lambda}{\lambda}\right)^2 + \frac{1}{m}}$$

and $se\lambda$ is the standard error of the number of amplifiable DNA molecules.

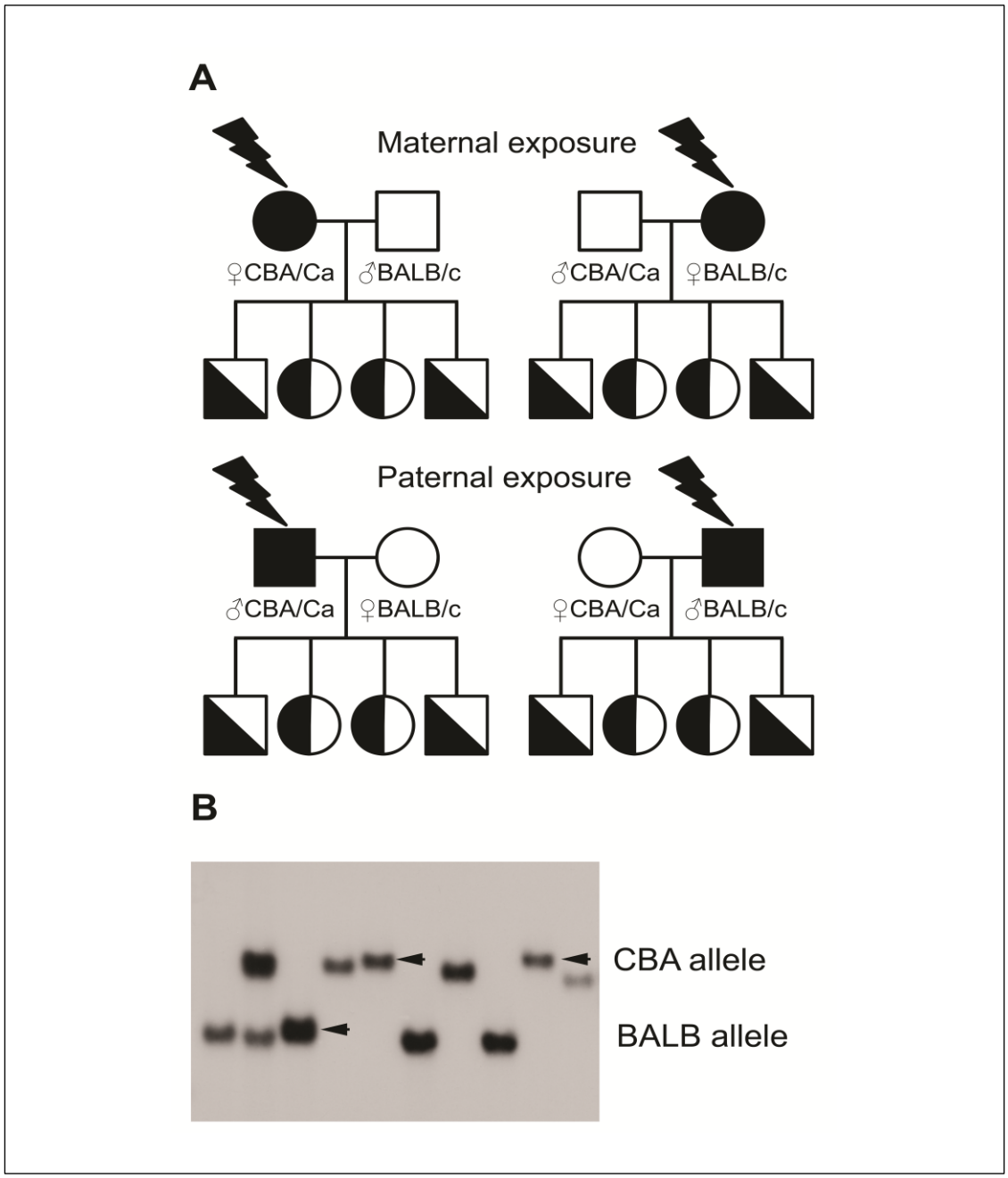


Figure 4.1. Experimental design of transgenerational study.

A) Experimental design of the current study aimed to compare the transgenerational effects of maternal and paternal irradiation during adulthood. The irradiated F_0 parents (shown in black) were mated to control partners and tissue samples were taken from their non-exposed F_1 offspring.

B) ESTR mutation detection by SM-PCR. Mutations at the CBA/Ca and BALB/c alleles are indicated by arrows.

4.3 Results

To analyse the transgenerational effects of parental irradiation, ESTR mutation frequencies were estimated in DNA samples extracted from sperm, bone marrow and brain of the offspring of controls and irradiated parents. Due to substantial difference in the allele size, the parental origin of ESTR mutants found in the offspring of control and irradiated females can be un-ambiguously established.. This approach allowed establishing the effects of parental irradiation at the alleles derived from the exposed and non-exposed parents.

4.3.1 ESTR mutation frequencies in the offspring of reciprocal crosses

Using SM-PCR, the ESTR mutation frequency was estimated in different groups of the F_1 male offspring conceived either by control parents or irradiated males and females. Given that in all groups the offspring were derived from two reciprocal crosses, ESTR mutation frequencies were separately estimated for each subgroup. Table 4.1 summarises ESTR mutational data. As within each group, the frequency of ESTR mutation did not significantly differ in the germline and somatic tissues of offspring of two reciprocal crosses, the data were aggregated.

Table 4.1. ESTR mutation frequencies in the offspring of two reciprocal crosses (♂CBA/Ca x ♀BALB/c and ♂BALB/c x ♀CBA/Ca)

Tissue, group, cross	**No. mutations**	**No. Progenitors**	**Frequency ± s.e.**	***t*** [*]	**Prob** [*]
Sperm					
Control					
♂CBA x ♀BALB F_1	14	372	0.0377 ± 0.0103	-	-
♂BALB x ♀CBA F_1	13	338	0.0385 ± 0.0109	0.05	0.9601
F_1 irradiated females					
♂CBA x ♀BALB F_1	17	403	0.0421 ± 0.0105	-	-
♂BALB x ♀CBA F_1	13	410	0.0317 ± 0.0090	0.76	0.4475
F_1 irradiated males					
♂CBA x ♀BALB F_1	32	436	0.0734 ± 0.0136	-	-
♂BALB x ♀CBA F_1	31	342	0.0907 ± 0.0172	0.79	0.4298
Bone marrow					
Control					
♂CBA x ♀BALB F_1	10	282	0.0355 ± 0.0115	-	-
♂BALB x ♀CBA F_1	14	307	0.0456 ± 0.0125	0.59	0.5554
F_1 irradiated females					
♂CBA x ♀BALB F_1	11	443	0.0248 ± 0.0076	-	-
♂BALB x ♀CBA F_1	14	341	0.0410 ± 0.0112	1.19	0.2344
F_1 irradiated males					
♂CBA x ♀BALB F_1	27	467	0.0578 ± 0.0116	-	-
♂BALB x ♀CBA F_1	36	366	0.0984 ± 0.0174	1.94	0.0527
Brain					
Control					
♂CBA x ♀BALB F_1	6	241	0.0249 ± 0.0103	-	-
♂BALB x ♀CBA F_1	9	165	0.0544 ± 0.0187	1.38	0.1684
F_1 irradiated females					
♂CBA x ♀BALB F_1	11	347	0.0317 ± 0.0098	-	-
♂BALB x ♀CBA F_1	9	251	0.0358 ± 0.0122	0.26	0.7949
F_1 irradiated males					
♂CBA x ♀BALB F_1	23	325	0.0708 ± 0.0155	-	-
♂BALB x ♀CBA F_1	33	295	0.1120 ± 0.0208	1.59	0.1123

[*] Student's test and probability for difference between the offspring of reciprocal crosses. Number of progenitor corresponds to the number of amplifiable molecules.

4.3.2 Effect of maternal irradiation on transgenerational instability

To analyse the transgenerational effects of parental irradiation, ESTR mutation frequencies have been estimated in DNA samples extracted from sperm, bone marrow and brain of the offspring of controls and irradiated parents. Then these estimates were cross-compared between the three groups included in this study, namely: the offspring of control parents, the offspring of irradiated females and the offspring of irradiated males. Within each exposed group, ESTR mutation frequencies were separately established at the alleles derived from the irradiated and non-irradiated parents. A summary of ESTR mutation data is presented in Table 4.2.

According to the SM-PCR data, ESTR mutation frequency in the germline of F_1 offspring of irradiated females (sperm) did not significantly differ from that in control. The same result was obtained when the frequencies of mutation were compared at the alleles derived from the irradiated mothers and non-irradiated fathers. The analysis of ESTR mutation frequencies in DNA samples extracted from the F_1 somatic tissues also failed to detect any significant differences between controls and the offspring of irradiated females. It was therefore concluded that maternal irradiation during adulthood did not affect the stability of their first-generation offspring.

Table 4.2. Summary of SM-PCR data

Tissue, group	No mutations	No progenitors	Frequency ± s.e.	Ratio*	*t*†	Prob†
Sperm						
Control						
- paternal allele	14	339	0.0413 ± 0.0113	-	-	-
- maternal allele	13	371	0.0350 ± 0.0099	-	-	-
Total	27	710	0.0380 ± 0.0075	-	-	-
F_1 of irradiated females						
- paternal allele	11	406	0.0271 ± 0.0833	0.65	1.02	0.3081
- maternal allele	19	407	0.0467 ± 0.0110	1.33	0.78	0.4356
Total	30	814	0.0369 ± 0.0069	0.97	0.12	0.9045
F_1 of irradiated males						
- paternal allele	31	379	0.0818 ± 0.0155	1.98	2.11	0.0352
- maternal allele	32	399	0.0802 ± 0.0149	2.29	2.52	0.0119
Total	63	778	0.0801 ± 0.0108	2.13	3.28	0.0011
				2.20††	3.45	0.0006
Bone marrow						
Control						
- paternal allele	9	282	0.0319 ± 0.0108	-	-	-
- maternal allele	15	306	0.0489 ± 0.0130	-	-	-
Total	24	589	0.0408 ± 0.0085	-	-	-
F_1 of irradiated females						
- paternal allele	12	376	0.0319 ± 0.0094	1.00	0.00	1.0000
- maternal allele	13	408	0.0318 ± 0.0090	0.65	1.08	0.2805
Total	25	785	0.0319 ± 0.0065	0.78	0.83	0.4067
F_1 irradiated males						
- paternal allele	34	413	0.0824 ± 0.0149	2.58	2.74	0.0063
- maternal allele	29	420	0.0690 ± 0.0134	1.41	1.07	0.2850
Total	63	833	0.0756 ± 0.0100	1.85	2.65	0.0081
				2.37††	3.67	0.0002

* Ratio to control.

† Student's test and probability for difference from mutation frequency in controls.

†† Difference between the offspring of irradiated males and females.

Table 4.2 (continued)

Tissue, group	No mutations	No progenitors	Frequency ± s.e.	Ratio*	*t*†	Prob†
Brain						
Control						
- paternal allele	7	195	0.0306 ± 0.0139	-	-	-
- maternal allele	8	211	0.0379 ± 0.0137	-	-	-
Total	15	406	0.0370 ± 0.0097	-	-	-
F_1 of irradiated females						
- paternal allele	11	300	0.0366 ± 0.0113	1.02	0.04	0.9681
- maternal allele	9	298	0.0302 ± 0.0103	0.80	0.45	0.6529
Total	20	598	0.0335 ± 0.0076	0.91	0.28	0.7795
F_1 of irradiated females						
- paternal allele	30	263	0.1141 ± 0.0222	3.17	2.98	0.0030
- maternal allele	26	356	0.0730 ± 0.0150	1.73	1.73	0.0842
Total	56	619	0.0904 ± 0.0128	2.45	3.33	0.0009
				2.46††	3.41	0.0007

* Ratio to control.

† Student's test and probability for difference from mutation frequency in controls.

†† Difference between the offspring of irradiated males and females

4.3.3 Effect of paternal irradiation on the transgenerational instability

In contrast to maternal irradiation, ESTR mutation frequency was significantly elevated in the offspring of irradiated male mice (Table 4.2). Specifically, more than a 2-fold increase was found in DNA samples extracted from sperm. ESTR mutation frequencies were equally elevated at the alleles derived from the irradiated fathers and non-irradiated mothers. The same was also true for the brain tissue. Thus, a 2.5-fold increase in the total frequency of ESTR mutation was found in this group. Similar to the sperm data, ESTR mutations were equally elevated at the alleles derived from the irradiated and control parents. The results obtained on the bone marrow samples were in agreement with these data. Again, in the offspring of irradiated males the total frequency of ESTR mutation significantly exceeded that of controls, and showed the same pattern regarding the effects of paternal exposure at the alleles derived from the irradiated fathers and non-irradiated mothers. Taken together, these results show that paternal irradiation during adulthood can destabilise the F_1 genome.

A comparison of the data presented here and the results of previous studies on the transgenerational effects of maternal and paternal irradiation *in utero* is shown on Figure 4.2.

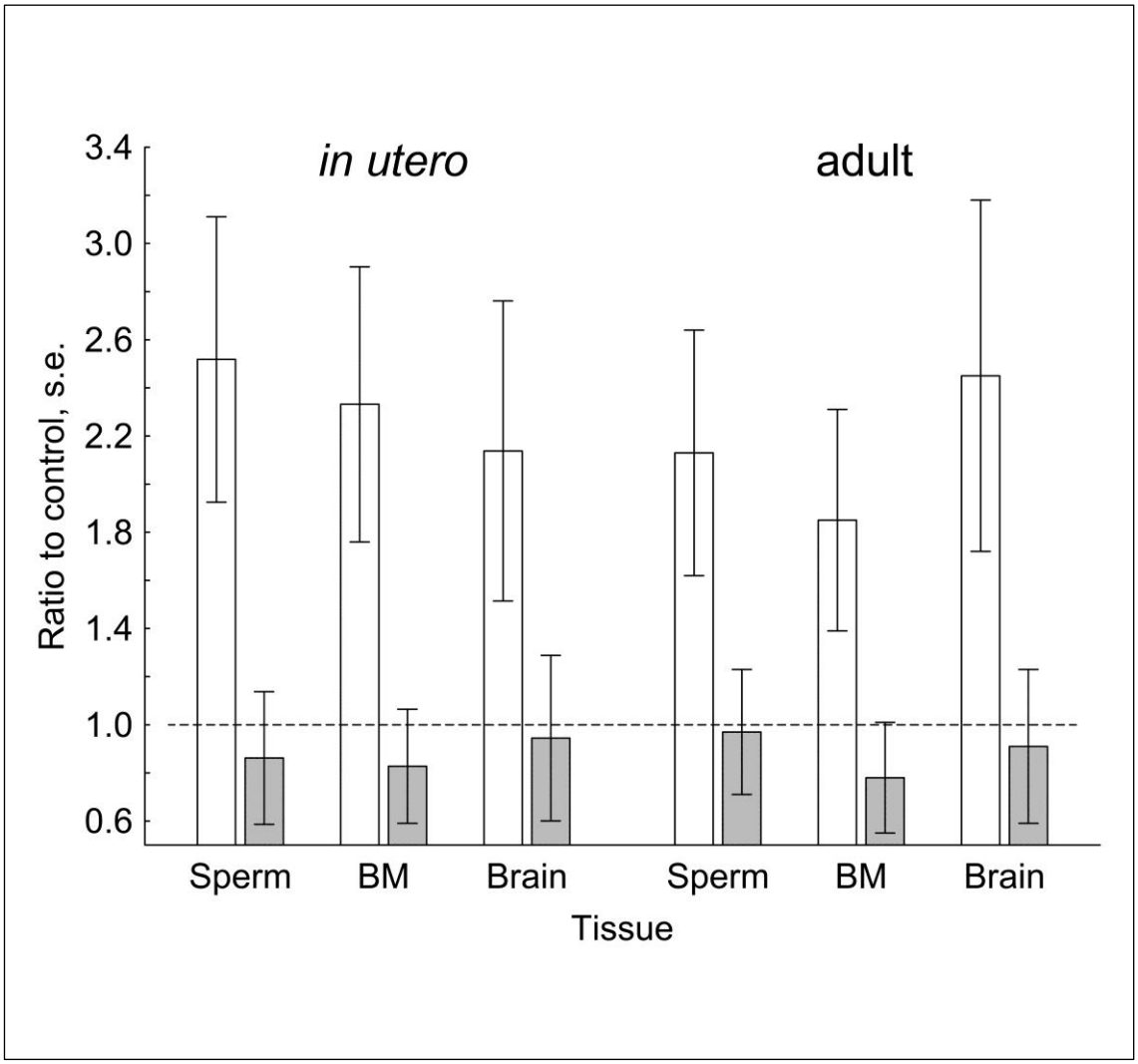


Figure: 4.2. The effect of maternal and paternal exposure (1Gy of acute X-rays) on the manifestation of transgenerational instability in the offspring of parents irradiated either *in utero* or during adulthood. The F_1 of irradiated males represented by the open bars; the offspring of irradiated females represented by the shaded bars. Data for the effects of *in utero* irradiation are taken from Barber *et al.* (2009).

4.3.4 Conclusions

The SM-PCR analysis of the ESTR mutation frequencies in the germline and somatic tissues of non-exposed first-generation offspring of irradiated males and females exposed during adulthood has revealed that:

- ESTR mutation frequencies in the F_1 offspring of irradiated females were indistinguishable from that in controls.
- In contrast, paternal irradiation resulted in significant transgenerational destabilisation of the F_1 genome. Elevated frequency of ESTR mutation were detected in the germline (sperm) and in two somatic tissues tested (bone marrow and brain).
- The elevated ESTR mutations were observed at the alleles derived from the irradiated fathers and non-exposed mothers.

4.4 Discussion

The data obtained in previous studies have shown that acute paternal exposure to ionising radiation and chemical mutagens results in transgenerational destabilisation of the genomes of their offspring (Dubrova at al., 2000, 2008; Barber *et al.*, 2002, 2006, 2009; Glen and Dubrova, 2012). Moreover, these transgenerational effects are manifested in non-exposed offspring regardless of the stage of spermatogenesis at which the ionising radiation has been delivered. In other words, the radiation induced destabilisation signal has persisted through the male germline at whatever stage of spermatogenesis irradiated; spermatogonia (Barber *et al.*, 2006), spermatids (Barber *et al.*, 2002), or mature sperm (Hatch *et al.*, 2007). Recently, a study by Barber *et al.* (2009) has evaluated the ability of this radiation-induced signal to pass through male or female germline of *in utero* irradiated mice. They found that the foetal male exposure leads to transgenerational instability that destabilises the genome of the non-exposed offspring whereas the *in utero* exposure of female mice did not affect genome stability of their offspring. Given the long period of time elapsed between the *in utero* exposure and the mating during adulthood, the lack of transgenerational effects in the offspring of irradiated females might be explained by erasure of the radiation-induced signal (likely to be epigenetic), during the complicated process of oogenesis, which was not the case in spermatogenesis. This study was designed to establish whether this may be the case, especially since the mating of females was very short (one week), following irradiation. In other words, the study aimed to establish whether the maternal irradiation during adulthood could result in transgenerational effects in their non-exposed offspring, affecting their genome stability.

4.4.1 Summary of findings

Analysis of the effects of adult acute irradiation of male and female mice on the genome stability of their non-exposed offspring has established number of findings. The first finding to emerge from this study was that in the offspring of irradiated males, the frequency of ESTR mutation was significantly elevated across all tissues (Figure 4.2; Table 4.2). In other words, paternal irradiation resulted in transgenerational genomic instability that manifested in both germline (sperm) and somatic cells (brain and bone marrow) of their F_1 offspring. This finding is consistent with the previously published work that reported an elevated ESTR mutation rates both in the somatic and germline cells of the offspring of irradiated fathers in almost all stages of spermatogenesis (Dubrova at al., 2000, 2008; Barber *et al.*, 2002, 2006, 2009; Hatch *et al.*, 2007; Glen and Dubrova, 2012).

The second observation was that following paternal irradiation the ESTR mutation rates were similarly elevated in both the irradiated paternally-inherited alleles and the non-exposed maternally inherited alleles (Table 4.2). This finding has supported the previously published work by Niwa suggesting that the radiation induced instability signal following paternal irradiation may act in *trans*, affecting the unexposed maternally derived ESTR allele (Niwa *et al.*, 2001; Shiraishi *et al.*, 2002). These data also lend further support for the hypothesis that radiation-induced transgenerational effects in the offspring of irradiated male mice are attributed to a genome-wide destabilisation signal, that manifests in many, if not all tissues.

In sharp contrast to the paternal exposure, maternal irradiation did not affect ESTR mutation rates of their offspring, resulting in ESTR mutation frequencies that were

indistinguishable from those of controls across all tissues (Figure 4.2; Table 4.2).. In a previous study, Barber *et al.* (2009) also failed to establish significant elevation in the ESTR mutation rates in either somatic (brain, bone marrow) or germline (sperm) in the offspring of *in utero* irradiated female mice compared to controls. Data from both studies have therefore, revealed that maternal irradiation does not affect genome stability of non-exposed offspring regardless of the stage of irradiation (Figure 4.2).

4.4.2 Radiation exposure affects the epigenetic landscape of both male and female germ cells

There is a large body of evidence that radiation-induced genomic instability in somatic cells and transgenerational instability is the same phenomenon but with different manifestations (Karotki *et al.*, 2012). Indeed, an early study by Carls and Schiestl (1999) has reported that the pink eye mutation (p^{un}) could be reverted before or after fusion of premeiotically-irradiated-sperm. In addition, a recent study by Aypar *et al.* (2011b) has reviewed the epigenetic mechanisms that may be implicated in the radiation-induced genomic instability and concluded that these mechanisms are likely to be similar to those manifesting in the directly-exposed animals. Therefore, the mechanism of transgenerational instability in the offspring of irradiated male mice could be explained by the hypothesis that paternal irradiation can alter the epigenetic landscape of germ cells and that newly-generated epigenetic marks might be passed to the offspring, and thereby destabilising their genomes in many tissues (Dubrova *et al.*, 2003). In agreement with this hypothesis, numerous studies have reported epigenetic alterations associated with exposure to ionizing radiation. For example, Koturbash *et al.* (2006) reported significant reduction in the level CpG methylation in thymus of the non-exposed offspring of irradiated male C57Bl/6 mice derived from irradiated sperm.

This DNA hypomethylation was accompanied with a substantial decrease in DNA methyltransferases as well as methyl-CpG-binding protein MeCP2. This global genome hypomethylation was viewed in some contexts as a sign of genome destabilization (Chen *et al.*, 1998). Histone modifications have also been reported as a consequence of radiation exposure. For instance, Barber *et al.* (2006) observed a persistent phosphorylation of H2AX (γ-H2AX foci) that was associated with highly elevated mutation rates as well as DNA lesions, in both somatic and germline cells of the F_1 offspring derived from irradiated sperm.

According to this hypothesis, the lack of transgenerational effects following maternal irradiation has two explanations: the first explanation is the absence of such epigenetic alterations in the germline of directly-exposed females; the second explanation is the inability of females to pass the instability signal (altered epigenetic landscape) to their offspring. However, there is a growing body of evidence that the irradiation of female mice does also affect their epigenetic landscape. Indeed, like irradiated males, various epigenetic alterations have been recorded in different organs and tissues of female mice exposed to ionising radiation. For example, radiation-induced DNA hypomethylation was observed both in the liver and spleen of irradiated C57/Bl6 female mice (Pogribny *et al.*, 2004). Also, global DNA hypomethylation accompanied with a decline in the levels of maintenance (DNMT1) and novel (DNMT3a and 3b) methyltransferases as well as the methyl-binding protein MeCP2 have been observed in rat mammary glands following acute X-irradiation (Pogribny *et al.*, 2005; Raiche, *et al.*, 2004). Given the above-mentioned evidences, the findings of this study have strongly suggested that exposure to ionising radiation may equally affect the epigenetic landscape of paternal and maternal genomes. Accordingly, it may be more possible that 'the inability' of

females to pass the instability signal (altered epigenetic landscape) to their offspring is the more likely scenario.

4.4.3 Radiation-induced epigenetic alteration can pass via male germline but not female germline

Additional evidence for this hypothesis has come from the work of Barber *et al.*, (2009). The directly *in utero* irradiated males and females showed similarly elevated ESTR mutation frequencies in their germline and somatic tissues. However, they showed different manifestations of the radiation-induced instability signal in their offspring (Figure 4.2). The offspring of *in utero* irradiated males was genetically unstable, whereas the maternal irradiation did not affect the genome stability of their offspring (Barber *et al.*, 2009). These findings have revealed that the epigenetic marks induced during foetal irradiation were able to survive the epigenetic reprogramming during the rest of developmental stages in both males and females, destabilising their genomes, which was detected as elevated ESTR mutation rates across multiple tissues (Barber *et al.*, 2009). Another line of evidence comes from the same study (Barber *et al.*, 2009) where *in utero* exposure have shown that the patterns of ESTR mutation induction in the germline of irradiated males and females are very similar regarding mutation rates and spectra (Figure 4.3).

The *in utero* irradiated females' lack of the transgenerational effect could be explained by the inability of the radiation-induced epigenetic signal to survive the epigenetic reprogramming during development. If this would be the case, then the offspring of the irradiated female mice during adulthood might manifest genomic instability, to some extent, in their tissues. However, the results of the current study have established that

the ESTR mutation frequencies of controls and F1 of irradiated female were indistinguishable (Figure 4.2).

The results of the current as well as that of the *in utero* study have established sharp contrast between the exposed males and females regarding their permissibility to propagate the instability signal to their F_1 offspring (Figure 4.2). This difference in the manifestation of the transgenerational instability in male and female germline raises the possibility that propagation of genomic instability in the offspring of irradiated males and females might be related to the early post-fertilisation events, which could somehow modify (keep or erase) the radiation-induced *de novo* epigenetic signals. Many publications have reported that starting from the first moment of fertilisation, the male and female genomes are not treated in the same way regarding the removal of epigenetic marks (Smallwood and Kelsey, 2012; Reik *et al.*, 2001; Morgan *et al.*, 2005).

For instance, during penetration of sperm into the oocyte at fertilisation, the sperm and the egg nuclei are very dissimilar. The egg is arrested in the metaphase of meiosis II and the maternal genome is packaged with nucleosomes. On the other hand, the paternal genome is packaged with protamines, which start to replace rapidly by histone proteins while the maternal genome remained packaged with nucleosomes (Feil, 2009; Morgan *et al.*, 2005). Shortly after substitution of protamines with histones, the paternal genome undergoes genome-wide active DNA demethylation before DNA replication (Smallwood and Kelsey, 2012; Reik *et al.*, 2001; Morgan *et al.*, 2005), probably by mechanisms involving oxidation into 5-hydroxymethylcytosine by oocyte-derived Tet3 DNA-dioxygenase (Gu *et al.*, 2011). In contrast, the maternal genome is demethylated passively via absence of DNA methylation maintenance during DNA replication, which

results in progressive loss of methylation at each cycle of DNA replication during cleavage (Smallwood and Kelsey, 2012).

Therefore, it could be speculated that the asymmetric epigenetic reprogramming during early fertilisation may unequally treat the radiation-induced epigenetic signal (Barber *et al.*, 2009). In other words, the active DNA demethylation of the paternal genome occurring a few hours post fertilisation may be able to keep more of the radiation-induced epigenetic modifications compared to the passive demethylation occurring in the maternal genome. However, this hypothesis remains highly speculative and clearly needs further analysis to shed more light on the pattern of transmission of epigenetic marks through the paternal and maternal germline.

4.4.4 Conclusions

The findings from the current study and the *in utero* study (Barber *et al.*, 2009) have established some important points:

- There is a prominent difference between the manifestation of the transgenerational instability in the offspring of irradiated male and female mice, regardless stage of exposure.
- The asymmetric epigenetic reprogramming in the first hours of fertilisation can offer explanation for the differential manifestation of the transgenerational instability in male and female germline.
- However, further studies are needed to understand the pattern of transmission of the epigenetic marks through male and female germline.

5 EXPRESSION PROFILING OF THE OFFSPRING OF MATERNALLY AND PATERNALLY IRRADIATED MICE

5.1 Introduction

It is well established that the phenomenon of transgenerational instability cannot be explained by Mendelian segregation of radiation-induced mutations, causing an overall destabilisation of F_1 genome. Instead, there is large and growing body of evidence implying that this phenomenon is attributed to as yet unknown epigenetic mechanisms. Gene expression profiling can offer an efficient tool to delineate the pathways that can potentially deregulate and affect genome integrity in the non-exposed offspring of irradiated parents and hence, mediate the manifestation of genomic instability across generations.

Previous work has established that adult maternal irradiation not affect the genome stability of their F_1 offspring, whereas the F_1 offspring of irradiated fathers were genetically unstable. This part of the project is a pilot study that aims to compare the pattern of gene expression of the F_1 offspring of irradiated males and females to establish whether the expression profile emphasises the established sharp contrast in manifestation of TI. This analysis is also an attempt to gain insights into the reasons behind the lack of transgenerational effects in the offspring of irradiated females as well as genetic pathways that can potentially compromise genome stability in the offspring of irradiated males.

In this study the expression profiles were analysed using whole-genome expression arrays (NimbleGen 12x135K multiplex expression array platform).This technique is based on the reverse transcription of total mRNA samples, which are labelled with the Cy3 and hybridised to the array. The intensity of hybridisation provides a measure of mRNA concentrations, thus estimating the efficiency of transcription in any tissue.

5.2 Experimental design

To compare the expression profile of F_1 offspring of irradiated male and female mice, CBA/Ca and BALB/c mice were irradiated and one week later, mated with control mice of the opposite sex (Figure 3.2). Kidneys were collected from eight-week old male offspring belonging to the three different groups: the first group included the offspring of non-irradiated parents, the second group included offspring of irradiated females, and the third group included offspring of irradiated males. RNA samples were extracted from 6 mice per each group, reverse transcribed into cDNA, labelled with Cy3 and hybridised to the microarray chip. The hybridised chips were scanned and further analysed to produce the expression pattern of each group. To minimise inter-experiment variation, each RNA sample was hybridized to two different arrays and the mean value for the two independent technical replicates was used.

5.3 Results

The mean values and standard errors of the mean of log-transformed levels of gene expression were estimated for each group (control, maternal, and paternal). Given that the distribution of the log-transformed values is close to normal distribution, the

Student's *t*-test was used to compare the control and irradiated groups. Similar to the majority of microarray studies, transcripts showing at least 1.5-fold up- or down-regulation of expression levels among the offspring of irradiated parents at the probability level $p<0.01$ was considered significantly altered. The pattern of gene expression was visualised using either volcano plots or heat-maps.

5.3.2 The effects of maternal irradiation on the expression profiles

A comparison of the pattern of gene expression in the offspring of control and irradiated females has revealed a number of significantly deregulated loci. For example, in the maternally irradiated group, 93 transcripts were significantly up-regulated whereas the expression of 42 was significantly decreased. (Figure 5.1; Tables 5.1 and 5.2). Hierarchical clustering of the differentially expressed genes has revealed a clear differentiation between all the offspring of control and irradiated females (Figure 5.2).

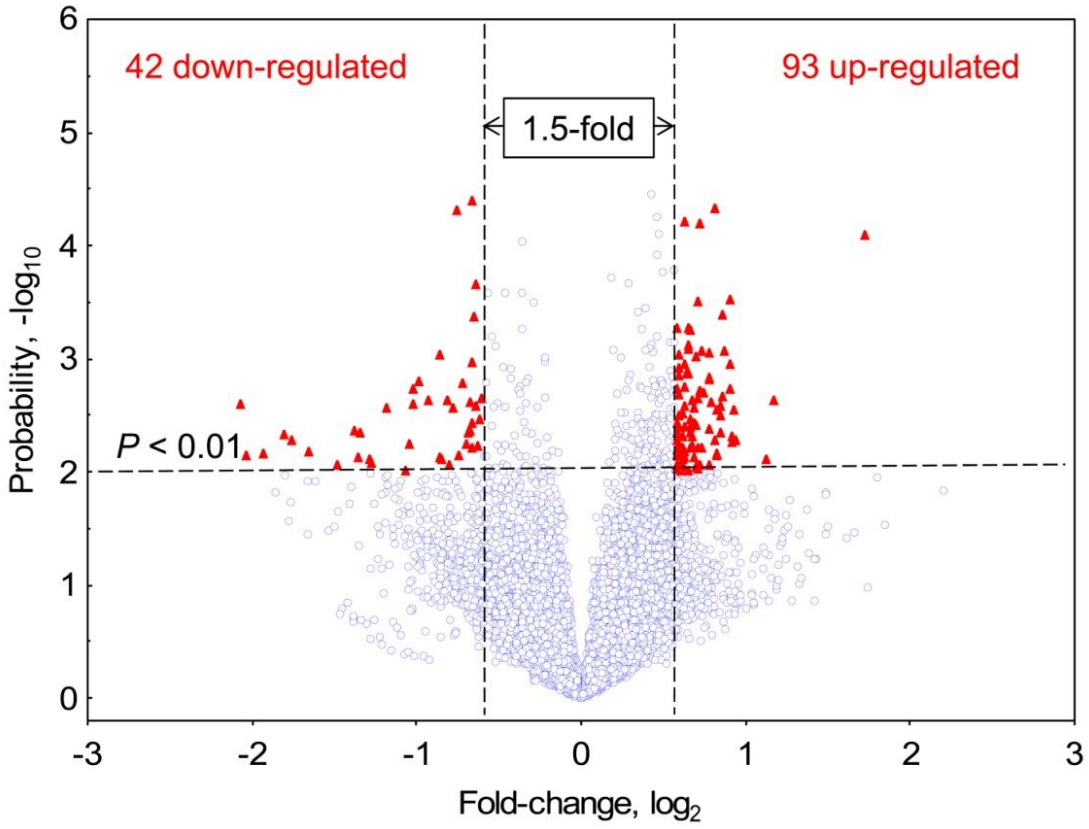


Figure 5.1. Volcano plot showing the effects of maternal irradiation on the pattern of gene expression in their first-generation offspring compared to the offspring of non-irradiated parents. The $-\log_{10}$ transformed values of probability from the *t*-test are plotted against the $\log_2$ transformed values of fold-change.

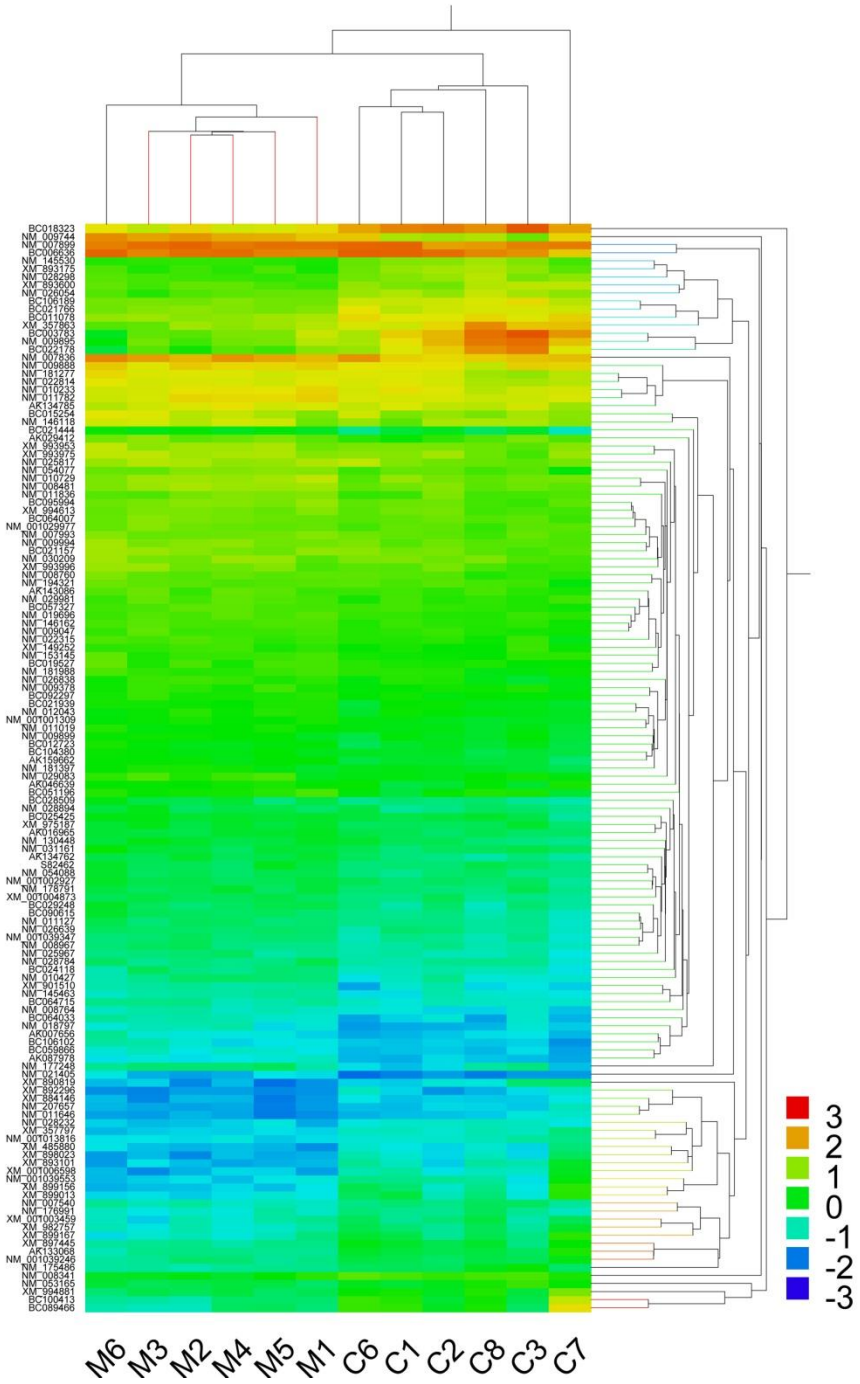


Figure 5.2. Heat map showing the clustering of the offspring of control and irradiated females according to the pattern of their gene expression. Each row represents single gene and each column represents single sample. The expression ratio graded from green (no differences) upwards to the red (the highly up-regulated) or downwards to the blue (the highly down-regulated). M1-M6 and C1-C7 denote the offspring irradiated and control females, respectively.

Table 5.1. List of significantly up-regulated genes in the offspring of irradiated females

Accession number	**Symbol**	**Gene name**	**Control ± sem**	**F_1 ± sem**	**Fold-change**	***t***	**Prob**
NM_009744	*Bcl6*	B cell leukemia/lymphoma 6	11.32 ± 0.26	13.06 ± 0.07	3.32	6.35	8.35×10^{-5}
BC021444	*Cpxm2*	Carboxypeptidase X 2 (M14 family)	8.07 ± 0.33	9.20 ± 0.07	2.18	3.30	0.0080
NM_010427	*Hgf*	Hepatocyte growth factor	6.88 ± 0.20	7.82 ± 0.18	1.93	3.55	0.0053
NM_054077	*Prelp*	Proline arginine-rich end leucine-rich repeat	9.98 ± 0.21	10.92 ± 0.11	1.91	3.92	0.0029
BC064033	*Fam180a*	Family with sequence similarity 180, member A	6.22 ± 0.24	7.14 ± 0.09	1.90	3.58	0.0050
AK159662	*Srpx*	Sushi-repeat-containing protein	8.44 ± 0.15	9.35 ± 0.07	1.88	5.39	0.0003
BC028509	*Slpi*	Secretory leukocyte peptidase inhibitor	7.62 ± 0.13	8.54 ± 0.17	1.88	4.18	0.0019
AK134762	*Vipr1*	Vasoactive intestinal peptide receptor 1	7.62 ± 0.17	8.53 ± 0.11	1.88	4.50	0.0011
BC090615	*Larp6*	La ribonucleoprotein domain family, member 6	7.40 ± 0.17	8.27 ± 0.09	1.84	4.69	0.0009
NM_008481	*Lama2*	Laminin, alpha 2	10.42 ± 0.13	11.29 ± 0.11	1.83	5.18	0.0004
AK029412	*Polr3e*	Polymerase (RNA) III (DNA directed) polypeptide E	9.91 ± 0.18	10.78 ± 0.11	1.82	4.09	0.0022
NM_029083	*Ddit4*	DNA-damage-inducible transcript 4	9.08 ± 0.09	9.94 ± 0.20	1.81	3.97	0.0026
BC029248	*Retnla*	Resistin like alpha	7.50 ± 0.20	8.36 ± 0.12	1.81	3.64	0.0046
NM_010729	*Loxl1*	Lysyl oxidase-like 1	10.50 ± 0.17	11.35 ± 0.15	1.80	3.84	0.0033
NM_018797	*Plxnc1*	Plexin C1	6.06 ± 0.18	6.89 ± 0.18	1.78	3.35	0.0074
XM_993975	*Gm8951*	Predicted gene 8951	10.67 ± 0.20	11.50 ± 0.09	1.78	3.91	0.0029
NM_026838	*Srpx2*	Sushi-repeat-containing protein, X-linked 2	9.09 ± 0.21	9.92 ± 0.14	1.78	3.38	0.0070
NM_007836	*Gadd45a*	Growth arrest and DNA-damage-inducible 45 alpha	12.28 ± 0.08	13.09 ± 0.09	1.76	6.79	4.78×10^{-5}
NM_146118	*Slc25a25*	Solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	10.91 ± 0.14	11.73 ± 0.19	1.76	3.53	0.0055
XM_993996	*3110040M04Rik*	RIKEN cdna 3110040M04 gene	10.10 ± 0.14	10.90 ± 0.14	1.74	4.02	0.0024
BC015254	*Cxcr7*	Chemokine (C-X-C motif) receptor 7	10.92 ± 0.14	11.71 ± 0.16	1.73	3.68	0.0042
BC095994	*Egflam*	EGF-like, fibronectin type III and laminin G domains	10.20 ± 0.15	10.98 ± 0.11	1.73	4.29	0.0016
BC024118	*Shisa2*	Shisa homolog 2 (Xenopus laevis)	6.98 ± 0.13	7.77 ± 0.20	1.72	3.23	0.0090
NM_145463	*Shisa2*	Shisa homolog 2 (Xenopus laevis)	6.70 ± 0.12	7.32 ± 0.13	1.54	3.45	0.0062
XM_993953	*Gm8947*	Predicted gene 8947	10.54 ± 0.12	11.32 ± 0.12	1.72	4.66	0.0009

Table 5.1. continued

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
NM_025817	*Tril*	TLR4 interactor with leucine-rich repeats	10.62 ± 0.15	11.40 ± 0.10	1.72	4.33	0.0015
NM_181277	*Col14a1*	Collagen, type XIV, alpha 1	11.39 ± 0.18	12.14 ± 0.04	1.69	4.13	0.0020
BC019527	*Smoc2*	SPARC related modular calcium binding 2	9.41± 0.08	10.15 ± 0.14	1.67	4.67	0.0009
NM_022315	*Smoc2*	SPARC related modular calcium binding 2	9.47 ± 0.16	10.20 ± 0.06	1.66	4.16	0.0019
NM_001039347	*Kcnd3*	Potassium voltage-gated channel, Shal-related family, member 3	7.25 ± 0.19	7.99 ± 0.09	1.67	3.44	0.0063
NM_009994	*Cyp1b1*	Cytochrome P450, family 1, subfamily b, polypeptide 1	10.28 ± 0.09	11.02 ± 0.07	1.66	6.54	6.52×10^{-5}
NM_130448	*Pcdh18*	Protocadherin 18	8.02 ± 0.18	8.75 ± 0.13	1.66	3.23	0.0090
BC106102	*Sytl3*	Synaptotagmin-like 3	6.08 ± 0.14	6.80 ± 0.18	1.64	3.19	0.0096
BC021157	*Hpgd*	Hydroxyprostaglandin dehydrogenase 15 (NAD)	10.38 ± 0.14	11.10 ± 0.11	1.64	4.05	0.0023
NM_146162	*Tmem119*	Transmembrane protein 119	9.61 ± 0.11	10.33 ± 0.07	1.64	5.36	0.0003
BC051196	*Retn*	Resistin	9.08 ± 0.15	9.79 ± 0.14	1.64	3.44	0.0063
NM_153145	*Abca8a*	ATP-binding cassette, sub-family A (ABC1), member 8a	9.43 ± 0.12	10.14 ± 0.10	1.63	4.59	0.0010
NM_181988	*Rerg*	RAS-like, estrogen-regulated, growth-inhibitor	9.36 ± 0.17	10.06 ± 0.08	1.62	3.72	0.0040
NM_011836	*Lamc3*	Laminin gamma 3	10.07 ± 0.17	10.77 ± 0.14	1.62	3.23	0.0091
BC059866	*Shc2*	SHC (Src homology 2 domain containing) transforming protein 2	6.08 ± 0.14	6.78 ± 0.11	1.62	3.95	0.0027
BC021939	*Ogn*	Osteoglycin	8.91 ± 0.17	9.60 ± 0.08	1.62	3.75	0.0038
NM_008760	*Ogn*	Osteoglycin	9.92 ± 0.16	10.60 ± 0.06	1.60	4.04	0.0023
NM_025967	*D16Ertd472e*	DNA segment, Chr 16, ERATO Doi 472, expressed	7.09 ± 0.16	7.78 ± 0.13	1.61	3.34	0.0075
XM_001004873	*Colec11*	Collectin sub-family member 11	7.68 ± 0.15	8.36 ± 0.08	1.61	4.04	0.0024
BC104380	*Colec11*	Collectin sub-family member 11	8.60 ± 0.13	9.26 ± 0.05	1.58	4.70	0.0008
NM_021405	*Cst10*	Cystatin 10 (chondrocytes)	5.46 ± 0.13	6.14 ± 0.15	1.61	3.46	0.0061

Table 5.1. continued

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
NM_181397	*Rftn1*	Raftlin lipid raft linker 1	8.72 ± 0.13	9.40 ± 0.14	1.60	3.58	0.0050
BC012723	*Igfbp6*	Insulin-like growth factor binding protein 6	8.60 ± 0.16	9.27 ± 0.08	1.59	3.71	0.0041
NM_008967	*Ptgir*	Prostaglandin I receptor (IP)	7.25 ± 0.14	7.91 ±0.12	1.59	3.63	0.0046
NM_007899	*Ecm1*	Extracellular matrix protein 1	13.02 ± 0.12	13.69 ± 0.07	1.59	4.95	0.0006
NM_029981	*Adamtsl2*	ADAMTS-like 2	9.70 ± 0.13	10.36 ± 0.12	1.59	3.80	0.0035
NM_011127	*Prrx1*	Paired related homeobox 1	7.45 ± 0.17	8.12 ± 0.07	1.59	3.71	0.0041
BC025425	*Lpar1*	Lysophosphatidic acid receptor 1	8.26 ± 0.18	8.92 ± 0.08	1.59	3.45	0.0063
NM_011782	*Adamts5*	A disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif, 5 (aggrecanase-2)	11.63 ± 0.08	12.29 ± 0.13	1.58	4.37	0.0014
NM_026639	*Art4*	ADP-ribosyltransferase 4	7.44 ± 0.20	8.10 ± 0.04	1.58	3.17	0.0100
NM_019696	*Cpxm1*	Carboxypeptidase X 1 (M14 family)	9.70 ± 0.09	10.36 ± 0.10	1.58	4.99	0.0005
BC057327	*Gucy1a3*	Guanylate cyclase 1, soluble, alpha 3	9.70 ± 0.11	10.35 ± 0.08	1.58	4.76	0.0008
AK143086	*Klhl25*	Kelch-like 25 (Drosophila)	9.77 ± 0.08	10.42 ± 0.12	1.56	4.38	0.0014
NM_001001309	*Itga8*	Integrin alpha 8	8.85 ± 0.10	9.49 ± 0.10	1.56	4.50	0.0011
NM_028894	*Lonrf3*	LON peptidase N-terminal domain and ring finger 3	7.85 ± 0.11	8.49 ± 0.15	1.56	3.40	0.0068
NM_022814	*Svep1*	Sushi, von Willebrand factor type A, EGF and pentraxin domain containing 1	11.45 ± 0.14	12.08 ± 0.04	1.55	4.22	0.0018
NM_054088	*Pnpla3*	Patatin-like phospholipase domain containing 3	7.76 ± 0.05	8.39 ± 0.08	1.55	6.57	6.27E-05
NM_009047	*Rem1*	Rad and gem related GTP binding protein 1	9.56 ± 0.11	10.19 ± 0.12	1.55	3.95	0.0027
NM_178791	*Vstm4*	V-set and transmembrane domain containing 4	7.83 ± 0.13	8.46 ± 0.11	1.55	3.70	0.0041
BC006636	*Fbln5*	Fibulin 5	13.00 ± 0.18	13.63 ± 0.05	1.55	3.33	0.0076
NM_028784	*F13a1*	Coagulation factor XIII, A1 subunit	7.30 ± 0.13	7.92 ± 0.14	1.54	3.23	0.0090
XM_994613	*Cpm*	Carboxypeptidase M	10.19 ± 0.16	10.82 ± 0.10	1.54	3.30	0.0080
AK007656	*Cd209b*	CD209b antigen	6.15 ± 0.11	6.77 ± 0.14	1.54	3.58	0.0050
NM_031161	*Cck*	Cholecystokinin	8.11 ± 0.07	8.73 ± 0.14	1.54	3.86	0.0031

Table 5.1. continued

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
NM_012043	*Islr*	Immunoglobulin superfamily containing leucine-rich repeat	8.99 ± 0.14	9.61 ± 0.13	1.53	3.22	0.0091
NM_008764	*Tnfrsf11b*	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	6.50 ± 0.16	7.11 ± 0.11	1.53	3.18	0.0099
NM_030209	*Crispld2*	Cysteine-rich secretory protein LCCL domain containing 2	10.41 ± 0.13	11.01 ± 0.13	1.53	3.35	0.0074
S82462	*Gata6*	GATA binding protein 6	7.91 ± 0.08	8.52 ± 0.14	1.52	3.84	0.0033
NM_007993	*Fbn1*	Fibrillin 1	10.22 ± 0.12	10.83 ± 0.08	1.52	4.35	0.0014
NM_011019	*Osmr*	Oncostatin M receptor	8.87 ± 0.11	9.47 ± 0.07	1.52	4.63	0.0009
XM_149252	*Fam65c*	Family with sequence similarity 65, member C	9.37 ± 0.14	9.98 ± 0.09	1.52	3.59	0.0050
BC064715	*Tmem45a*	Transmembrane protein 45a	6.89 ± 0.07	7.49 ± 0.13	1.52	4.10	0.0021
BC092297	*Cdc42ep3*	CDC42 effector protein (Rho gtpase binding) 3	9.16 ± 0.08	9.76 ± 0.11	1.52	4.44	0.0013
AK134785	*Pdgfrb*	Platelet derived growth factor receptor, beta polypeptide	11.41 ± 0.13	12.01 ± 0.12	1.51	3.41	0.0066
AK046639	*Eif4g3*	Eukaryotic translation initiation factor 4 gamma, 3	8.90 ± 0.14	9.50 ± 0.07	1.51	3.71	0.0041
XM_975187	*Zcchc24*	Zinc finger, CCHC domain containing 24	8.18 ± 0.13	8.78 ± 0.12	1.51	3.49	0.0058
AK016965	*Wdr66*	WD repeat domain 66	8.14 ± 0.07	8.73 ± 0.10	1.51	4.98	0.0006
NM_009378	*Thbd*	Thrombomodulin	9.25 ± 0.11	9.84 ± 0.14	1.51	3.42	0.0065
BC064007	*S1pr3*	Sphingosine-1-phosphate receptor 3	10.20 ± 0.11	10.79 ± 0.09	1.51	4.19	0.0019
NM_194321	*Fxyd1*	FXYD domain-containing ion transport regulator 1	9.89 ± 0.18	10.49 ± 0.05	1.51	3.20	0.0095
NM_009888	*Cfh*	Complement component factor h	11.87 ± 0.15	12.46 ± 0.07	1.51	3.64	0.0046
NM_001002927	*Penk*	Preproenkephalin	7.91 ± 0.13	8.50 ± 0.09	1.51	3.76	0.0037
NM_009899	*Clca1*	Chloride channel calcium activated 1	8.79 ± 0.16	9.37 ± 0.08	1.50	3.25	0.0088
AK087978	*Dntt*	Deoxynucleotidyltransferase, terminal	6.05 ± 0.10	6.63 ± 0.13	1.50	3.53	0.0055
NM_001029977	*Gm4788*	Predicted gene 4788	10.14 ± 0.15	10.72 ± 0.09	1.50	3.35	0.0073
NM_010233	*Fn1*	Fibronectin 1	11.64 ± 0.14	12.22 ± 0.11	1.50	3.36	0.0072

Abbreviations: F_1 - the offspring of irradiated females; sem – standard error of mean; *t* – Student's test; Prob – probability of difference between the offspring of control and irradiated parents.

Table 5.2. List of significantly down-regulated genes in the offspring of irradiated females

Accession number	**Symbol**	**Gene name**	**Control ± sem**	**F_1 ± sem**	**Fold-change**	***t***	**Prob**
BC022178	*Cish*	Cytokine inducible SH2-containing protein	12.10 ± 0.33	10.04 ± 0.40	4.18	3.99	0.0026
BC003783	*Cish*	Cytokine inducible SH2-containing protein	12.55 ± 0.37	10.62 ± 0.43	3.79	3.38	0.0070
NM_009895	*Cish*	Cytokine inducible SH2-containing protein	12.36 ± 0.33	10.61 ± 0.37	3.37	3.53	0.0054
XM_899156	*LOC434113*	Vomeronasal 2, receptor 44	7.81 ± 0.46	6.15 ± 0.15	3.15	3.41	0.0067
XM_899167	*LOC434113*	Vomeronasal 2, receptor 44	8.33 ± 0.32	6.98 ± 0.25	2.55	3.33	0.0077
XM_899013	*LOC624512*	Vomeronasal 2, receptor33	7.94 ± 0.44	6.46 ± 0.11	2.78	3.25	0.0088
XM_893101	*LOC628368*	Vomeronasal 2, receptor, pseudogene 37	7.32 ± 0.33	5.94 ± 0.18	2.59	3.66	0.0044
XM_001006598	*LOC668964*	Predicted gene 10922	7.28 ± 0.35	5.94 ± 0.12	2.53	3.62	0.0047
XM_890819	*LOC625353*	Vomeronasal 2, receptor 35	6.98 ± 0.33	5.69 ± 0.21	2.43	3.31	0.0078
NM_001039553	*4930467E23Rik*	RIKEN cdna 4930467E23 gene	7.58 ± 0.35	6.31 ± 0.16	2.41	3.27	0.0084
XM_357863	*Irs2*	Insulin receptor substrate 2	12.13 ± 0.20	11.09 ± 0.22	2.05	3.50	0.0058
AK133068		Predicted gene 10683	8.57 ± 0.21	7.56 ± 0.14	2.02	3.99	0.0026
XM_897445	*LOC622981*	Predicted gene 6379	8.57 ± 0.22	7.56 ± 0.11	2.02	4.17	0.0019
XM_982757	*LOC666285*	Predicted gene 8024	7.97 ± 0.19	6.99 ± 0.13	1.97	4.28	0.0016
NM_175486	*6430571L13Rik*	RIKEN cdna 6430571L13 gene	8.40 ± 0.20	7.48 ± 0.10	1.90	4.04	0.0023
XM_357797	*Gm1447*	Vomeronasal 1 receptor 119	7.10 ± 0.15	6.24 ± 0.11	1.81	4.63	0.0009
XM_892296	*LOC627618*	Vomeronasal 2, receptor, pseudogene 122	6.35 ± 0.24	5.49 ± 0.08	1.81	3.34	0.0075
BC018323	*Dbp*	D site albumin promoter binding protein	12.96 ± 0.16	12.16 ± 0.11	1.75	4.04	0.0024

Table 5.2. continued

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
BC106189	*Acvr2b*	Activin receptor IIB	11.60 ± 0.10	10.85 ± 0.05	1.68	6.76	0.0000
NM_008341	*Igfbp1*	Insulin-like growth factor binding protein 1	9.91 ± 0.10	9.17 ± 0.19	1.66	3.36	0.0073
NM_145530	*Rhov*	Ras homolog gene family, member V	10.59 ± 0.16	9.87 ± 0.06	1.64	4.25	0.0017
NM_011646	*Try4*	Trypsin 4	6.30 ± 0.16	5.60 ± 0.12	1.62	3.50	0.0058
NM_053165	*Clec2h*	C-type lectin domain family 2, member h	9.12 ± 0.18	8.45 ± 0.06	1.60	3.63	0.0046
BC021766	*Clec2h*	C-type lectin domain family 2, member h	11.61 ± 0.04	10.95 ± 0.08	1.57	6.92	0.0000
NM_207657	*5031410I06Rik*	RIKEN cdna 5031410I06 gene	6.33 ± 0.15	5.66 ± 0.11	1.59	3.65	0.0045
NM_026054	*2810474O19Rik*	RIKEN cdna 2810474O19 gene	11.08 ± 0.10	10.42 ± 0.13	1.58	4.02	0.0024
NM_176991	*Adam28*	A disintegrin and metallopeptidase domain 28	7.57 ± 0.14	6.91 ± 0.11	1.58	3.74	0.0038
NM_028298	*Zfp655*	Zinc finger protein 655	10.83 ± 0.13	10.17 ± 0.06	1.57	4.53	0.0011
NM_028232	*Sgol1*	Shugoshin-like 1 (S. Pombe)	6.82 ± 0.09	6.17 ± 0.09	1.57	5.15	0.0004
XM_884146	*LOC619775*	Vomeronasal 2, receptor, pseudogene 134	6.31 ± 0.09	5.67 ± 0.13	1.55	3.95	0.0027
BC011078	*Fus*	Fusion, derived from t(12;16) malignant liposarcoma (human)	11.91 ± 0.06	11.28 ± 0.09	1.55	5.60	0.0002
NM_001013816	*LOC434459*	Predicted gene 5622	7.06 ± 0.15	6.43 ± 0.10	1.54	3.48	0.0059
XM_893600	*LOC245174*	Zinc finger protein 937	11.01 ± 0.14	10.40 ± 0.07	1.53	3.79	0.0035
NM_007540	*Bdnf*	Brain derived neurotrophic factor	7.78 ± 0.12	7.18 ± 0.09	1.52	4.07	0.0023

Abbreviations: F_1 – the offspring of irradiated females; sem – standard error of mean; *t* – Student's test; Prob – probability of difference between the offspring of control and irradiated parents

When the expression profile of the offspring of irradiated females was compared with the controls, 135 transcripts showed significant changes. Among them, 125 transcripts (102 genes) represented genes with known function.

Using the BioProfiling.de toolkit, the loci in the offspring of irradiated females showing significant changes in the pattern of gene expression were subjected to the gene ontology (GO) analysis. In total, six functional groups of genes related to the structure and functions of the extracellular matrix showed significant enrichment (Table 5.3).

The first group that included 32 gene encoded proteins located in the extracellular region which included *Bdnf, Cck, Cfh, Col14a1, Ecm1, Fbn1, Fn1, Igfbp1, Igfbp6, Lama2, Loxl1, Ogn, Tnfrsf11b, Penk1, Slpi, Adamts5, Fbln5, Lamc3, Islr, Cpxm2, Cpxm1, Retnla Retn, Smoc2, Svep1, Srpx2, Colec11, F13a1, Adamtsl2, Crispld2, Prelp,* and *Au040377.* In addition, *Col14a1, Ecm1, Fbn1, Fn1, Lama2, Loxl1, Ogn, Tnfrsf11b, Adamts5, Lamc3, Smoc2, Adamtsl2, Prelp,* and *Au040377* were categorised as proteinaceous extracellular matrix. Of these, 7 differentially expressed genes code proteins that belong to the extracellular matrix (*Fbn1, Fn1, Lama2, Loxl1, Adamts5, Lamc3, Smoc2, Adamtsl2, Crispld2,* and *Au040377*). Another group included 7 genes (*Fn1, Tnfrsf11b, Fbln5, Smoc2, Crispld2, Itga8,* and *Au040377*) implicated in extracellular matrix organisation. The eleven genes *Cck, Cfh, Ecm1, Hgf, Igfbp1, Igfbp6, Thbd, Fbln5, Cpxm2, Cpxm1,* and *Retn* comprise the fifth group that have a role in the extracellular space. The final group included 5 genes (*Fn1, Lama2, Lamc3, Smoc2,* and *Au040377*) that have a role in the organisation of the basement membrane.

Another significantly enriched GO category included gene coding proteins located in the extracellular matrix. Eleven of the differentially expressed genes were assigned to this functional pathway: *Col14a1, Fn1, Lama2, Fbln5, Lamc3, Cpxm2, Cpxm1, Svep1, Srpx2, Pcdh18,* and *Itga8*. The *Cfh, Fn1, Smoc2, Crispld2,* and *Prelp* genes are implicated in the non-covalent binding with heparin or any member of the glycosaminoglycans.

There was a significant deregulation of genes belonging to the GO categories involved in the organisation and/or the maintenance of the cell membrane among the offspring of irradiated females. Among them 32 genes code proteins located in plasma membrane (Table 5.3). In addition, 8 significantly deregulated genes (*Clca1, Edg3, Osmr, Pdgfrb, Tmem45a, Kcnd3, Pcdh18,* and *Clec2h*) code integral membrane proteins, 15 genes code plasma membrane proteins, and 17 genes are implicated in signalling from receptors located on the surface of the cell via molecules located within the cell. The rest of the significantly deregulated genes belong to a number of GO categories implicated in protein binding, peptidase activity, binding, calcium ion binding, metallopeptidase activity, and proteolysis.

In summary, it should be noted that the majority of the differentially expressed genes following maternal irradiation were grouped into functional classes that have a role in the structure or function of the plasma membrane, extracellular matrix, and cell adhesion. In other words, most of them belonged to the functional pathways that are involved in cell-to-cell communication and organisation of the extracellular compartment. Interestingly, none of those genetic pathways have been implicated in the maintenance of genome stability.

Table 5.3. Gene ontologies for 135 transcripts modulated differentially in the offspring of irradiated females

GO term	Description	Odds ratio	*p*-value	Genes
GO:0005576	Extracellular region	6.29	1.18×10^{-14}	*Bdnf, Cck, Cfh, Col14a1, Ecm1, Fbn1, Fn1, Igfbp1, Igfbp6, Lama2, Loxl1, Ogn, Tnfrsf11b, Penk1, Slpi, Adamts5, Fbln5, Lamc3, Islr, Cpxm2, Cpxm1, Retnla Retn, Smoc2, Svep1, Srpx2, Colec11, F13a1, Adamtsl2, Crispld2, Prelp, Au040377*
GO:0005578	Proteinaceous extracellular matrix	16.21	1.70×10^{-10}	*Col14a1, Ecm1, Fbn1, Fn1, Lama2, Loxl1, Ogn, Tnfrsf11b, Adamts5, Lamc3, Smoc2, Adamtsl2, Prelp, Au040377*
GO:0031012	Extracellular matrix	25.69	5.65×10^{-9}	*Fbn1, Fn1, Lama2, Loxl1, Adamts5, Lamc3, Smoc2, Adamtsl2, Crispld2, Au040377*
GO:0030198	Extracellular matrix organization	25.00	1.10×10^{-5}	*Fn1, Tnfrsf11b, Fbln5, Smoc2, Crispld2, Itga8, Au040377*
GO:0007155	Cell adhesion	7.02	0.0004	*Col14a1, Fn1, Lama2, Fbln5, Lamc3, Cpxm2, Cpxm1, Svep1, Srpx2, Pcdh18, Itga8*
GO:0005615	Extracellular space	5.47	0.0046	*Cck, Cfh, Ecm1, Hgf, Igfbp1, Igfbp6, Thbd, Fbln5, Cpxm2, Cpxm1, Retn*
GO:0005604	Basement membrane	19.53	0.0049	*Fn1, Lama2, Lamc3, Smoc2, Au040377*
GO:0006508	Proteolysis	5.13	0.0100	*Adam28, Hgf, Adamts5, Cpxm2, Cpxm,1 Cpm, Lonrf3*
GO:0016020	Membrane	1.67	0.0100	*Acvr2b, Cmkor1, Cyp1b1, Adam28, Edg3, Edg2, Osmr, Pdgfrb, Ptgir, Rem1, Thbd, Plxnc1, Fxyd1, Tmem45a, Kcnd3, Svep1, 1200009o22rik, Cd209b, Cpm, Pcdh18, 2310015n21rik, Clec2h, Adpn, Abca8a, Tmem46, Slc25a25, Rhov, Tmem119, Rerg, 6430571l13rik, Itga8, Cdc42ep3*

Table 5.3. continued

GO term	Description	Odds ratio	*p*-value	Genes
GO:0005515	Protein binding	2.00	0.0100	*Acvr2b, Bcl6, Bdnf, Cck, Cish, Gadd45a, Fn1, Gata6, Loxl1, Ogn, Tnfrsf11b, Pdgfrb, Islr, Kcnd3, 1200009o22rik, Pcdh18, Lonrf3, Sytl3, Prelp, Synpo2, Klhl25, Shc2, Eif4g3, Cdc42ep3, Au040377, Irs2*
GO:0005509	Calcium ion binding	4.44	0.0100	*Fbn1, Thbd, Fbln5, Smoc2, Svep1, Pcdh18, Slc25a25*
GO:0008201	Heparin binding	16.09	0.0100	*Cfh, Fn1, Smoc2, Crispld2, Prelp*
GO:0008237	Metallopeptidase activity	9.39	0.0100	*Adam28, Adamts5, Cpxm1, Cpm, Adamtsl2*
GO:0005488	Binding	3.75	0.0100	*Fbn1, Hpgd, Thbd, Cd209b, 2310033k02rik, Colec11, Lonrf3, Clec2h, Slc25a25, Eif4g3*
GO:0007165	Signal transduction	2.65	0.0100	*Acvr2b, Cmkor1, Edg2, Tnfrsf11b, Osmr, Pdgfrb, Ptgir, Rem1, Thbd, Plxnc1, Smoc2, Cd209b, Clec2h, Rhov, Rerg, Itga8, Irs2*
GO:0008233	Peptidase activity	3.94	0.0100	*Adam28, Slpi, Try4, Adamts5, Cpxm1, Cpm, Adamtsl2*
GO:0005887	Integral to plasma membrane	5.83	0.0100	*Clca1, Edg3, Osmr, Pdgfrb, Tmem45a, Kcnd3, Pcdh18, Clec2h*
GO:0005886	Plasma membrane	1.95	0.0500	*Cfh, Cish, Cmkor1, Adam28, Edg3, Edg2, Ptgir, Thbd, Kcnd3, Cpm, Pcdh18, 2310015n21rik, Clec2h, Abca8a, Rhov*
GO:0046872	Metal ion binding	1.75	0.0800	*Acvr2b, Bcl6, Cyp1b1, Adam28, Gata6, Loxl1, Dntt, Adamts5, Cpxm1, Kcnd3, Cd209b, Cpm, 2310047a01rik, F13a1, Lonrf3, Rhov*
GO:0008270	Zinc ion binding	2.20	0.1500	*Bcl6, Adam28, Gata6, Adamts5, Cpxm2, Cpxm,1 Cpm, 2310047a01rik, Lonrf3, Adamtsl2*
GO:0004872	Receptor activity	1.69	0.1600	*Acvr2b, Cmkor1, Edg3, Edg2, Tnfrsf11b, Osmr, Pdgfrb, Ptgir, Thbd, Plxnc1, Cd209b, Clec2h, Itga8, Irs2*

5.3.3 The effects of paternal irradiation on the expression profile

A comparison of the pattern of gene expression in the offspring of controls and irradiated male mice has revealed a number of significantly deregulated loci. The expression of 74 transcripts was significantly altered in the offspring of irradiated males, with 50 of them down-regulated and 24 up-regulated (Figure 5.3; Tables 5.4 and 5.5). Similar to the effects of maternal irradiation, hierarchical clustering of the differentially expressed genes revealed a clear differentiation between all the offspring of controls and irradiated males (Figure 5.4).

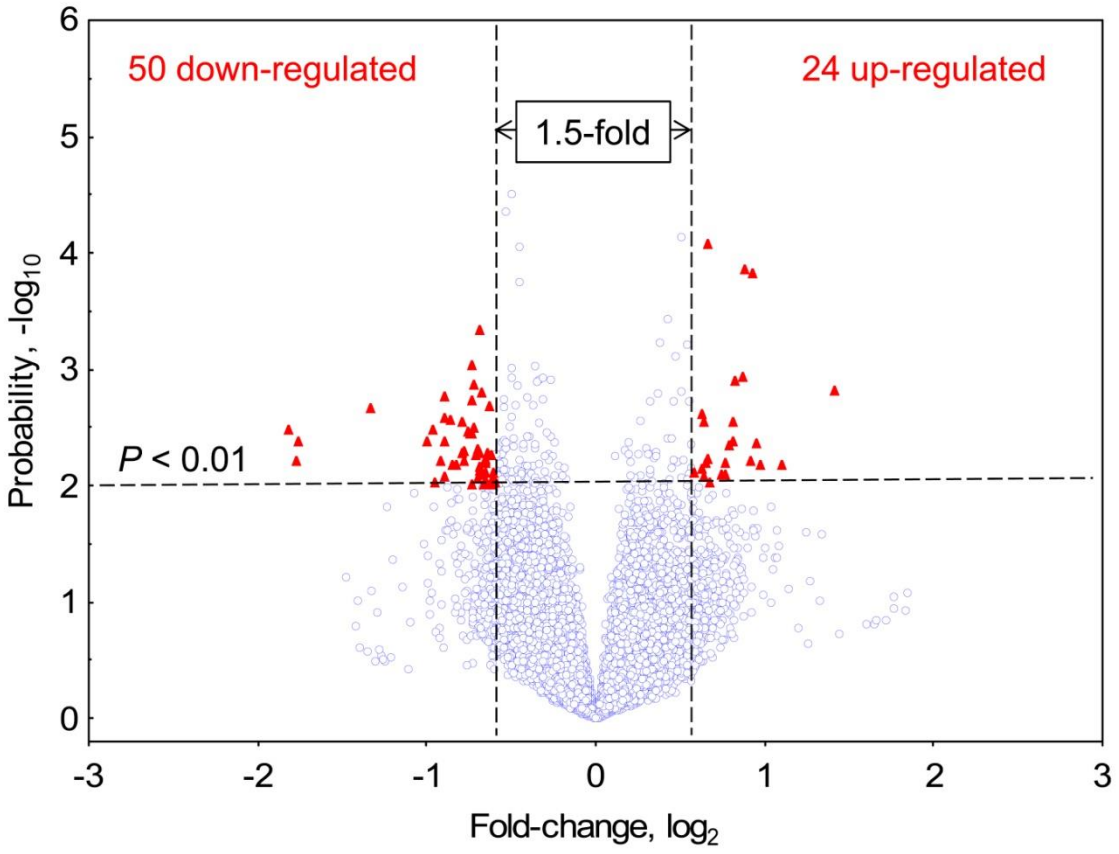


Figure 5.3. Volcano plot showing the effects of paternal irradiation on the pattern of gene expression in their first-generation offspring compared to the offspring of non-irradiated parents. The $-\log_{10}$ transformed values of probability from the *t*-test are plotted against the $\log_2$ transformed values of fold-change.

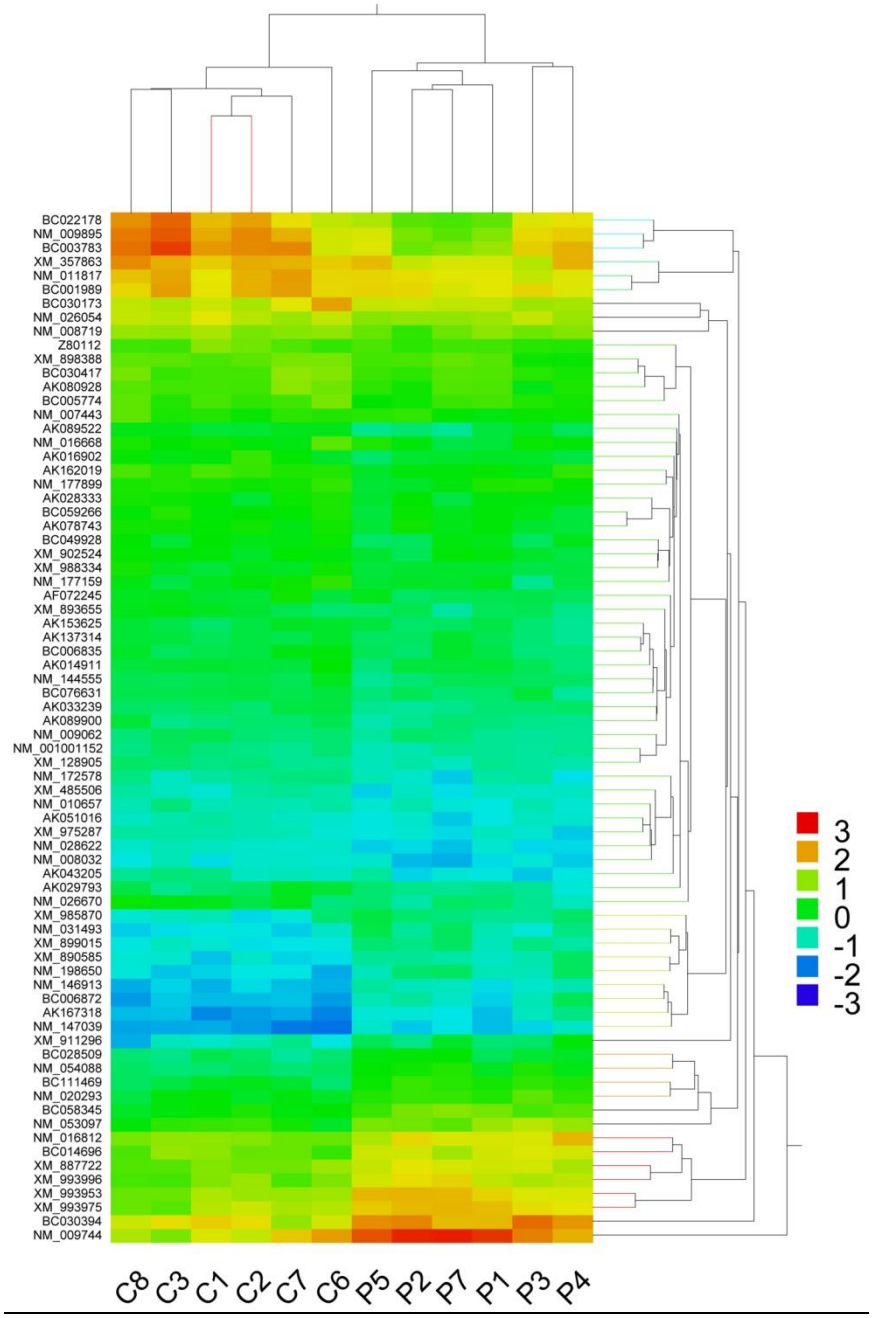


Figure 5.4. Heat map showing the clustering of the offspring of control and irradiated males according to the pattern of their gene expression. Each row represents single gene and each column represents single sample. The expression ratio graded from green (no differences) upwards to the red (the highly up-regulated) or downwards to the blue (the highly down-regulated). P1-P7 and C1-C7 denote the offspring irradiated and control males, respectively.

In contrast to the offspring of irradiated females, a completely different set of GO categories showed significant enrichment in this group (Table 5.6). They included the nucleus, DNA binding, and regulation of transcription as well as the intracellular compartment. In other words, the deregulation of a number of transcripts belonging to these functional categories could be implicated in the observed F_1 genomic destabilisation following paternal irradiation.

The most striking finding was that the highest enrichment was found for 19 genes involved in the function of cell nucleus. This group included *Bcl6, Dnase1, Aff2, Gsta2, Itch, Hivep3, Mbd2, Npas2, Zfpn1a4, Gadd45g, Banp, Fign, Upf3a, Pitpnc1, Luzp5, C79407, Vgll4, Zfp458,* and *D330038o06ri*.

Another group consisted of 7 genes known to have a role in DNA binding, including *Bcl6, Hivep3, Mbd2, Npas2, Zfpn1a4, Banp,* and *C79407*. Another enriched GO category is implicated in the regulation of transcription and included an overlapping set of transcripts *Bcl6, Hivep3, Mbd2, Npas2, Zfpn1a4, Banp,* and *Vgll4*. As individual transcripts, these genes have shown a statistical significant difference with more than 1.5 fold change. However, the enrichment of these GO categories failed to achieve statistical significance.

Furthermore, four genetic pathways involved in intracellular activity as well as metabolic activities were also deregulated in the offspring of irradiated fathers without achieving statistically significance as functional genetic pathways. The first gene class includes 9 genes involved in metabolic processes (*Cel, Dnase1, Gsta2, Slpi, Fign, Exod1, Ddx23, Adpn,* and*Hmgcs1*). The second category comprises 5 genes *Cel, Gsta2,*

Rgs4, Hmgcs1, and Irs2 that are involved in the cytosol. In addition, there are also 7 genes *Bcl6, Itch, Hivep3, Zfpn1a4, Exod1, Pitpnc1,* and *Plekhg3* that are grouped into a class of genes involved in the intracellular compartment. The GO category implicated in the hydrolase activity (*Cel, Dnase1, Exod1, Ddx23, Agbl3,* and *Adpn*) was also marginally enriched. Finally, six differentially expressed genes (*Cxcr4, Npas2, Rgs4, Olfr1348, Olfr1414,* and *Irs2*) were implicated in the signal transduction pathway.

Table 5.4. List of significantly up-regulated genes in the offspring of irradiated males

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
NM_009744	*Bcl6*	B cell leukemia/lymphoma 6	11.32 ± 0.26	12.75 ± 0.20	2.68	4.29	0.0016
NM_053097	*Cml3*	Camello-like 3	9.18 ± 0.23	10.16 ± 0.17	1.97	3.41	0.0067
BC014696	*Cml3*	Camello-like 3	10.12 ± 0.20	10.87 ± 0.10	1.68	3.28	0.0082
BC006872	*Cel*	Carboxyl ester lipase	5.79 ± 0.13	6.76 ± 0.23	1.95	3.65	0.0045
NM_054088	*Pnpla3*	Patatin-like phospholipase domain containing 3	7.76 ± 0.05	8.69 ± 0.15	1.91	5.90	0.0002
NM_198650	*Slc22a20*	Solute carrier family 22 (organic anion transporter), member 20	6.18 ± 0.14	7.11 ± 0.23	1.90	3.45	0.0062
XM_993953	*Gm8947*	Predicted gene 8947	10.54 ± 0.12	11.43 ± 0.09	1.85	5.95	0.0001
BC058345	*Lrp12*	Low density lipoprotein-related protein 12	8.86 ± 0.12	9.74 ± 0.16	1.84	4.49	0.0012
XM_993996	*3110040M04Rik*	RIKEN cdna 3110040M04 gene	10.10 ± 0.14	10.93 ± 0.12	1.78	4.41	0.0013
XM_993975	*Gm8951*	Predicted gene 8951	10.67 ± 0.20	11.49 ± 0.08	1.77	3.91	0.0029
NM_031493	*Xlr5c*	X-linked lymphocyte-regulated 5C	6.33 ± 0.10	7.13 ± 0.20	1.74	3.63	0.0046
AK167318	*Lrrc43*	Leucine rich repeat containing 43	5.62 ± 0.12	6.39 ± 0.19	1.71	3.42	0.0065
NM_016812	*Banp*	BTG3 associated nuclear protein	10.35 ± 0.12	11.12 ± 0.21	1.71	3.28	0.0083
BC030394	*Dnase1*	Deoxyribonuclease I	11.36 ± 0.16	12.04 ± 0.14	1.61	3.20	0.0094
XM_899015	*Xlr5b*	X-linked lymphocyte-regulated 5B	6.48 ± 0.10	7.15 ± 0.16	1.59	3.47	0.0061
XM_887722	*Alms1-ps2*	Alstrom syndrome 1, pseudogene 2	10.21 ± 0.08	10.88 ± 0.07	1.59	6.33	0.0001
BC028509	*Slpi*	Secretory leukocyte peptidase inhibitor	7.62 ± 0.13	8.28 ± 0.14	1.58	3.43	0.0064
NM_147039	*Olfr1414*	Olfactory receptor 1414	5.45 ± 0.15	6.10 ± 0.06	1.57	3.91	0.0029
XM_985870	*Xlr4a*	X-linked lymphocyte-regulated 4A	6.74 ± 0.17	7.39 ± 0.09	1.56	3.26	0.0086
NM_020293	*Cldn9*	Claudin 9	8.61 ± 0.15	9.24 ± 0.12	1.55	3.35	0.0074
NM_146913	*Olfr1348*	Olfactory receptor 1348	5.93 ± 0.12	6.56 ± 0.10	1.55	4.02	0.0025

Abbreviations: F_1 the offspring of irradiated males; sem standard error of mean; *t* – Student's test; Prob - probability of difference between the offspring of control and irradiated parents

Table 5.5. List of significantly down-regulated genes in the offspring of irradiated males

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
BC022178	***Cish***	Cytokine inducible SH2-containing protein	12.10 ± 0.33	10.29 ± 0.34	3.50	3.81	0.0034
BC003783	***Cish***	Cytokine inducible SH2-containing protein	12.55 ± 0.37	10.79 ± 0.36	3.39	3.44	0.0064
NM_009895	***Cish***	Cytokine inducible SH2-containing protein	12.36 ± 0.33	10.61 ± 0.34	3.37	3.68	0.0042
NM_026670	***Zmym1***	Zinc finger, MYM domain containing 1	8.48 ± 0.26	7.15 ± 0.19	2.51	4.08	0.0022
AK029793	***Agbl3***	ATP/GTP binding protein-like 3	8.00 ± 0.18	7.01 ± 0.20	1.99	3.69	0.0042
XM_357863	***Irs2***	Insulin receptor substrate 2	12.13 ± 0.20	11.18 ± 0.22	1.93	3.19	0.0096
AK016902	***Eri2***	Exoribonuclease 2	8.92 ± 0.20	8.01 ± 0.18	1.88	3.44	0.0063
AK162019	***Vgll4***	Vestigial like 4 (Drosophila)	9.51 ± 0.12	8.62 ± 0.17	1.85	4.23	0.0018
XM_893655	***Gm6654***	Predicted pseudogene 6654	8.34 ± 0.21	7.45 ± 0.18	1.85	3.26	0.0086
AK080928	***Plekhg3***	Pleckstrin homology domain containing, family G (with rhogef domain) member 3	9.95 ± 0.16	9.06 ± 0.18	1.85	3.69	0.0042
NM_011817	***Gadd45g***	Growth arrest and DNA-damage-inducible 45 gamma	12.01 ± 0.21	11.13 ± 0.08	1.84	3.96	0.0027
BC001989	***Gadd45g***	Growth arrest and DNA-damage-inducible 45 gamma	11.99 ± 0.21	11.21 ± 0.05	1.71	3.57	0.0051
AK043205	***Dlc1***	Deleted in liver cancer 1	7.11 ± 0.18	6.25 ± 0.12	1.81	3.93	0.0028
NM_177159	***9530091C08Rik***	RIKEN cdna 9530091C08 gene	8.81 ± 0.16	7.97 ± 0.19	1.79	3.41	0.0067
NM_172578	***Mis18bp1***	MIS18 binding protein 1	7.29 ± 0.11	6.47 ± 0.22	1.77	3.39	0.0069
BC030417	***Plekhg3***	Pleckstrin homology domain containing, family G (with rhogef domain) member 3	10.04 ± 0.18	9.26 ± 0.13	1.72	3.54	0.0053
BC049928	***Ddx23***	DEAD (Asp-Glu-Ala-Asp) box polypeptide 23	8.78 ± 0.09	8.01 ± 0.21	1.71	3.44	0.0063
NM_008719	***Npas2***	Neuronal PAS domain protein 2	10.58 ± 0.10	9.84 ± 0.17	1.67	3.77	0.0036
XM_128905	***Taf4b***	TAF4B RNA polymerase II, TATA box binding protein (TBP)-associated factor	7.53 ± 0.11	6.80 ± 0.14	1.66	4.18	0.0019

Table 5.5. Continued

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
AF072245	***Mbd2***	Methyl-cpg binding domain protein 2	8.55 ± 0.17	7.83 ± 0.09	1.66	3.76	0.0037
NM_026054	***2810474O19Rik***	RIKEN cdna 2810474O19 gene	11.08 ± 0.10	10.36 ± 0.12	1.65	4.62	0.0009
NM_028622	***Lce1c***	Late cornified envelope 1C	6.72 ± 0.07	6.00 ± 0.15	1.64	4.37	0.0014
AK078743	***Hmgcs1***	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	9.03 ± 0.08	8.32 ± 0.17	1.64	3.84	0.0033
BC006835	***Gigyf2***	GRB10 interacting GYF protein 2	8.35 ± 0.12	7.64 ± 0.16	1.63	3.51	0.0056
BC059266	***Fign***	Fidgetin	9.01 ± 0.08	8.32 ± 0.18	1.62	3.58	0.0050
AK014911	***Micalcl***	MICAL C-terminal like	8.41 ± 0.05	7.72 ± 0.21	1.61	3.24	0.0089
AK153625	***Pitpnc1***	Phosphatidylinositol transfer protein, cytoplasmic 1	8.24 ± 0.11	7.55 ± 0.16	1.61	3.54	0.0054
AK033239	***Upf3a***	UPF3 regulator of nonsense transcripts homolog A	7.93 ± 0.07	7.25 ± 0.11	1.60	5.09	0.0005
BC076631	***Ncapg2***	Non-SMC condensin II complex, subunit G2	8.15 ± 0.04	7.47 ± 0.20	1.60	3.28	0.0083
NM_177899	***Zfp866***	Zinc finger protein 866	9.27 ± 0.04	8.59 ± 0.20	1.60	3.33	0.0076
NM_016668	***Bhmt***	Betaine-homocysteine methyltransferase	9.14 ± 0.13	8.46 ± 0.15	1.60	3.38	0.0071
XM_988334	***Gm13570***	Predicted gene 13570	8.71 ± 0.13	8.04 ± 0.09	1.59	4.28	0.0016
AK137314	***Gm19537***	Predicted gene, 19537	8.22 ± 0.05	7.57 ± 0.20	1.57	3.18	0.0098
XM_485506	***Ajap1***	Adherens junction associated protein 1	6.99 ± 0.09	6.34 ± 0.17	1.57	3.30	0.0080
BC030173	***Gm10639***	Predicted gene 10639	11.32 ± 0.18	10.67 ± 0.06	1.56	3.45	0.0062
XM_902524	***Itch***	Itchy, E3 ubiquitin protein ligase	8.81 ± 0.10	8.16 ± 0.16	1.56	3.42	0.0066
Z80112	***Cxcr4***	Chemokine (C-X-C motif) receptor 4	9.89 ± 0.17	9.25 ± 0.07	1.56	3.46	0.0061
NM_144555	***AB041803***	Cdna sequence AB041803	8.13 ± 0.06	7.51 ± 0.17	1.55	3.53	0.0055
NM_001001152	***Zfp458***	Zinc finger protein 458	7.55 ± 0.10	6.92 ± 0.12	1.54	4.10	0.0022
NM_008032	***Aff2***	AF4/FMR2 family, member 2	6.59 ± 0.10	5.97 ± 0.14	1.53	3.53	0.0055
NM_010657	***Hivep3***	Human immunodeficiency virus type I enhancer binding protein 3	7.06 ± 0.11	6.45 ± 0.15	1.52	3.20	0.0095

Accession number	Symbol	Gene name	Control ± sem	F_1 ± sem	Fold-change	*t*	Prob
NM_009062	***Rgs4***	Regulator of G-protein signaling 4	7.74 ± 0.11	7.14 ± 0.15	1.52	3.26	0.0086
AK051016	***Haus6***	HAUS augmin-like complex, subunit 6	6.88 ± 0.12	6.27 ± 0.14	1.52	3.30	0.0080
NM_007443	***Ambp***	Alpha 1 microglobulin/bikunin	9.41 ± 0.16	8.82 ± 0.09	1.50	3.21	0.0094

Abbreviations: F_1 – the offspring of irradiated males; sem – standard error of mean; *t* – Student's test; Prob – probability of difference between the offspring of control and irradiated parents

Table 5.6. Gene ontologies for 74 transcripts modulated differentially in the offspring of irradiated males

GO term	Description	Odds ratio	*p*-value	Genes
GO:0005634	Nucleus	2.37	0.0100	*Bcl6, Dnase1, Aff2,Gsta2, Itch, Hivep3, Mbd2, Npas2, Zfpn1a4, Gadd45g, Banp, Fign, Upf3a, Pitpnc1, Luzp5, C79407, Vgll4, Zfp458, D330038o06rik*
GO:0008152	Metabolic process	2.69	0.0400	*Cel, Dnase1, Gsta2,Slpi, Fign, Exod1, Ddx23, Adpn, Hmgcs1*
GO:0005829	Cytosol	5.23	0.0600	*Cel, Gsta2, Rgs4, Hmgcs1, Irs2*
GO:0005622	Intracellular	3.21	0.0600	*Bcl6, Itch, Hivep3, Zfpn1a4, Exod1, Pitpnc1, Plekhg3*
GO:0006350	Transcription	2.39	0.2300	*Bcl6, Hivep3, Mbd2, Npas2, Zfpn1a4, Banp, Vgll4*
GO:0003677	Dna binding	2.32	0.2400	*Bcl6, Hivep3, Mbd2, Npas2, Zfpn1a4, Banp, C79407*
GO:0045449	Regulation of transcription	2.32	0.2400	*Bcl6, Hivep3, Mbd2, Npas2, Zfpn1a4, Banp, Vgll4*
GO:0016787	Hydrolase activity	2.14	0.3900	*Cel, Dnase1, Exod1, Ddx23, Agbl3, Adpn*
GO:0004871	Signal transducer activity	2.07	0.4100	*Cxcr4, Npas2, Rgs4, Olfr1348, Olfr1414, Irs2*

5.3.4 Comparison of the effects of maternal and paternal irradiation on the pattern of gene expression in their offspring

The global pattern of gene expression in the offspring of irradiated males and females was cross-compared. The differences between these two groups substantially exceeded those between the corresponding groups and controls. Thus, the expression of 246 transcripts significantly differed. Hierarchical clustering of the differentially expressed genes has revealed a clear differentiation between all of the offspring of irradiated males and females (Figure 5.7).

Among the 246 transcripts showing significant differences between the offspring of irradiated males and females, 132 transcripts were assigned to the known 17 GO categories. Twelve GO categories showed significant enrichment. They included those implicated in the cell adhesion, zinc ion binding, metallopeptidase activity, peptidase activity, proteinaceous extracellular matrix, extracellular region, proteolysis, metal ion binding, extracellular space, signal transduction, G-protein coupled receptor signalling pathway, receptor activity and the nucleus. The deregulation of three GO categories may potentially destabilise the F_1 genomes following paternal irradiation.

The signal transduction GO category (p = 0.01, odds ratio = 2.29) included 19 genes showing significant differences between the two groups (*Arntl, Cnr1, Srgap2, Npas2, P2rx1, Pdgfra, Per2, Prkg1, Stat1, Cart1, V2r5, Gng13, Colec12, Cntnap4, Npbwr1, Itga8, Olfr1230, Olfr1263,* and *Gpr31c*). The closely related signal transducer category (p = 0.01, odds ratio = 2.20) included largely overlapped set of 15 genes (*Arntl, Cnr1, Npas2, Per2, Rgs4, Stat1, V2r5, Gng13, Npbwr1, Olfr1287, Olfr1302, Olfr1258, Gipr, Olfr1252,* and *Gpr31c*). Another important functional group included 29 genes coding

the nuclear proteins (p = 0.03, odds ratio = 1.54: *Arntl, Bcl6b, Cenpa, Dbp, Dnmt3b, Lox, Mmp2, Ints6, Npas2, Per2, Prrx1, Pou5f1, Rbm3, Snai2, Stat1, Cart1, Gadd45g, Polr3e, Upf3a, Mcm10, Rkhd3, 5830484a20rik, Foxp2, Synpo2, Prc1, Brip1, Zfp458, Obox5,* and *9630020c08rik*). As a number of these proteins are involved in DNA repair and cell cycle regulation, their deregulation can also destabilise the genomes of offspring of irradiated male mice.

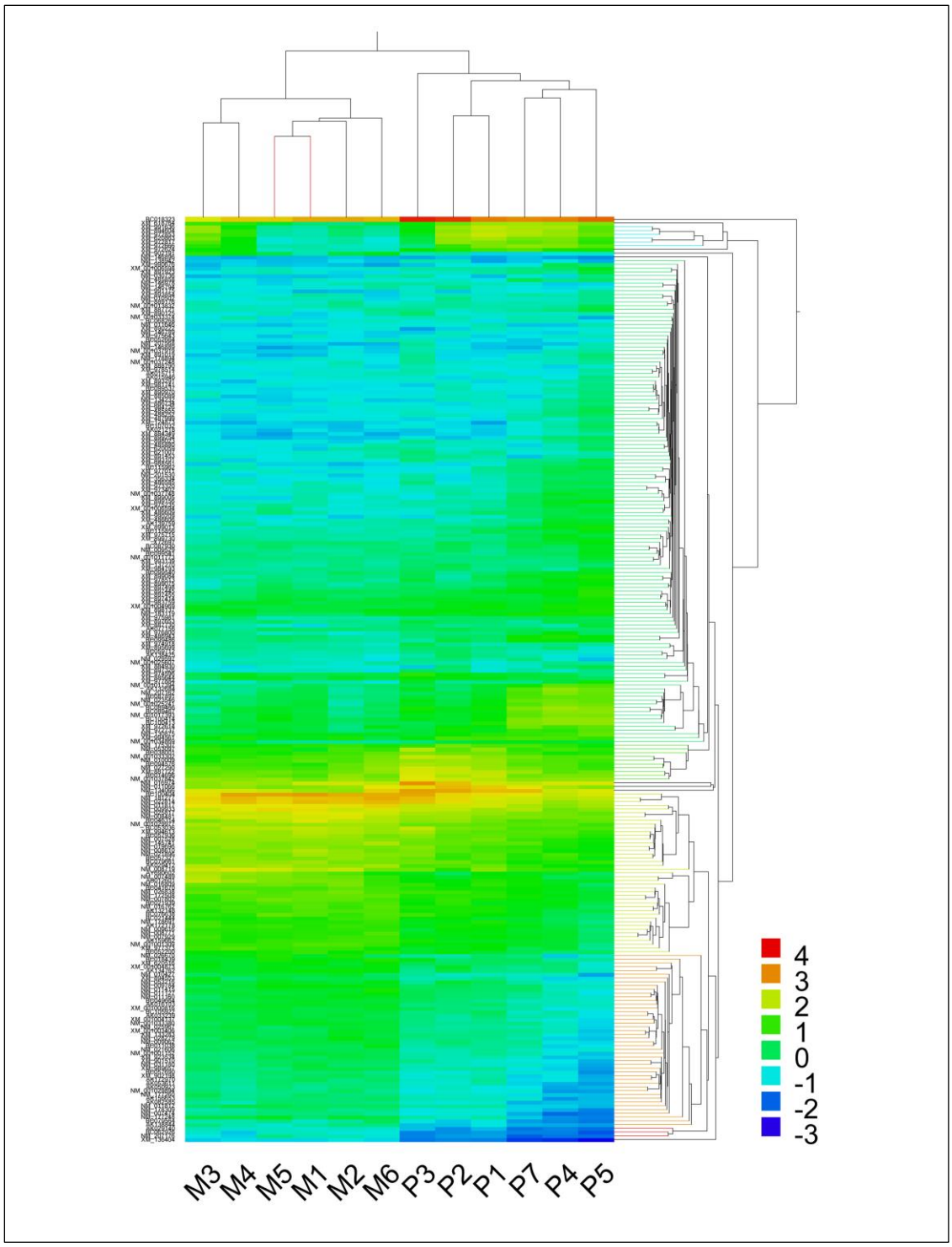


Figure 5.7: Heat map showing the clustering of the offspring of irradiated males and females according to the pattern of their gene expression. Each row represents single gene and each column represents single sample. The expression ratio graded from green (no differences) upwards to the red (the highly up-regulated) or downwards to the blue (the highly down-regulated). M1-M6 and P1-P7 denote the offspring irradiated females and females, respectively.

5.3.5 Conclusions

A comparison of the pattern of gene expression in the non-exposed first-generation offspring of control and irradiated parents has shown that:

- Parental irradiation significantly affects the pattern of expression in the non-exposed first-generation offspring.
- The pattern of gene expression in the offspring of irradiated males and females dramatically differs, showing no overlap between up- and down-regulated genes.
- Functional analysis and grouping into GO classes revealed that nearly half of the differentially expressed genes in the paternally irradiated group are grouped into pathways that are known to be implicated in genome instability. On the other hand, almost all of the differentially expressed genes following maternal irradiation can be grouped into gene classes that are not implicated in the maintenance of genome stability.
- The results of this work therefore provide further evidence for observed differences in the manifestation of transgenerational instability in the offspring of irradiated males and females.

5.4 Discussion

Chapter 4 part of this thesis established marked differences in the manifestation of transgenerational instability in the offspring of irradiated male and female mice. It was shown that in contrast to the offspring of irradiated males where the ESTR mutation rate was significantly elevated, maternal irradiation did not affect the genomic stability of their first-generation offspring. To gain insights into the mechanisms underlying such a difference in the transgenerational effects of paternal and maternal irradiation, a pilot study was designed to establish whether this dissimilarity may be attributed to some yet unknown changes affecting the pattern of gene expression in the offspring.

The analysis of the expression profiles in both groups of offspring has revealed markedly different expression patterns following paternal and maternal exposure. This remarkable dissimilarity in the transcriptional response was particularly pronounced following the gene ontology analysis, which revealed significant enrichment of completely different functional sets of genes in the two groups. It should be noted that no single gene among the 102 loci differentially expressed genes in the offspring of irradiated females can implicated in the maintenance of genome stability. In contrast, the observed deregulation of a number of genes in the offspring of irradiated males can potentially destabilise their genome. A detailed description of the function of these genes is presented below.

5.4.1 Nuclear matrix and regulation of gene expression

5.4.1.1 *Banp*

The expression of *Banp1* gene coding the *SMAR1* or scaffold/matrix attachment region binding protein1 is significantly down-regulated in the offspring of irradiated males (Table 5.4). This protein belongs to the nuclear matrix gene ontology group – a dynamic structural network that contains chromatin and ribonucleoprotein domains providing the structural environment for gene function (Berezney and Coffey, 1977; Smith *et al.*, 1984) by binding the nuclear proteins and assembling them into functional complexes involved in biochemical processes including DNA replication, repair and transcription (Zink *et al.*, 2004; Bode *et al.*, 2003). It also has a role in the spatial organisation of chromatin loop domains which are formed by attaching chromatin to specific DNA sequences called matrix attachment region (MARS) in the nuclear matrix (Bode *et al.*, 2003; Malonia, *et al.*, 2011). Disturbances in the chromatin structure can alter gene expression and genomic stability. Therefore, proteins that control chromatin organisation in the nuclear matrix are implicated in genomic instability and malignancy transformation. *SMAR1* or scaffold/matrix attachment region binding protein1 is a nuclear matrix associated proteins (Malonia, *et al.*, 2011; Chattopadhyay, *et al.*, 2000) with DNA binding ability that enables it to act as transcription modulator and chromatin modifier. Via its interaction with several important transcription factors such as *p53* and *NF-κB*, *SMAR1* modulates the expression of many genes involved in variety of cellular pathways.

Indeed, *SMAR1* plays a role in genome maintenance. It has been reported that *SMAR1* is able to interact with protein p53 which acts as a G1/S or G2/M check point regulator

and imposes both G1/S and G2/M arrests (Rampalli *et al.*, 2005; Singh, *et al.*, 2007). *SMAR1* is a stress responsive protein and its expression is induced upon treatment with chemotherapeutic agents like doxorubicin (Singh *et al.*, 2007). Treatment of cells that express wild type *p53* with DNA damaging agents such as doxorubicin leads to parallel induction of *p53* and *SMAR1* expression. It has also been shown that *SMAR1* and *p53* positively control each other in a positive feedback loop where the p53 protein initiates *SMAR1* transcription and *SMAR1* in turn stabilises *p53* to enable cell cycle arrest at G1/S or G2/M transition. *SMAR1* also has a role in damage induced apoptosis. It has been reported that depending on the extent of DNA damage, *SMAR1* modulates *p53* activity to decide the cell fate either towards arrest or apoptosis (Sinha *et al.*, 2010). And by its role in negative regulation of cyclin D1 expression, *SMAR1* can inhibit cell cycle progression. It interacts with histone deacetylase complex 1 (*HDAC1*), *SIN3* and pocket retinoblastoma to form a multi-protein repressor complex which is recruited to the MAR site on cyclin D1 promoter leading to more condensed chromatin at the gene site repressing its transcription. The reduced level of cyclin D1 abolishes its binding to CDK4/6 kinases which finally leads to inhibition of the synthesis of other cyclins needed for cell cycle progression (Klein and Assoian, 2008).

5.4.1.2 *Aff2*

The expression of the *Aff2* gene is significantly down-regulated in the offspring of irradiated males. The mammalian *Aff2*/*Fmr2* gene is X-linked and its protein AFF2/FMR2 is mainly localised to nuclear speckles which represent the sites for storage and modification of immature mRNA (pre-mRNA) splicing factors (Melko *et al.*, 2011) which very important for genome maintenance. Due to its high affinity to bind the G quadruplex RNA-forming structure, AFF2/FMR2 protein modulates

alternative splicing (Bensaid *et al.*, 2009). In addition, its over-expression along with *AFF3* and *AFF4* members of the family interfere with the organisation and biogenesis of nuclear speckles (Melko *et al.*, 2011). Silencing of this gene in humans results in non-syndromic intellectual disability (ID) and FRAXE mental retardation (Melko *et al.*, 2011; Gecz *et al.*, 1996; Gu *et al.*, 1996).

5.4.1.3 *Mbd2*

The gene *Mbd2* codes testis specific methyl-CpG binding protein MBD2 is down-regulated in the offspring of irradiated males. This protein is located in the nucleus and is involved in DNA methylation. DNA methylation has a role in inactivation of genetic imprinting during embryogenesis (Bourc'his *et al.*, 2001; Hata *et al.*, 2002), repression of transposons (Bourc'his and Bestor, 2004), and chromosomal stability (Eden *et al.*, 2003) with CpG methylation specifically being very important for chromatin structure and function. Repression of methylated genes is mediated by methyl cytosine binding domain proteins (MBD) including MDB2 which recruits histone deacetylases (HDAC) and transcription repressors to the chromatin. Compared to the other MCBPs, MBD2 targets numerous tumour suppressor genes and binds the densely methylated DNA with higher affinity (Berger and Bird, 2005; Fraga *et al.*, 2003; Tan and Nakielny, 2006; Mian *et al.*, 2011).

The MDB2 protein is also involved in maintaining the stability of the genome. Thus, Pogribny *et al.*, (2005) reported that fractionated low dose irradiation of C57/BL6 mice resulted in accumulation of DNA damage as indicated by γ-H2AX foci associated with global DNA hypomethylation in the thymus. This reduced methylation was accompanied with a decrease of methyltransferases as well as methyl-binding proteins

MBD2 and MeCP2.The results of this study indicates that the down-regulation of Mdb2 gene in the offspring of irradiated males (Table 5.5) can potentially contribute the transgenerational destabilisation of their genomes.

5.4.1.4 *Upf3a*

The *Upf3a* gene encodes a nuclear protein which along with other factors mediates the surveillance of mRNA via elimination of mRNA containing prematurely terminated codons through a process called nonsense-mediated mRNA decay (NMD)(Chamieh, *et al.*, 2008; Kervestin and Jacobson, 2012). Therefore, NMD serves as a quality control mechanism for degradation of faulty mRNA that can produce truncated proteins (Holbrook *et al.*, 2004). DNA damaging agents or mis-incorporation of nucleotides during either replication or transcription can result in a frame-shift or non-sense mutation that can lead to the formation of a premature translation termination codon which is mainly eradicated by NMD. Moreover, about 35% of alternatively spliced mRNAs are down-regulated by NMD (Matsuda *et al.*, 2008; Lareau *et al.*, 2004; Lewis *et al.*, 2003). The deregulation of this gene in the offspring of irradiated males may therefore affect the stability of their genomes.

5.4.2 Genes involved in cell cycle control and DNA damage response

5.4.2.1 *Bcl6*

The *Bcl6* gene encoding a transcriptional repressor B-cell lymphoma 6 protein is significantly up-regulated in the offspring of irradiated males (Table 5.4). The *Bcl6* gene is mapped to chromosome 3q27 (Miki *et al.*, 1994). In non-Hodgkin B-cell lymphoma, a chromosomal translocation involving the 5' non-coding region of the *BCL6* gene alters its expression (Kurosu *et al.*, 2003; Bastard *et al.*, 1994; Ohno and

Fukuhara, 1997). The BCL6 protein is implicated in the repression of ataxia telangiectasia and Rad3-related (*ATR*) genes in primary human centroblasts and lymphoma cells (Ranuncolo *et al.,* 2007). Due to its role in monitoring of the timing of replication forks, ATR is crucial for the proper S-phase organisation during the cell cycle (Shechter *et al.,* 2004). During DNA replication, *ATR* can trigger intra-S-phase checkpoints that are essential for guarding genome integrity (Costanzo, 2003). *ATR* is also involved in the cellular responses to single- and double-strand DNA breaks including those induced by γ-irradiation (Ranuncolo *et al.,* 2007; Unsal-Kaçmaz, 2002; Myers and Cortez, 2006). As such, the attenuation of *ATR* activity conceivably compromises those effects and could enhance cellular proliferation in the presence of mutations (Fernandez-Capetillo *et al.,* 2004) which characterises genome instability and the cancer phenotype.

Kurosu *et al.* (2003) have reported that *Bcl6* overexpression did not affect cell proliferation of lymphoma cell lines treated with the chemotherapeutic drug etoposide. However, etoposide-induced apoptosis was significantly inhibited. In addition, *Bcl6* overexpression inhibited the increased levels of reactive oxygen species (ROS) that are generated by chemotherapeutic drugs. It is noteworthy that, production of ROS is typically associated with radiation exposure.

5.4.2.2 *Gadd45g*

The three growth arrest and DNA damage *Gadd45* genes *Gadd45a, Gadd45b, Gadd45g* are known to be involved in response to physiological or environmental stress and are induced by a variety of genotoxic agents (Fornace *et al.,* 1992; Fornace *et al.,* 1989; Zhan *et al.,* 1994). One of these genes, the *Gadd45g,* was significantly down-regulated

in the offspring of irradiated males as demonstrated of this study. The results of numerous studies suggests the involvement of *Gadd45* genes in modulation of cell-cycle control, genome stabilisation (Hollander and Fornace, 2002), stress-induced apoptosis (Sheikh *et al.*, 2000), as well as DNA repair (Zhan, 2005), and DNA demethylation (Barreto *et al.*, 2007). Vairapandi *et al.* (2002) have reported that the Gadd45b and Gadd45g proteins can specifically interact both *in vitro* and *in vivo* and inhibit Cdk1/cyclinB1, the key regulator of the G2/M transition in the cell cycle, the same role as described earlier for *Gadd45a* (Zhan *et al.*, 1999). Moreover, all three Gaad45 proteins are involved in the activation of the G2/M checkpoint in response to UV-induced DNA damage. The authors highlighted that the three Gadd45 proteins collaborate in controlling the cell cycle checkpoints in the case of stress.

5.4.2.3 *Itch*

The *Itch* gene is significantly down-regulated in the offspring of irradiated males. The Itch protein is one of the HECT-E3-upiquitin ligases that are involved in immune response as well as several signalling pathways regulating cellular proliferation (Rotin and Kumar, 2009). *Itch*-deficiency was found in the non-agouti-lethal 18H or Itchy mice, showing dark skin colour as well as severe immunological disorder (Melino *et al.*, 2008; Perry *et al.*, 1998). The *Itch* gene modulates the ubiquitination of several factors that participate in cellular responses such as the DNA damage response including p73 and p63 (Bernassola *et al.*, 2008; Melino *et al.*, 2008). It has been reported that the amount of p73 and p63 protein expression is controlled by *Itch*-mediated ubiquitination (Rossi *et al.*, 2005; Rossi *et al.*, 2006). It should be noted that in some tumour cells, the p73 and p63 proteins behave like p53 as they accumulate in response to γ- irradiation or exposure to chemotherapeutic drugs. It has been found that p73 and p63 are targets for

Itch-mediated ubiquitination and degradation (Hansen *et al.*, 2007). In other words, the induction and transcriptional activation of p73 and p63 in response to genotoxic insults is modulated at the post-translational level, at least partially, by DNA damage-caused *Itch* down-regulation (Hansen *et al.*, 2007; Rossi *et al.*, 2005), thus implying that the down-regulation of this gene may affect F_1 genome stability.

5.4.2.4 *Npas2*

In the offspring of irradiated males the expression of the *Npas2* gene is significantly down-regulated. The *Npas2* gene encodes Neuronal PAS domain-containing protein 2 transcriptional factor NPAS2. This protein is an essential component of the circadian rhythm system where it is involved in dimerization of the BMAL1 protein (Zhou *et al.*, 1997; Reik *et al.*, 2001; DeBruyne *et al.*, 2007). According to the results of microarray studies, the pattern of expression of up to 20% of mammalian genes is modulated by this system (Akhtar *et al.*, 2002; Storch *et al.*, 2002; Duffield *et al.*, 2002). It is well documented that the circadian clock system controls activity of a variety of cellular processes including cell cycle, response to genotoxic stress, and genome integrity (Kondratov and Antoch, 2007; Shadan, 2007). For example, the expression of cell cycle progression modulators cyclins A, B1, D1, E, the *p53* gene, as well as *Mdm2*, *Wee1*, *c-Myc*, and *Gadd45* genes is tightly regulated by the circadian rhythm system (Bjarnason *et al.*, 2001; Fu *et al.*, 2002). In addition, the *Wee1* and *c-Myc* genes were found to be direct transcriptional targets for the circadian clock protein complex CLOCK-BMAL1 (Fu *et al.*, 2002; Matsuo *et al.*, 2003). Moreover, for many of these genes, the expression pattern is altered in tissues of mice carrying mutations affecting the structure and activity of circadian genes (Gauger and Sancar, 2005; Miller *et al.*, 2007; Fu *et al.*, 2002; Matsuo *et al.*, 2003). Hoffman *et al.* (2008) provided evidence that the circadian

gene *Npas2* has a role in DNA damage response to mutagen exposure *in vitro*. The authors reported that that cells depleted of NPAS2 activity via RNA interference failed to delay their cell cycle in response to mutagen treatment and showed compromised DNA repair. In addition the knockdown of *Npas2* significantly reduced the expression of several cell cycle and DNA repair genes (Hoffman *et al.*, 2008). The findings of the abovementioned studies indicate that the compromised expression of *Npas2* in the offspring of irradiated male mice observed in this study might be implicated in genome instability.

5.4.3 Genes involved in chromosome assembly and dynamics

5.4.3.1 *Mis18bp1*

The central component of centromeric chromatin is the histone H3 variant CENP-A which mediates the recruitment of numerous proteins that are essential for the formation of the constitutive centromere-associated network (CCAN) which is itself crucial for kinetochore specification (Foltz *et al.*, 2006; Obuse *et al.*, 2004; Okada *et al.*, 2009; Hori *et al.*, 2008; Cheeseman and Desai, 2008). The centromere defines the formation of kinetochore and interconnection of sister chromatids and during cell division, the chromosome interacts with spindle fibres through the kinetochore which is essential for the proper segregation of chromatids (Gonçalves Dos Santos Silva, *et al.* 2008). Therefore, the presence of a functional centromere is crucial for genome stability. The *Mis18bp1* gene which is significantly down-regulated in the offspring of irradiated males, encodes MIS18 binding protein 1. This protein plays an important role in preparation of centromeric chromatin for CENP-A deposition (Fujita *et al.*, 2007; Hayashi *et al.*, 2004; Lagana *et al.*, 2010) and therefore, indirectly involved in maintaining genome integrity through cell division. In mouse embryonic stem cells, the

MIS18 binding protein 1 is associated with centromeric chromatin during distinct cell cycle stages with highest abundance at the centromere from anaphase until late G1 phase (Dambacher *et al.*, 2012).

5.4.3.2 *Fign*

The down-regulated gene *Fign* encodes the Fidgetin (FIGN) protein- a member of gene super family ATPases-associated with diverse cellular activities (Frickey and Lupas, 2004). The presence of *Fign* mutation in mice impairs embryonic development, resulting in the absence or reduction semicircular canals, defected retinal neural epithelium and malformed bones (Yang *et al.*, 2005). Recently data suggest that the FIGN protein plays an important role in mitosis, especially in spindle microtubule movement 'flux'. For example, in *Drosophila melanogaster* FIGN attaches to mitotic centrosomes and modulates the microtubule flux (Zhang *et al.*, 2007) which is crucial for the equal segregation of the replicated chromosomes (Khodjakov and Kapoor, 2005). In addition, in *C. elegans* the loss of *Fign* function resulted in the accumulation of proliferative nuclei in the germline as well as the early embryo which is indicative of the role played by *Fign* in the modulation of progression through mitosis (Luke-Glaser *et al.*, 2007). Mukherjee *et al.* (2012) have recently shown that the human *Fign* gene regulates many aspects of mitosis through modulation of mitotic spindle architecture via its ability to work as a potent microtubule serving and polymerizing enzyme. The authors found that human Fidgetin targets to centrosomes. Also, the velocity of pole-ward tubulin flux and chromatid-to-pole motion was significantly reduced following siRNA treatment.

5.4.3.3 *Ncapg2*

The *Ncapg2* gene also known as *Luzp5* and was down-regulated in the offspring of irradiated fathers as demonstrated in this study. This gene encodes for the non-structural maintenance chromosome (non-SMC) G2 subunit of condensin II, Condensin II, with condensin I plays a significant role in the assembly of chromatin fibres into bivalent chromosomes (Lee *et al.*, 2011). Condensin II is located inside the nucleus during interphase and is required for chromosome condensation in early prophase before disintegration of the nuclear envelope (Hirota *et al.*, 2004). In late prophase, condensin I and II were found to be arranged in a distinct pattern alternatively along the chromatid axes and a proportion of condensing II was seen to be enriched proximal to the inner region of the kinetochore (Ono *et al.*, 2003; Ono *et al.*, 2004). Lee *et al.* (2011) have reported a similar role of condensin complexes in mouse oocytes. Ono *et al.* (2003) have also reported that depletion of condensin I or II-specific subunits via RNA interference results in abnormal chromosomal morphology in HeLa cells. A study by Wood *et al.* (2008) has established a role of condensin II in homologous recombination repair mechanism of DNA DSBs which is essential for genome stability. The authors have revealed that condensin II depleted cells have defective HR however; condensin II was not required for G2/M checkpoint activation. Taken together, the abovementioned results indicate that *Ncapg2*down-regulation may potentially destabilise the genomes of first-generation offspring of irradiated male mice.

In conclusion, the results of this pilot study have provided important insights into the mechanisms underlying the phenomenon of radiation-induced transgenerational instability. First of all, the data showing remarkable differences in the transcriptional response between the offspring of irradiated males and females provide offer a plausible

explanation for the lack of transgenerational effects of maternal irradiation. The fact that none of the genes significantly deregulated in this group of mice can be implicated to the maintaining of genome stability can alone explain the results presented in the previous chapter showing that maternal irradiation does not affect ESTR mutation rates in their offspring. In contrast, a substantial number of genes significantly deregulated in the offspring of irradiated males belong to functional groups directly involved in maintaining of the stability of genome.

6 SUMMARY AND FUTURE WORK

This thesis presents the results of three inter-related studies aimed to establish the long-term genetic effects of maternal irradiation in mice.

The first study was conducted to establish the effect of radiation exposure on ESTR mutation induction in the germline of female mice exposed to acute X-rays during adulthood. The results of this study shows that maternal irradiation during adulthood targeting dictyate oocytes in maturing follicles does not affect ESTR mutation rate in the germline. In addition, they also show that the spectra of radiation-induced and spontaneous mutation in the paternal and maternal germline are likely to be similar. Given the fact that ovary of adult female mice contains only meiotically arrested dictyate oocytes at different stages of maturity, which would not undergo any further rounds of DNA replication, it is likely that directly exposed (F_0) adult female mice irradiation would not affect ESTR stability in their germline. On the other hand, testis of adult mice consists of highly replication-proficient stem cells and spermatogonia, as well as non-dividing haploid spermatids/sperm cells. The analysis of ESTR mutation induction in the male germline has shown that it is highly stage-specific, with some late stages showing evidence for ESTR mutation induction.

In the second part of this project, the transgenerational effects of maternal irradiation during adulthood were investigated. The results of this project show that maternal irradiation during adulthood does not destabilise the genome of their offspring. These data further confirm the results of previous study (Barber *et al*., 2009) on the effects of *in utero* irradiation in mice and therefore imply that, in contrast to paternal exposure,

the transgenerational effects of maternal irradiation are likely to be negligible. The results of current study therefore provide further evidence for the manifestation of transgenerational effects in the germline and somatic tissue of first-generation offspring of irradiated male mice. On the other hand, the data presented in this thesis clearly show that regardless of the stage of oogenesis exposed, maternal irradiation does not destabilise the F_1 genomes.

The third part of this project represents a pilot study aimed to compare the pattern of gene expression in the first-generation offspring of irradiated males and females. According to the results, the pattern of gene expression in the offspring of irradiated males and females drastically differs. Most importantly, none of the genes showing significantly deregulated in the offspring of irradiated females can be implicated to the maintaining of genome stability. These results offer a plausible explanation for the lack of transgenerational effects of maternal irradiation. The fact that expression of these genes is altered in the offspring of irradiated males whose genomes are unstable and such radiation induced instability signal could alter the promoter methylation and/or chromatin architecture, which eventually alter the expression pattern of these genes. This finding, therefore, make noteworthy contribution towards better understanding the epigenetic nature of the radiation induced instability signal. Therefore, gene expression profiling can serve as a body for future studies to enrich our understanding of the nature of radiation induced signal and hence, unveiling different mechanisms underlying the phenomenon of transgenerational instability.

In conclusion, the results presented in this thesis provide new evidence for striking differences in the manifestation of long-term effects of paternal and maternal exposure

to acute low-LET irradiation in mice. Although the comparison of mouse and human data may appear to be problematic, the results of this study may indicate that the genetic risk of paternal irradiation in humans can exceed that following maternal exposure.

6.1 Future work

The data from this thesis suggest that maternal irradiation does not result in transgenerational instability in their offspring. Given the results of previous studies on the transgenerational effects of paternal exposure to alkylating agent ethylnitrosourea (Dubrova *et al.*, 2008) and mutagenic anti-cancer drugs (Glen and Dubrova, 2012), it therefore remains to be established whether exposure to these chemicals can destabilise the offspring genome.

A special consideration should be taken to the asymmetrical epigenetic reprogramming of male and female genomes as early as post-fertilisation. This stage seems to be very crucial to keep or erase of epigenetic marks that could pass gametogenesis during process of unequal treatment of male and female genomes epigenetically. Further studies are needed to delineate how epigenetic marks that passed the male germline could escape being erased during early development. For example, studying the mechanism of active DNA demethylation of the paternal genome compared to the passive demethylation of maternal genome can increase our knowledge about the epigenetic nature of radiation induced instability signal and how it works.

According to the third part of this thesis, the gene expression profiling may provide a new era to understand the mechanisms underlying transgenerational instability.

However, further analysis is needed to expand the comparison to include different mouse strains and analysing expression patterns in different organs. There is also a definite need to analyse the differentially expressed genes, especially those implicated in genome integrity, to understand the reasons behind their altered expression in terms of epigenetics. For instance, to analyse their promoter methylation as well as histone modification that could compromise whose expression. This may help to enhance our understanding how the radiation induced signal triggers genomic instability.

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