The Dose and Dose-Rate Effects of Paternal Irradiation on Transgenerational Instability in Mice

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Safeer Kamil Mughal

University of Leicester
Abstract

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Of the non-targeted, delayed consequences of exposure to ionising radiation, genomic instability is a particular area of concern, especially with regard to its manifestation in the non-exposed offspring of irradiated parents. However, further analysis of these effects and their implications is mainly limited by our understanding of the underlying mechanisms and the lack of reliable data for humans. As of yet, transgenerational instability has only been consistently demonstrated in animal models using high, acute doses of ionising radiation (> 1 Sv).

To investigate the effects of low-dose acute and low dose-rate (chronic) irradiation and whether or not they are capable of destabilizing the genomes of the unexposed offspring, we exposed male BALB/c mice to a range of $\gamma$-ray doses (10-100 cGy) and dose-rates (chronic and acute), and mated them to unexposed BALB/c females 10 weeks post-irradiation. The mutation frequency at the Ms6-hm locus was established in DNA samples extracted from the sperm of directly exposed mice, as well as from the sperm and brains of their F1, using the single-molecule PCR technique. A linear dose-response was observed for direct exposure across the range of acute doses, with a doubling dose of 57 cGy. Furthermore, 100 cGy of acute $\gamma$-rays was shown to be more mutagenic than chronic exposure to the same accumulated dose. However, acute exposure to 10-25 cGy failed to manifest genomic instability in the derived offspring. This was also true of low dose-rate exposure to 100 cGy. Only acute paternal exposure to 50 and 100 cGy resulted in transgenerational instability, to a similar extent for both doses. Analogous results were found for both tissues.

Taken together, this would imply the presence of a stress-like response where a threshold of acute dose determines the onset of transgenerational instability. Our results also suggest that children whose fathers are subject to most forms of human exposure to ionizing radiation would be safe from the effect.

The results of this thesis have also separately been published as:

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‘He who has not thanked people has not thanked God’

The Prophet Muhammad (pbuh)

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Chapter 1 – Exposure to Ionising Radiation

1.1 General Introduction to Ionising Radiation

Ionising radiation refers to the transmission of electromagnetic waves (photons) or subatomic particles that carry enough energy to ionise atoms and break chemical bonds in matter. They do this by displacing electrons or hadrons from target atoms and producing charged free radicals (Kudryashov, 2008). The radiation itself is produced by radioactive decay of unstable particles, nuclear reactions, extremely high temperature or particle acceleration. Of the photons that constitute the electromagnetic spectrum, only X-rays and γ-rays contain enough energy to always be considered ionising. They differ only in their origin, with the former emitted from the electron shells and the latter from the nucleus (Grupen, 2005). Particle, or corpuscular, radiation may include electrons (β-particles or accelerated electrons), α-particles (Helium nuclei) and free protons or neutrons (Kudryashov, 2008). Photons and particles with energies above 10 electron volts (eV) are generally considered ionising. This equates to the typical binding energy of an outer electron to an atom or molecule: $1.6 \times 10^{-18}$ joules (J).

Charged particles that result from ionisation may actively form compounds that disturb processes in exposed biological material. The extent of this damage is in part related to the amount of energy deposited at a given target. This is called the absorbed dose and is measured by the SI unit the gray (Gy), which represents the amount of joules (J) of energy deposited per kilogram of matter. However, equal doses of different types of radiation may also produce varying levels of damage. This is due to the linear energy transfer (LET) of a form of ionising radiation, which determines the mean energy loss per unit of path length (keV/µm) along the ionisation track of an
ionising particle. In this way, X-rays, γ-rays and β-particles are considered low-LET ionising radiation with more widely dispersed energy deposition patterns and more penetrating tracks in matter (Grupen, 2005). Conversely, larger particles such as neutrons, protons, α-particles and heavy ions are high-LET radiations with denser tracks of energy deposition. This difference has a profound effect on the complexity of damage produced in target matter and in living tissue relates to varying degrees of biological effectiveness (Goodhead, 1988). To take this into account the absorbed dose may be multiplied by a weighting factor (dependant on radiation type) to obtain the biologically equivalent dose as measured by the SI unit the sievert (Sv). Given the low LET of ionising photon and electron radiation, their relative biological effectiveness will mean a weighting factor of 1 and so 1 Gy of each of these forms will equate to 1 Sv. However, high-LET radiation may be up to 20 times as effective as this, with as little as 0.05 Gy required to produce the same equivalent dose and, therefore, biological effect (ICRP, 2003). Furthermore, different organs and tissues exhibit different sensitivities to irradiation, requiring another weighting factor dependant on the exposed targets (Kudryashov, 2008). An effective dose, also measured in Sv, is thus calculated for the consequences of irradiation to individual tissues, where whole-body exposure has the cumulative weighting factor of 1 (Table 1).
<table>
<thead>
<tr>
<th>Organs</th>
<th>ICRP Tissue Weighting Factors</th>
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<tr>
<td>Remainder of body</td>
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Table 1 – Summary of tissue weighting factors reflecting differing sensitivities to radiation damage (ICRP, 2007). To calculate the effective dose to a given organ, the equivalent dose is multiplied by the respective weighting factor.
1.2 The Nature of Human Exposure

Due to the high levels of energy carried by ionising radiation it has many practical uses, which have been utilised for many medical, research, industrial and military purposes. Furthermore, it is ubiquitous in the environment, produced by naturally occurring radioactive materials and cosmic rays from the sun and outer space. Therefore, human exposure to ionising radiation may come from a variety of naturally occurring and artificial sources, each differing in the type of radiation emitted and its energy, intensity, duration and physical form. It is important to understand the nature of this exposure and to assess the threat ionising radiation may pose to biological organisms. The major sources of human exposure to ionising radiation are described in this chapter.

1.2.1 Background Exposure

Exposure from natural sources or background radiation refers to the sources of ionising radiation present in the environment, to which populations may ordinarily be exposed (Figure 1). One of the major natural sources is from cosmic rays, particles originating in outer space that bombard the Earth with primary charged ions and secondary radiation from interactions in the atmosphere. Exposure may vary worldwide, but delivers a mean annual dose of 0.39 mSv (UNSCEAR, 2000). The other main contributor to background radiation is from terrestrial sources of radionuclides such as uranium, thorium and potassium isotopes that occur naturally and are distributed throughout most materials. External sources in building materials, rocks and soil may deliver an average human dose of 0.48 mSv per year (UNSCEAR, 2000). However, consumption of food and water may also build up internal levels of radioisotopes such as potassium-40 ($^{40}$K) and carbon-14 ($^{14}$C) to levels that add around
0.29 mSv to the annual natural background. An additional 1.26 mSv per year is typically absorbed from the air, mainly from accumulated radon gas (\(^{222}\)Rn) released from the decay of radium-226 (\(^{226}\)Ra) and uranium-238 (\(^{238}\)U) in soil (UNSCEAR, 2000). Collectively, these sources of ionising radiation generate a worldwide annual effective natural background dose of around 2.4 mSv per person, generally ranging between 1 and 13 mSv. This may be considerably higher in populations inhabiting areas with particularly high levels of terrestrial radiation. For example, the city of Ramsar, Iran has a network of hot springs with naturally dissolved radium-226, where residents may receive annual background doses of up to 260 mSv (Ghiassi-nejad et al., 2002). Similarly, a markedly higher contribution from cosmic ray radiation will be observed for individuals who are routinely exposed to more intense radiation in the upper troposphere. In this way, airline crews are typically exposed to an additional 2.2 mSv per year (Feng et al., 2002). Even so, for the majority of the world’s population, natural background radiation represents approximately 80 % of the total effective background dose received per person (UNSCEAR, 2000). The remaining 20 % is acquired from artificial sources that may affect the general population, such as medical exposures and the global distribution of nuclear fallout.

**Sources of Background Radiation**

![Pie chart showing the relative contribution of each source of background radiation](image)

Figure 1 – Sources of background radiation. The relative contribution of each source is shown for an average annual human exposure.
1.2.2 Exposure from Medical Sources

One of the most widespread uses of ionising radiation is for medical purposes. Radiographical imaging procedures remain the most common use, where a typical chest X-ray may deliver 0.05-0.4 mSv to the patient (UNSCEAR, 2000). This is higher for fluoroscopy-based, real-time imaging that may use an effective dose up to 10-50 mSv per procedure. However, the largest patient doses from diagnostic medical radiation come from computed tomography (CT) scans. Effective doses may range between 1.5 and 27 mSv per procedure and the absorbed dose to target organs can often be much higher (UNSCEAR, 2000). For example, a cardiac CT angiogram may typically deliver 40-100 mGy to blood vessels throughout the body (Hall and Brenner, 2008). Radionuclides may also be combined with chemical compounds to form radiopharmaceuticals for use as tracers in the diagnosis and treatment of various diseases. The average effective dose for most of these nuclear medicine procedures varies between 0.3 and 20 mSv (Mettler et al., 2008). Generally, these medical uses of ionising radiation are comparable to the average annual background radiation dose of around 3 mSv (UNSCEAR, 2000).

However, by far the highest doses of medical radiation are used in external beam radiation therapy, primarily in the treatment of cancer to kill or control malignant cells. Such radiotherapy typically uses low-LET X-rays or cobalt-60 ($^{60}\text{Co}$) γ-rays to deliver shaped radiation beams to the tumour, aimed from several intersecting angles. This ensures a large absorbed dose to the target while minimising the dose to the surrounding, healthy tissue. In this way, radiotherapy is a major treatment modality for cancer patients and is second only to surgery in its ability to cure tumours. Typical curative doses for solid tumours may range from 60-80 Gy, delivered in 1.8-2 Gy acute fractions per day (UNSCEAR, 2000). For lymphomas the cumulative dose after
fractionated treatment may reach 20-40 Gy. Even so, the out-of-field, side scatter dose to healthy tissue will rarely exceed 10 cGy (0.1 Gy) per procedure, or 100 cGy (1 Gy) in total (UNSCEAR, 2000; Tawn et al., 2005; Mazonakis et al., 2007; Di Betta et al., 2010), especially with the use of additional shielding (Nazmy et al., 2007).

It should also be noted that medical professionals are exposed to ionising radiation, albeit attenuated via adequate shielding. In this way, healthcare workers such as X-ray technicians, radiologists, nurses and physicians may be exposed to less than 1 mSv per year (UNSCEAR, 2000; Mostafa et al., 2002).

1.2.3 Exposure from Nuclear Weapons

Following the 1945 bombings of Hiroshima and Nagasaki, Japan there were around 280,000 survivors, of which dose estimates are available for 94,000 in the Life Span Study as determined by the latest DS02 dosimetry of the Radiation Effects Research Foundation (Young and Kerr, 2005). This system takes into account individual proximity to the respective hypocentres and shielding of organs by the body and building structures. Although, the effective dose received by some of these individuals may have been in excess of 4 Gy, the survivor cohort is predominately a low exposure population, with an average dose of 300 mSv and a mean weighted colon dose of 210 mSv. Furthermore, the maximum doses due to radioactive fallout in the surrounding area have been estimated at 10-30 mSv in Hiroshima and 200-400 mSv in Nagasaki. Exposure to $\gamma$-rays comprised the majority of the atomic bomb radiation with fast neutrons contributing only 1-2 % of the total dose.

Nuclear fallout from atmospheric testing of atomic weapons is also a major source of exposure for local populations in the path of radioactive clouds produced by the explosions. Following the 456 nuclear tests at the Semipalatinsk test site in
Kazakhstan between 1949 and 1989, 111 of which were atmospheric, residents of the surrounding area were exposed to an average 100 cGy of low-LET ionising radiation (Takada et al., 1999). There are claims that inhabitants of the nearby Dolon village may have received doses in excess of 1 Sv (Gusev et al., 1997). Moreover, dosimetry for local populations is further complicated by internal exposure to low-LET emitting radionuclides such as iodine-131 ($^{131}$I), caesium-137 ($^{137}$Cs) and strontium-90 ($^{90}$Sr), which accumulate in the thyroid, whole body and bone marrow, respectively, after mimicking the chemical interactions of related biological ions (Gordeev et al., 2002; Shoikhet et al., 2002). Similarly, around 100 atmospheric tests were performed at the Nevada test site in the U.S. between 1951 and 1962 (Till et al., 1995). The resulting estimate for average thyroid dose for exposed children living in Washington County, Utah, was 170 mGy, 50 mGy in Lincoln County, Nevada and 13 mGy for those living in Graham County, Arizona.

1.2.4 Exposure from Nuclear Power Industry

Human exposure to ionising radiation may also come from its increased use in nuclear facilities for the production of electricity. Nuclear power stations, fuel-reprocessing plants and the highly radioactive waste they produce are a major source of exposure to workers and surrounding populations, especially following several high profile accidents that occurred over the last 60 years. A large international study recently collected external low-LET dosimetry information from 154 nuclear facilities across 15 countries for 407,391 workers who had been employed for at least one year (Cardis et al., 2005). The average effective dose was found to be 19.4 mSv, with 90 % of workers receiving total doses below 50 mSv and less than 0.1 % exposed to doses in excess of 500 mSv. Furthermore, since the commencement of operations at the
Sellafield nuclear reprocessing site, Cumbria, UK in 1951 an estimated 200 kg of plutonium has been deposited in marine sediments of the Irish Sea from discharged radioactive waste, with local cattle and fish contaminated with isotopes such as technetium-99 ($^{99}$Tc), plutonium-239 ($^{239}$Pu) and caesium-137 ($^{137}$Cs) (Gray et al., 1995). Large quantities of radioactive material were also released from the plant following the Windscale fire of its Unit 1 nuclear reactor in 1957. Workers at the site have subsequently been exposed to an average external dose of 138 mSv (Cardis et al., 1995) and 190 mSv by internal exposure to $^{239}$Pu (Omar, Barber and Smith, 1999).

Plutonium emits $\alpha$-particles that do not penetrate far into tissue. However, when taken into the body, high-LET sources may be extremely hazardous because they are allowed to interact with many cells as they are transported throughout the body.

The first facility for the production of plutonium in the former Soviet Union was the Mayak Production Association in the Chelyabinsk Oblast, beginning operations in 1948. Due to deliberate dumping of radioactive waste into the Techa River between 1948-1951, the 1957 Kyshtym explosion following the failure of a nuclear waste cooling system and an incident in 1967 when wind dispersed radioactive dust over nearby Ozyorsk, the surrounding environment and population were heavily contaminated (Degteva et al., 2000). Internal exposure to radionuclides, particularly $^{90}$Sr, is estimated to have irradiated local residents with an average 0.3 Sv, with external $\gamma$-ray exposure of up to 0.4 Sv. Furthermore, the workers of the facility itself have been exposed to an average accumulated dose of 0.8 Gy of $\gamma$-radiation (Shilnikova et al., 2003). Inhalation of plutonium is also believed to have contributed to a significant internal exposure for these individuals (Kreisheimer et al., 2003; Gilbert et al., 2004).
However, the most catastrophic nuclear power plant disaster to occur was in 1986 at the Chernobyl facility in Ukraine. When a sudden power output surge during an electrical test at Unit 4 of the plant provoked an emergency shutdown attempt, an even greater power spike ruptured the reactor vessel (NEA, 2002). The resultant explosions exposed the reactor’s graphite moderator to air, which caused it to ignite and send dense clouds of highly radioactive smoke into the atmosphere over much of the western Soviet Union and Europe. Using biological dosimetry it was determined that staff may have been exposed to up to 16 Sv from external irradiation and thyroid doses, primarily due to inhalation of $^{131}$I, up to 20 Sv (UNSCEAR, 1988). Doses to recovery operation workers are not known for certain, but have been estimated at an average of 170 mSv in 1986 and 15 mSv for those working in 1989 (Sevan’kaev et al., 1995). Evacuees from the 30 km-exclusion zone were calculated to have a mean effective dose of 17 mSv from external low-LET irradiation (Likhtarev, Chumack and Repin, 1994) and thyroid doses in young children as high as 1 Sv (Balonov et al., 2003). In nearby populations beyond this zone, including in other regions of the former Soviet Union such as Belarus and Russia, irradiation was primarily external from $^{137}$Cs deposited on the ground (NEA, 2002). This was largely due to proper control of foodstuffs that minimised internal exposure to radionuclides. Whole body doses to these individuals between 1986 and 1989 have been measured at an average 40 mSv, ranging from 5 to 250 mSv. Furthermore, the worldwide annual dose was estimated to be 0.04 mSv per person, which has since dropped to below 2 μSv (UNSCEAR, 2000).

More recently, the Tohoku earthquake and subsequent tsunami in 2011 cut power to the coolant systems of the Fukushima I Nuclear Power Plant in Japan and led to the Fukushima Daiichi nuclear disaster. The accident was originally classified as a level 4
on the International Nuclear Event Scale, but was later raised to the maximum level 7 of the Chernobyl disaster as large amounts of radiochemicals, particularly $^{131}$I and $^{137}$Cs, were released into the air and sea (Black, 2011; Brumfiel and Fuyuno, 2012). As of yet, it is too early to determine the full individual and global effects of the accident, but preliminary reports suggest that only 146 of the 20,115 workers and contractors at the plant were exposed to doses greater than 100 mSv (Brumfiel, 2012) and 99.3 % of the residents of Fukushima and neighbouring prefectures received below 10 mSv of ionising radiation (WHO, 2012).
1.3 Investigating the Risk to Human Populations

As shown, human exposure to ionising radiation predominantly involves low doses (< 100 mSv) of low-LET sources that are accumulated over an extended period of time (chronic exposure). Irradiation by higher effective doses is very uncommon, especially for those delivered quickly in a short burst (acute exposure). This information is vital to understanding how ionising radiation affects us biologically and how our use of it may put us at risk.

1.3.1 Health Implications

Exposure to ionising radiation is generally harmful to biological tissue as the structural damage induced by such high energy disrupts the equilibrium and normal functioning of various systems. This can often be lethal to irradiated cells and, potentially, whole organisms. The effects may be stochastic, such that the probability of their onset increases with dose, but their severity does not. This would include the risk of cancer attributed to radiation-induced mutations and chromosome aberrations (Anand et al., 2008), teratogenesis (De Santis et al., 2005), and other disorders such as heart disease (Stewart and Fajardo, 1992). Conversely, other conditions may be deterministic in that a reliable threshold dose determines their onset, above which their severity increases with dose. Radiation burns begin at around 2 Gy (Valentin, 2000) and acute radiation sickness from whole-body absorbed doses above 1 Gy describes a range of illnesses that get progressively worse with increasing dose, with mortality almost certain from 6 Gy onwards (Bushberg, 2009). Other than acute effects, perhaps the consequences of most concern are those mutagenic effects that may pose a threat to the children of exposed individuals. Genetic mutations in the exposed germ line
would be inherited by the offspring of those still capable of reproducing, extending the burden of irradiation.

To investigate the impact of exposure to ionising radiation a wealth of data has been collected from epidemiological studies and laboratory experimentation. The findings from this vast body of work are monitored by various advisory committees such as the International Commission on Radiological Protection (ICRP) and the United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) to assess and estimate the risk to human health. These bodies produce regular reports to update current consensuses on issues including the sources and effects of ionising radiation so that governments and organisations around the world may establish accurate safety standards. In the UK such efforts have formed the basis of the Ionising Radiations Regulations 1999 (IRR99) that defines effective dose limits and imposes duties on employers to ensure that radiation exposures to employees and the general public arising from work activities are kept as low as reasonably possible. The national independent watchdog, the Health Safety Executive (HSE), enforces this legislation.

1.3.2 The Limitations of Epidemiological Studies

Even so, a robust understanding of the effects of ionising radiation is generally limited by the relative lack of exposed human populations with well-detailed irradiation histories and sufficient information on confounding lifestyle factors. While medical or occupational exposure is closely monitored, dosimetry for environmentally or accidentally irradiated individuals can only be measured retrospectively with little certainty. In addition, the route of exposure, whether internal or external, may profoundly affect the biological consequences of irradiation. Difficulties can also arise in epidemiological studies when selecting appropriate control groups. Consideration
must be taken to ensure that variables such as age, ethnicity and lifestyle factors do not distort comparison between control and exposed individuals. Furthermore, stochastic effects may be especially problematic in that they are not immediately identifiable and may require investigation of large populations over considerable time to give sufficient statistical power to detect them.

1.3.3 The Use of Model Systems

In order to overcome these obstacles, the effects of ionising radiation may alternatively be investigated in model systems. This would ensure controllable exposure to known doses of specific sources of radioactivity, enabling a proper understanding of the dose response of a given radiological effect. Additionally, model systems provide exposed populations that are genetically homogenous to controls and may be kept in an identical environment. For the genetic risk to whole organisms, an added advantage would be in the ease of time and effort for producing and monitoring multiple generations in animal models. In this way, the effects of exposure to ionising radiation have been explored in a variety of systems, from *Drosophila* (Muller, 1927) to rodent models (Searle, 1974) and, more recently, the Japanese Medaka fish (Shimada *et al.* 2001). These animals have short gestation periods and are reproductively prolific, providing a large sample population in a short period of time. This also makes transgenic organisms relatively easy to produce, allowing researchers to manipulate the genetic sequence of an animal model.
Chapter 2 – Dose and Dose-Rate Effects of Direct Exposure

2.1 DNA Damage Response

A central pillar of radiation biology and risk assessment is the target theory. Independently developed by two sets of authors in the 1940s (Lea, 1946; Timofeeff-Ressovsky and Zimmer, 1947), the contemporary model predicts that the biological effects of irradiation are primarily attributed to damage at DNA target sites hit either directly by ionising radiation or indirectly by free radicals formed from the ionisation of water (Ballarini, 2010). Although any type of molecule in a cell may be damaged following exposure, DNA is thought to be the critical target. This is because damage to even a single gene can often have highly detrimental consequences that may also be inherited by the daughter cells of survivors. A wide variety of DNA damage is initially produced following exposure to ionising radiation, including base damage, bulky adducts and single- and double-strand breaks (Frankenberg-Schwager, 1990). Such damage will hinder transcription of target genes and may block cellular replication or even kill the cell. Therefore, recognition and repair of radiation-induced DNA damage is essential.

Base damage and bulky, helix-distorting lesions that affect only one DNA strand are routinely repaired by base excision (BER) and/or nucleotide excision (NER) repair pathways, that use the undamaged strand as a template to guide correction of the damaged strand (Hall and Giaccia, 2011). Similarly, single-strand breaks may be rapidly repaired by the same DNA repair machinery (Marintchev *et al*., 1999). This involves XRCC1, which forms a complex with DNA ligase III, polymerase beta and poly (ADP-ribose) polymerase to correct the gap. However, double-strand DNA breaks and persistent single-strand breaks pose a more substantial challenge to the irradiated cell. Approximately 40 initial double-strand breaks are created in an
irradiated cell following exposure to 1 Gy of low linear energy transfer (low-LET) irradiation (Frankenberg-Schwager, 1990), which may produce extensive genome rearrangements if left un-repaired (Hall and Giaccia, 2011). To deal with damage of this nature the cell often undergoes cell cycle arrest at G1 and G2 checkpoints that control entry to the DNA-replicating S-phase and the cell-dividing mitosis (Weinert, 1998). This prevents cells with high levels of damage from entering these critical phases and provides more time for DNA repair.

Single-strand breaks may cause this checkpoint activation when replication protein A (RPA) coats single-stranded DNA to prevent it from winding back on itself and forming secondary structures (Wold, 1997). This recruits a protein complex of ataxia telangiectasia and Rad3-related protein (ATR) and its interacting protein, ATRIP (Zou and Elledge, 2003). Through this interaction, ATR is activated (Figure 2.1), in turn phosphorylating the kinase CHK1, which triggers a signal transduction cascade that ultimately arrests the cell cycle (Sancar et al., 2004).

Figure 2.1 – Cell-cycle checkpoint activation following single-strand break detection. Exposure to ionising radiation produces single-strand breaks, which become coated with RPA. A complex of ATR and ATRIP binds to this structure and phosphorylates CHK1. The subsequent S-phase or G2 arrest allows the cell time to deal with the damage.
Similarly, radiation-induced double-strand breaks are bound by the proteins MRE11A, RAD50 and NBS1 that form the MRN complex and tether the broken ends of the break (Figure 2.2) (Paull and Lee, 2005). This recruits ataxia telangiectasia mutated (ATM), which interacts with the NBS1 subunit and phosphorylates the histone H2AX on serine 139 to form γH2AX (Huang et al., 2004). This activation generates binding sites for a variety of adapter proteins that possess the BRCT domain, such as MDC1 that also associates with MRE11A and ATM to promote further MRN complex assembly and amplify the damage signal (Goldberg et al., 2003). MDC1 also interacts with the effector kinase CHEK2, which in turn phosphorylates CDC25A, marking it for degradation. In this way, inhibitory phosphate groups are not removed from cyclin-dependant kinase 2 (CDK2) and its inactivity may result in cell cycle arrest (Falck et al., 2001).

Figure 2.2 – Cell-cycle checkpoint activation following double-strand break detection. The MRN protein complex tethers the ends of a radiation-induced double-strand break and recruits the ATM master kinase. Histone H2AX is phosphorylated to produce γH2AX, which recruits MDC1 and p53 to arrest the cell cycle or initiate apoptosis.
This produces a rapid response to radiation-induced DNA damage (Figure 2.2). However, the ATM mediated formation of γH2AX foci may also elicit a more long-term cell cycle arrest or even initiate cellular senescence or apoptosis to eliminate the damaged cell if the threat to genomic integrity is too high (Naka et al., 2004; Lee et al., 2001). This may be done through phosphorylation of the cell cycle regulator and tumour suppressor protein p53, with interaction through its binding protein 53BP1 (Wang et al., 2002). The latter contains the BRCT domain that binds to activated γH2AX, but may be primarily recruited by association with bound MDC1 (Eliezer et al., 2009). Even so, it has been shown that the formation of 53BP1 foci following irradiation is not entirely dependent on ATM or NBS1 downstream activity and may also be directly induced much earlier in the damage response (Schultz et al., 2000; Yoo et al., 2005). Activation of p53 leads to the subsequent transcription of numerous target genes, including the CDK inhibitor p21 (Waga et al., 1994). Cell cycle arrest in this manner may also be regulated by the interaction of p21 with proliferating cell nuclear antigen (PCNA), a DNA polymerase processivity factor (Fotedar et al., 1996; Frouin et al., 2003). The interaction appears to prevent PCNA from promoting replication of long stretches of DNA, but not the short fragments involved in repair (Li et al., 1994).

In other cases the insult to the cell may be so detrimental that it initiates apoptosis (Figure 2.2). This programmed cell death may be induced via the p53-dependant activation of the IGF-BP3 and BAX genes (Buckbinder et al., 1995; Chipuk et al., 2004). The former inhibits insulin-like growth factor-1 that normally prevents the apoptotic response. Bax suppresses the function of the Bcl-2 protein, which has a similar effect. Alternatively, p53 may upregulate the activity of effector caspase
proteins, such as caspase-8, that initiate cleavage cascades that brings about cell death through mitochondrial dysregulation and intracellular signaling (Afshar et al., 2006).

Repair of double-strand breaks is mainly performed by two basic processes: non-homologous end-joining (NHEJ) and homologous recombination (HR). The former involves ligation of the cut ends of a break. Recognition of free DNA ends by the Ku heterodimer, consisting of Ku70 and Ku80, initiates the process (Lieber et al., 2003) and subsequently recruits the catalytic subunit of DNA protein kinase (DNA-PKcs). Once engaged, DNA-PKcs phosphorylates another Ku-bound protein, Artemis, activating its endonuclease activity (Drouet et al., 2006). Since breaks induced by ionising radiation are likely to have 5’ and 3’ overhangs and even hairpin structures, the ends may be processed via this activity prior to ligation. X family DNA polymerases such as Pol λ and Pol μ (Daley et al., 2005) fill in any resultant gaps and DNA ligase IV is recruited to form a complex with its cofactors XRCC4 and XLF and directly join the two ends (Wilson et al., 1997; Ahnesorg et al., 2006). When the overhangs are compatible, NHEJ is generally an accurate repair system (Budman and Chu, 2005). Nevertheless, it is potentially error-prone and may introduce mutations into the genome. Loss of damaged nucleotides at the break may produce deletion mutations in the religated sequences and the aberrant joining of non-matching strands may form translocations (Heidenreich et al., 2003; Lieber et al., 2010).

Alternatively, HR-based repair requires an undamaged strand to serve as a template. ATM-mediated phosphorylation of H2AX also recruits the caretaker protein BRCA1, which activates the nuclease activity of MRE11 (Zhong et al., 1999). The DNA ends are subsequently processed to generate single-stranded DNA with 3’ overhangs in an action called resection (Hall and Giaccia, 2011). BRCA2, attracted by bound BRCA1, next facilitates the loading of RAD51 onto the RPA and RAD52-
coated overhangs created by this process (Sugiyama and Kantake, 2009). The RAD51-covered DNA filament initiates strand invasion into an undamaged homologous DNA molecule and forms a Holiday junction of intertwined strands (Hall and Giaccia, 2011). A DNA polymerase extends the resected ends to fill the gap by copying the homologous template and the Holiday junction is resolved to complete the process. HR is an error-free process because damaged strands are repaired by copying information directly from the undamaged homologous chromatids/chromosomes. However, no such template exists in the G1 stage of interphase that precedes DNA replication. Therefore, the error-prone NHEJ is primarily responsible for repair at this time; with HR playing a larger role after DNA replication has occurred (Hall and Giaccia, 2011).

Erroneous processing may also occur when S-phase cells that are actively replicating their DNA encounter damage, either through irradiation at that time or the presence of lesions that previously evade repair. In this situation, replication is likely to stall and arrest since many types of lesions block replication fork progression (Friedberg et al., 2006). However, the cell may avoid these mechanisms by down-regulating genomic integrity in an attempt to allow the cell to survive and repair the damage at a later time. The two main pathways of this DNA damage tolerance are translesion synthesis (TLS) and template switching.

Translesion synthesis involves the bypass of damaged bases by use of lower-fidelity DNA polymerases that lack proofreading capability and contain large active sites capable of accommodating distorted bases and mismatches (Waters et al., 2009). Following replication stalling, PCNA is ubiquitinated by a RAD6/RAD18 complex to provide a platform to recruit specialised TLS polymerases (Andersen et al., 2008). Even so, fidelity of the process is largely dependent on the lesion and which TLS
polymerase is used. Pol τ bypasses 6-4 pyrimidine adducts and abasic sites, but introduces a high frequency of mismatched bases (Tissier et al., 2000). Conversely, Pol η preferentially inserts two adenosine nucleotides opposite thymine dimers (McCulloch et al., 2004) and Pol κ accurately bypasses guanine adducts (Zhang et al., 2000). The synthesised sequence is then extended by the same TLS polymerase or, in the case of a mismatch, another such as Pol ζ. Finally, replicative polymerases are restored to the template and replication continues as normal.

Alternatively, the cell may avoid replicating template DNA in the immediate vicinity of the damage. Collectively known as template switching mechanisms, such strategies are error-free, suggesting they may be preferentially induced following replication arrest (Berdichevsky et al., 2002). To date, little is known about this process, but it is thought that the template containing the lesion is temporarily replaced by the undamaged template of the newly synthesised strand of the sister duplex (Michel et al., 2004). In one model, arrested replication reinitiates downstream of the lesion leaving a gap of approximately 1,000 nucleotides, which is resolved by strand invasion of the sister duplex in a similar manner to homologous recombination (Cox, 2002). A second alternative may potentially occur through ‘regression’ of the replication fork, where the original template strands re-anneal around the damage and the newly synthesised strands protrude as a short ‘chicken foot’ structure (Postow et al., 2001). This denotes a four-way structure, through which the two nascent strands are also annealed and DNA synthesis is continued beyond the sequence of the lesion using the newly synthesised molecule from the undamaged strand as a template. The process is concluded by reversal of the regressed fork to a normal configuration and the damaged site is practically bypassed. Furthermore, repositioning the blocking
lesion away from the replication fork may facilitate its repair by allowing access of repair enzymes (Atkinson and McGlynn, 2009).

In any case, repair of radiation-induced DNA damage and replication of the strands that contain them will result in one of three direct possibilities: restoration of the genome to its original integrity, permanent arrest of the cell cycle or the introduction of mutations into the genomic sequence (mainly deletions, translocations and point mutations). The mutation induction of the latter scenario is central to producing the detrimental stochastic effects of exposure to ionising radiation. Therefore, characterising the nature of this process is essential, especially with regards to any effects that may be inherited by the next generation.
2.2 Germ Line Mutagenesis

Considering the conversion of sites of DNA damage to mutations by low-fidelity repair and replication processes, it follows that tissues with a high mitotic index (rate of replication), such as in the male germ line, will manifest an increased onset of mutations following exposure to ionising radiation (Hall and Giaccia, 2011). Moreover, irradiation is likely to affect the variety of mutations; with partial deletions of chromosome regions following low-fidelity repair of radiation-induced double-strand breaks most likely to occur. This is in contrast to the single base, point mutations most commonly associated with spontaneous events.

According to target theory, mutation frequency should also increase with increasing absorbed dose: more energy causes more damage, which would in turn result in a higher yield of mutations. Furthermore, this would be affected by the dose-rate of exposure, since a given level of damage would be less of a burden to repair mechanisms if its incidence was spread out over a longer period of time. Conversely, with a more acute irradiation cells would have to deal with a more intense onset of damage. Therefore, studies of mutagenicity in model systems can compare the effects of various doses and dose-rates of radiation to those observed in tissue only exposed to background radiation (~3 mSv/year). Since background radiation is generally unavoidable these exposures are typically used for non-exposed controls in experimental studies.

2.2.1 Mutational End-Points

However, analysing mutation induction also requires an appropriate means of detection. Mutations occur in a variety of forms, from sequence alterations to chromosome aberrations, and the chosen end-point for an experimental design must be
sensitive and reliable to provide sufficient detection of them. This is especially relevant when examining heritable mutations in the exposed germ line. Such events are predominantly measured by detecting mutations occurring in the germ line of irradiated parents and inherited by their offspring. The following sections outline the most popular methods used to this effect.

2.2.1.1 Traditional Mutation Detection Assays

From the 1950s onwards the Russell laboratory produced some of the most reliable data on germ line mutation induction after developing the specific locus test (Russell, 1951). This system made use of a T (test) stock of mice that are homozygous recessive (/-) for seven loci with distinct phenotypic manifestation, such as coat colouring and morphological structures. Upon mating with control or irradiated wild-type mice, homozygous dominant (+/+), for the same loci, observable changes in the resultant offspring would accurately correspond to mutation induction in the wild-type germ line. Due to the objective and reproducible nature of this method, it provides a highly robust system of germ line mutation induction. However, since the mutation rate at these structural genes does not exceed $10^{-5}$ per generation (Schlager and Dickie, 1967), this assay requires the use of an exceptionally large number of mice parentally exposed to very high doses of ionising radiation (3-6 Gy). This considerably hinders its widespread use. Furthermore, the technique is solely applicable to the mouse model due to the need to expose mice with specific genotypes to excessively high irradiation and so does not provide a comparable means of investigating mutation induction in humans.

Similar problems have arisen when analysing the induction of dominant mutations that produce a visible phenotype. For example, detecting skeletal
malformations in the offspring of control and irradiated parents has otherwise
provided a reliable means of demonstrating the germ line mutagenicity of ionising
radiation (Bartsch-Sandhoff, 1974; Selby, 1979; Ehling, 1991). This is aided by the
large number of genes that contribute to the intricate development of the skeleton
throughout embryonic development, which provides a system representative of
radiation-induced damage throughout protein-coding regions of the genome.
Nevertheless, the low spontaneous frequency of these events (0.06 %) and difficulty of
performing detailed inspection of internal malformations that are not readily apparent
greatly restricts use of these methods (Searle, 1974).

Germ line mutation induction may also be measured using the dominant lethal
assay. Males are irradiated and mated with unexposed females, which are then
sacrificed 17 days into gestation. Upon assessment of the uterus, the number of viable
offspring is compared to the number of potential offspring, as determined by the
number of corpus lutea. In this way, several studies have reported that the incidence of
embryonic mortality increases with increasing paternal dose of exposure (Luning and
Searle, 1971; Searle and Beechey, 1981; Kirk and Lyon, 1984). Even so, this analysis
does not provide reliable and robust estimates of spontaneous or induced mutation
rates because it fails to take into account the induction of non-lethal mutations or the
effects of maternal health and environment.

The damage induced by exposure to ionising radiation may also result in changes
to chromosome structure; particularly following low-fidelity repair of double-strand
breaks produced prior to DNA replication. These aberrations may be scored by
conventional cytogenetic techniques or fluorescence in situ hybridisation (FISH) to
provide a clear system of monitoring germ line mutation induction (van Buul, 1983).
This may be detected as reciprocal translocations inherited by the F₁ offspring of
exposed fathers (Leonard and Deknudt, 1967; van Buul, 1983) or via direct analysis of irradiated sperm DNA (Generoso et al., 1984). Furthermore, though the data from human studies is limited, it is thought that such chromosomal analysis in model systems may be appropriately adjusted to allow reliable extrapolation to humans. However, this should be considered with caution due to inherent differences in the complex processes that create chromosomal aberrations in various mammalian species (van Buul, 1983).

An increase in reciprocal translocations in mice may also be associated with heritable semi-sterility of the male offspring of irradiated fathers (Lyon, Phillips and Searle, 1964). The analysis of this connection has been used to demonstrate germ line mutation induction that manifests as changes in fertility in the F_1 generation. Even so, the use of this method is limited by the complex breeding protocol of the large number of mice required. Moreover, the technique appears to lack the sensitivity needed to detect mutation induction following the chronic irradiation most relevant to human exposure (Phillips and Searle, 1964).

Another end-point that has been used to analyse germ line mutation induction is lacI mutations (Luke et al., 1997; Hoyes et al., 1998). This assay uses a λ shuttle vector that contains the target gene to create transgenic mouse constructs. lacI encodes the repressor of the lacZ gene that itself encodes the β–galactosidase enzyme. Once the strain is established, mice are exposed to ionising radiation and the vector may subsequently be recovered from the murine genomic DNA of the offspring. The extracts are then plated with bacteria on agar containing a β–galactosidase indicator. Transduction of phage DNA carrying lacI mutations will result in blue plaques indicative of β–galactosidase activity, as opposed to the clear plaques of progenitor
alleles. However, such studies have only demonstrated an increase in germ line mutation frequency following exposure to high doses of ionising radiation.

Together these predominantly phenotypic-based assays have provided strong *in vivo* evidence for germ line mutation induction caused by irradiation. Nevertheless, problems with the accuracy of many of them have greatly reduced their efficacy for characterizing the nature of this induction, particularly with regards to the low doses and dose-rates of ionising radiation most relevant to human exposure. At the dose-ranges used in these studies, irradiation may result in extensive DNA damage that induces cellular senescence or apoptosis in the exposed germ cells and evades detection, effectively reducing the mutagenic capability of the irradiation (Preston and Brewen, 1976). Furthermore, the lack of sensitivity inherent to most of the traditional methodology ensures the need for an extremely high number of individuals exposed to unusually high doses of ionising radiation, which is now becoming increasingly unacceptable. The high dose exposure, necessitated by this insensitivity, therefore often produces data that does not reflect the full extent of radiation-induced mutation induction.

The relevance of many of the end-points themselves is also questionable, with regards to their representation of genome wide events, their implications for humans and finding a corresponding means of mutation detection for use in human studies. This produces the need for an efficient system of mutation detection that corrects these inadequacies. The following section describes a set of DNA loci that ostensibly meets these criteria.
2.2.1.2 Tandem Repeat Loci as Markers of Mutation Induction

Repetitive DNA sequences form a major part of most eukaryotic genomes: up to 69% in the human genome (de Koning et al., 2011). Therefore, investigating mutation induction at repetitive DNA would be far more representative of radiation-induced, genome-wide mutation events than protein coding sequences, which constitute less than 2% of the genome. Of particular interest is the tandem-repeat family of repetitive sequences. This family is divided into three main classes, primarily based on the length of the constituent repeat units and the total size of the loci: satellite DNA, minisatellites and microsatellites. A fourth class, known as Expanded Simple Tandem Repeat loci (ESTRs), may be found in the mouse genome and possess characteristics of both mini- and microsatellites.

Due to the size of mammalian satellite DNA loci (up to 5 Mb in the largest centromeric regions) they are rarely useful for genotyping purposes. On the other hand, minisatellite loci (Table 2.1), with an array size ranging from 0.5 to 30 kb and made up of repeat units of 8-90 bp (Jeffreys, 1987), are a reasonable target for routine mutation detection. Minisatellites number in the thousands, distributed throughout the genome with a strong bias towards telomeric regions (Armour, 1999). These loci may show sequence variants within a given repeat array, but primarily differ in repeat copy number between alleles. Furthermore, the mutation rate at minisatellites varies greatly between specific loci (Turnpenny and Ellard, 2011). While many are shown to be relatively stable, with mutation rates less than 0.5% per gamete, some hypervariable minisatellites have been found to have mutation rates higher than 20%. It is these hypermutable sites that are of particular interest as an end-point for mutation induction analysis.
Male germ line mutations at minisatellite loci may be detected by gel electrophoresis and Southern blotting-hybridisation techniques via traditional pedigree-based analysis using genomic DNA of the offspring or by PCR-based approaches that directly examine rare mutation events in sperm DNA. However, due to the lack of oocytes for direct analysis, the pedigree approach is the only current means of maternal mutation detection. Though this provides a useful method to measure the rate at which new mutant allele lengths are observed, PCR amplification of a single DNA molecule is far more efficient and provides additional information about the processes acting at the loci (Jeffreys et al., 1990). This small-pool PCR (SM-PCR) involves the dilution of genomic DNA to the estimated concentration of 10-20 diploid genomes and the amplification of multiple aliquots per sample. The reactions each contain PCR products derived from the progenitor alleles and, in some cases, from mutant length variants. An understanding of the turnover processes that occur at these repeats may also be investigated via minisatellite variant repeat mapping by PCR (MVR-PCR) that makes use of the internal variants found in all hypermutable minisatellites (Jeffreys et al., 1991a; 1994; May et al., 1996). Such work details the distribution of variant repeats throughout a given array to chart the structure of alleles before and after mutation events.

Microsatellites are a much smaller form of tandem repeat array that may vary in size between 10 bp and 1 kb (Table 2.1), with repeating sequences of 1-6 bp and little internal variation (Turnpenny and Ellard, 2011). They have also been shown to have mutation rates orders of magnitude greater than protein coding genes and are distributed throughout the genomes of most organisms. These loci may be found in non-coding regions as well as in coding sequences where trinucleotide repeat arrays that code for repeated amino acids, and thus do not cause frame-shift mutations, are
most common. Another common form of microsatellite is the CA dinucleotides that are present around every 30,000 bp in the human genome. Mutations at microsatellite loci may also be detected by pedigree- and PCR-based electrophoretic approaches in a similar manner as for minisatellites.

<table>
<thead>
<tr>
<th></th>
<th>Minisatellites</th>
<th>Microsatellites</th>
<th>ESTRs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Repeat unit length</td>
<td>8 - 90 bp</td>
<td>1 - 6 bp</td>
<td>4 - 10 bp</td>
</tr>
<tr>
<td>Length of alleles</td>
<td>0.5 - 30 kb</td>
<td>10 bp – 1 kb</td>
<td>0.5 – 20 kb</td>
</tr>
<tr>
<td>Repeat homology</td>
<td>Often heterogenous</td>
<td>Mostly homogenous</td>
<td>Mostly homogenous</td>
</tr>
<tr>
<td>Hypothesized mechanism of mutation</td>
<td>Recombination/gene conversion events</td>
<td>Replication stress</td>
<td>Replication stress</td>
</tr>
</tbody>
</table>

Table 2.1 – Comparison of tandem repeat loci used for mutation detection. The murine ESTR array shares characteristics of both minisatellites and microsatellites that allow them to be successfully utilised in model investigation.

However, the mechanisms that give rise to mutations at these two forms of tandem repeat array differ significantly. Minisatellites themselves appear to have distinct mutation processes in somatic and germ line tissues (Shanks et al., 2008a). In somatic tissues, MVR-PCR-based investigation has shown that mutations were predominantly simple duplications/deletions of repeats, randomly distributed throughout the array without clustering (Jeffreys et al., 1997). Furthermore, mutants have been detected at heterogeneous repeats within the array (Jeffreys, 1997; Jeffreys et al., 1999). Taken together, this may suggest the involvement of recombination events or unequal sister-chromatid exchanges. Conversely, mutation processes in the germ line appear to be more complicated. Minisatellite mutation rates in sperm DNA are up to 250-fold higher than in somatic cells (Jeffreys et al., 1994). Moreover, MVR-
PCR analysis of sperm DNA reveals profound clustering of mutation events to one end of the repeat arrays with a strong bias to gains and losses of a small number of repeats, regardless of allele size. The types of rearrangements are also of a complex nature, varying between individual minisatelites and may include imperfect reduplications of donor and recipient alleles in addition to clustered duplications and deletions. For example, analysis of the MS32 minisatellite has revealed an intense and highly localised meiotic crossover hotspot in the upstream flanking region, which extends into the start of the locus (Jeffreys et al., 1998). Such hotspots would potentially drive minisatellite instability and account for the observed polarity of mutation events. Additionally, blocks of repeats have also been shown to transfer between arrays via a conservative process that does not affect the allele of the donor array (Jeffreys et al., 1999; Vergnaud and Denoeud, 2000). This is thought to reflect gene conversion events that occur frequently during meiosis and the repair of double-strand breaks by homologous recombination.

In contrast, the lack of internal variants in microsatellite arrays prevents PCR based scrutiny of the structure of mutation distribution. Instead mechanistic analysis has focused on microsatellite repeat types that are associated with human repeat expansion disorders. This has revealed a number of common features that characterise microsatellite mutation processes. Firstly, detection of mutant microsatellite alleles is not associated with the transfer of flanking markers, suggesting that the mutational events are intra-allelic and not due to recombination-based processes (Levinson and Gutman, 1987; Morral et al., 1991). Secondly, high somatic and germ line microsatellite mutation rates have been observed in individuals affected by disorders such as myotonic dystrophy and Huntington’s disease, with strong dependence on the size of the progenitor alleles (Telenius et al., 1994; Leeflang et al., 1995; Monckton et
al., 1995; Wong et al., 1995). Furthermore, mutations at microsatellite loci have been shown to accumulate with age in tissues with a high mitotic index, suggesting a replication-based process (Kenyon et al., 2012). Also important is the simple spectrum of mutation events at these loci. Human microsatellites mutate via gains or losses of repeats with a strong bias towards changes of a few units (Huang et al., 2002). Taken together, these factors strongly suggest that microsatellite loci primarily undergo replication slippage to produce mutant alleles. This refers to the aberrant realignment of the two DNA strands at repeat elements following the completion of replication (Mariappan et al., 1996; Chen et al., 1998). Most of the sequence length rehybridises, but a small section may a form hairpin loop structure that inhibits the action of DNA polymerase and leads to the alteration in the length of the subsequent arrays.

Murine ESTR loci are tandem repeat loci with characteristics of both mini- and microsatellites (Table 2.1) (Kelly et al., 1989; Gibbs et al., 1993; Bois et al., 1998). They have minisatellite-like array sizes between 0.5 and 20 kb and similar germ line mutation rates, up to 10%. However, like microsatellites, they are predominantly homogenous throughout a given array, with relatively short (4-10 bp) repeat units. ESTR mutants are readily detectable by both pedigree analysis and single-molecule PCR (SM-PCR). The former has been used to describe high spontaneous mutation rates for these loci in both somatic (Kelly et al., 1989) and germ line tissues (Bois et al., 1998). Furthermore, PCR analysis of the spontaneous and induced ESTR mutation spectrum has shown that they both involve the gains or losses of homogenous repeats with a small-mutation bias reminiscent of microsatellite arrays (Yauk et al., 2002; Barber et al., 2009). Indeed, it is widely believed that ESTR mutational events involve the same slipped-strand synthesis that produces the mutant alleles of the latter (Sadamoto et al., 1994; Dubrova et al., 1993; Yauk et al., 2002). Support for this
notion is provided by recent observations that spontaneous mutations accumulate in an age-dependant manner, almost exclusively in replication-proficient cells (Hardwick et al., 2009) and that the frequency and spectrum of ESTR mutation does not differ between different stages of spermatogenesis, showing recombination-based events to be unlikely (Shanks et al., 2008b)

It should also be noted that mutation induction at ESTR loci does not conform to the rules of targeted mutation. If the average spontaneous mutation rate per ESTR locus (0.055) is compared to that induced by paternal X-irradiation with 1 Gy (0.225), the mutation rate is observed to have been elevated by 0.170 (Dubrova et al., 1998). Given a mean ESTR array size of $5 \times 10^3$ bp out of an approximate $3 \times 10^9$ bp that makes up the murine genome, this would estimate an additional 100,000 sites of DNA damage following exposure. In spite of this, each Gy of X-rays has been shown to produce only 3,300 new lesions (Frankenberg-Schwager, 1990). This discrepancy has been consistently demonstrated and suggests that the high level of mutation induction observed at ESTR loci is primarily due to untargeted events that occur elsewhere in the genome (Sadamoto et al., 1994; Dubrova et al., 1998; Niwa and Kominami, 2001). How this occurs is largely unknown, but it is likely to reflect non-targeted, delayed mutational events following exposure to ionising radiation that are not accounted for by target theory (see Chapter 3).

### 2.2.2 Germ Line Mutation Induction

A large number of epidemiological and model studies have used these tandem repeat loci to investigate germ line mutation induction following ionising radiation. However, analysis of human populations has produced a highly conflicting body of work. Dubrova and colleagues (1996) examined families from Belarus after the
Chernobyl disaster who were exposed to less than 5 mSv a year and reported a 2-fold increase in mutation frequency for four different loci. A follow up study that added 41 families to the original cohort also showed this response using a range of minisatellite probes (Dubrova et al., 1997). Similar analysis of Ukrainian residents, exposed to an estimated 0.2-0.4 Sv, resulted in a sex-specific response where only paternal exposure increased germ line minisatellite mutation rates (Dubrova et al., 2002b).

However, studying the liquidators who responded to the disaster has failed to reveal significant increases in minisatellite mutation rates across a range of paternal doses up to 1.2 Sv (Livshits et al., 2001; Kiuru et al., 2003; Slebos et al., 2004). Moreover, other authors described the absence of germ line mutation induction at microsatellite loci in the families of Ukrainian and Belarusian response workers (Slebos et al., 2004; Furitsu et al., 2005; Ryo et al., 2006). Evidence for tandem-repeat array mutation induction in human populations has also been elusive following the atomic bombings in Japan. Kodaira and co-workers have consistently demonstrated the apparent inability of high doses from the survivor cohort to affect germ line mutation rates at either minisatellites (Kodaira et al., 1995; 2004; Satoh et al., 1996) or microsatellites (Satoh et al., 1996; Kodaira et al., 2010).

Nevertheless, subsequent studies have found evidence for germ line minisatellite mutation induction in other highly exposed populations. Significantly elevated minisatellite mutation frequencies were described in the male germ line for villagers that live along the Techa River who were exposed to an average 102 mSv, primarily by internal plutonium consumption due to radioactive material released from the Mayak facility (Dubrova et al., 2006; Akleev et al., 2007). Similarly, Dubrova et al. (2002a) examined residents of the Beskaragai district of Kazakhstan after the Semipalatinsk nuclear bomb tests using eight hypervariable minisatellite single locus
probes and detected a significant 1.8-fold increase in the germ line of men exposed to fallout doses greater than 1 Sv.

Even so, the multiple confounding factors that characterise epidemiological analysis (see Chapter 1.3.2) prevent a conclusive narrative of radiation-induced germ line mutation induction from forming. Use of ESTR loci in mouse studies has successfully demonstrated an increased germ line mutation rate for male mice exposed to a variety of irradiation profiles. Moreover, this has often enabled the identification of clear dose response trends and an estimation of the mutation-rate doubling dose in these groups. The doubling dose is that which induces an additional mutation rate per generation that matches the spontaneous rate itself. It offers an expression of the mutagenic capacity of a given agent.

2.2.2.1 Dose Responses

Dubrova and colleagues (1993) performed a pedigree-based analysis of approximately 200 mice paternally exposed to either 0.5 or 1 Gy of $\gamma$–radiation. In this way, a 2-fold increase in germ line mutation frequency was observed for two ESTR loci ($Ms6$-$hm$ and $Hm$-2). Subsequent studies have described this increase to show a clear linear dose response for 0.5-3 Gy of low-LET irradiation (Fan et al., 1995; Dubrova et al., 1998; 2000a; Dubrova, 2005) and high-LET based exposure in excess of 0.35 Gy (Niwa et al., 1996; Dubrova et al., 2000a). Through this work, Dubrova and colleagues have estimated the doubling dose of low-LET exposure to lie between 0.33 and 0.5 Gy for the CBA/H strain of mice (Dubrova et al., 1993; 1998) and at 0.88 Gy for BALB/c mice (Dubrova, 2005). The difference in doubling doses between the two strains is likely to reflect known polymorphisms in DNA-PKcs for the BALB/c mice (Fabre et al., 2011). These findings are supported by those of a wide range of studies that measure traditional endpoints of mutation induction, including heritable
chromosomal aberrations (Leonard and Deknudt, 1967), dominant lethal mutations (Searle and Beechey, 1981) and protein-coding genes (Russell and Kelly, 1982a). In this way, it is thought that low to intermediate acute doses produce a linear mutational response, as predicted by the model of target theory.

Furthermore, model investigation has proposed that the mutagenicity of irradiation is greatly reliant on the dose-rate of exposure (Phillips and Searle, 1964; Russell and Kelly, 1982b; van Buul, 1983). The Russell group demonstrated that the doubling dose at the genes of the specific locus test increases to 1 Gy for chronic irradiation as opposed to the mere 0.3 Gy required by high-intensity exposure (Russell and Kelly, 1982b). Even so, a more recent publication showed that between 0.5 and 1 Gy of low-LET ionising radiation, there are no differences in the efficiency of ESTR mutation induction, suggesting that a more detailed analysis is warranted (Dubrova et al., 2000a).

The straightforward, targeted response to radiation exposure is also complicated by reports of a phenomenon called radiation hormesis. This hypothesis proposes that low doses of ionising radiation that approach natural background levels produce a minute, easily-managed level of DNA damage, but also provide a beneficial, adaptive response through the activation of global repair responses that protect against disease or even subsequent exposure to a higher dose (Feinendegen, 2005). Hormesis theory is primarily based on the observations that enzymatic DNA repair mechanisms may be activated by doses as low as 10 mSv and that damaged/unrepaired cells are regularly removed via apoptosis (Tubiana et al., 2009). The response has been described in vitro whereby the mutagenicity of an acute γ-irradiation with 4 Gy is reduced up to 2-fold when previously exposed to γ-rays as low as 0.1 Gy (Azzam et al., 1994). Similar events have also been described in whole body mice models (Miyachi, 2000). Even so,
the effect is yet to be convincingly demonstrated in humans (Hwang et al., 2006) and remains highly controversial.

The dose-rate effect portrays a related phenomenon. As the intensity of exposure is lowered from high acute dose-rates to a more protracted treatment, the challenge to repair mechanisms becomes less severe. An increasing proportion of sublethal damage may be comfortably repaired by the cell, until a point when all such damage is actively managed (Hall and Giaccia, 2011). However, an inverse-dose-rate effect has been demonstrated in vitro using mammalian cells, whereby a further lowering of the dose-rate permits cells to progress through cell cycle checkpoints, allowing the accumulation of damage that escapes cellular detection (Vilenchik and Knudson, 2000). Nonetheless, it remains to be seen how these events affect the complex processes of mutation induction at tandem repeat loci, especially with regards to germ line mutagenesis.

2.2.2.2 Stage-Specific Responses

Another factor that has been shown to influence the germ line mutagenicity of ionising radiation is the irradiated stage of spermatogenesis that is investigated by the various studies. These stages may be individually analysed by understanding the duration of each stage and the time taken for the respective germ cells to develop into mature sperm in the ejaculate. This is well characterised in mice (see Table 2.2). It is also well established that these different stages show varying sensitivity to the damage produced by irradiation, reflected in the extent of cell killing (Searle, 1974). Nevertheless, there is a great discrepancy in the reports of mutation induction following these same stages of spermatogenesis. Traditional assays of germ line mutation induction have generally shown that post-meiotic spermatids are most
sensitive to irradiation (Ehling, 1971; Nomura, 1982; Favor, 1999). This supported by ESTR data collected by Niwa and colleagues (Sadamoto et al., 1994; Fan et al., 1995; Niwa et al., 1996). The explanation offered for this bias is the decreased capacity of the post-meiotic male germ line for repair. This would imply that a high level of induced lesions is carried to fertilisation where mutations are introduced in the embryo (Fan et al., 1995). However, if the mutations observed at ESTR loci are results of the processing of DNA damage it may also follow that such repair systems, intact at pre-meiotic spermatogonia and stem cells, sensitise cells to manifesting mutations. If this were the case, the results of studies by Dubrova and colleagues would be more appropriate. These authors have consistently demonstrated the high sensitivity of pre-meiotic male germ cells to radiation exposure and an actual lack of mutation induction for irradiated post-meiotic spermatids and spermatozoa (Dubrova et al., 1998; Yauk et al., 2002; Dubrova, 2005; Barber et al., 2009).
<table>
<thead>
<tr>
<th>Germ cell stage</th>
<th>Days taken to reach ejaculate</th>
<th>Corresponding mating scheme</th>
<th>Sensitivity to cell killing by IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial germ cells</td>
<td>Not known</td>
<td>Not known</td>
<td>Intermediate</td>
</tr>
<tr>
<td>A&lt;sub&gt;s&lt;/sub&gt; stem cells</td>
<td>42 +</td>
<td>&gt; 6 weeks</td>
<td>Low</td>
</tr>
<tr>
<td>Type A spermatagonia</td>
<td></td>
<td></td>
<td>High</td>
</tr>
<tr>
<td>Intermediate spermatagonia</td>
<td>35-37</td>
<td></td>
<td>Very high</td>
</tr>
<tr>
<td>Type B spermatagonia</td>
<td>34-36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary spermatocytes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preleptotene</td>
<td>33-35</td>
<td></td>
<td>Intermediate/low</td>
</tr>
<tr>
<td>Leptotene</td>
<td>32-33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zygote</td>
<td>30-32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pachytene</td>
<td>23-30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diplotene</td>
<td>22-23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diakinesis (Meiosis)</td>
<td>21-22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Secondary spermatocytes</td>
<td>21-22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spermatids</td>
<td>7-21</td>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>0-7</td>
<td>&lt; 1 week</td>
<td>Very low</td>
</tr>
</tbody>
</table>

Table 2.2 – Spermatogenesis in mice. Timings of the various stages of spermatogenesis and the corresponding mating schemes used in mouse models are shown. Information on the relative sensitivities to cell killing by ionising radiation is also provided. Adapted from Searle (1974).
2.3 Experimental Design

The present study seeks to clarify the dose response of direct exposure by investigating the influence of varying doses and dose-rates of low-LET ionizing radiation on mutation induction in the germ line of exposed male mice. Given that previous work has shown that expanded simple tandem repeat (ESTR) loci currently provide the most powerful tool for monitoring radiation-induced germ line mutation in mice (Dubrova et al., 1998), which is representative and reliable (Singer et al., 2006), they were chosen for use in this capacity. BALB/c inbred strains were used, which possess Ms6-hm ESTR alleles that are readily amplifiable by PCR and, having been used in previous studies of radiation-induced mutation, have well characterized ESTR mutation rates (Barber et al., 2006; Hatch et al., 2007; Shanks et al., 2008).

6-week-old males were exposed to a range of doses of γ-rays, 10, 25, 50 and 100 cGy, which were administered at a high dose-rate to achieve an acute irradiation range (see Chapter 4.2.1). This range was designed to consider the low to intermediate doses that characterize human exposure to ionizing radiation. All current X-ray-based radiotherapy regimens deliver a highly accurate beam, angled and shaped directly at the target tumour and with normal tissues shielded to ensure the lateral side scatter dose they receive is rarely in excess of 10 cGy per procedure (UNSCEAR, 2000). Even the maximum official estimates of dose due to fallout from the atomic blasts at Hiroshima and Nagasaki, 1-3 cGy and 20-40 cGy respectively, fall below or within the range considered by the present study and the average exposure to survivors has been estimated at 30 cGy (Young and Kerr, 2005). The chosen range also reflects the external effective doses to which survivors of the various groups affected by the Chernobyl disaster were exposed (see Chapter 1.2). Doses in excess of 100 cGy are very rare in terms of human exposure and increase the likelihood of apoptosis,
removing more and more germ cells from the gene pool (Radford, 1985). On another level, individuals exposed to higher acute doses begin to suffer progressively severe acute radiation syndromes (Bushberg, 2009) and those exposed become increasingly less likely to father additional children, making the germ line effects of these doses less relevant with increasing dose.

A chronic exposure of 100 cGy delivered over 2 weeks was also investigated to reflect human exposures that, even at the highest doses, are accumulated over an extended period of time, but with a much lower dose rate. The vast majority of public and occupational exposures to ionizing radiation occur in such a way (see Chapter 1.2). Generally, 100 cGy is also higher than the total absorbed dose to the shielded germ line accumulated by the end of a fractionated radiotherapy treatment (UNSCEAR, 2000). This daily irradiation has previously been shown to have a similar level of mutagenicity to low dose-rate chronic exposure (Russell and Kelly, 1982a; b; Lyon, Philips and Bailey, 1972; Tucker et al., 1998). In this way, the study hopes to analyse a low dose-rate that is representative of long-term human exposure to radiation.

The mice were culled 12 weeks after exposure for their caudal epididymides and sperm DNA was extracted. This ensured that extracted germ line DNA was derived from irradiated A| spermatogonia (Searle, 1974). Although there is no consensus, exposed spermatogonia (> 6 weeks before reaching the ejaculate) may provide the most consistent means of demonstrating germ line mutation induction (Dubrova et al., 1998; Yauk et al., 2002; Dubrova, 2005; Barber et al., 2009). Furthermore, stem cells at the older range of spermatogonia (> 10 weeks before reaching the ejaculate) are known to have a lower sensitivity to cell killing by ionising radiation (Searle, 1974).
As fewer cells are removed from the germ line, a more accurate understanding of mutation induction in these cells may be obtained.

ESTR mutation frequency was evaluated at the Ms6-hm locus by single-molecule PCR (SM-PCR) and then compared between irradiated and control mice (see Chapter 4). In this way, the germ line could be analysed directly, substantially reducing the time and number of mice required for pedigree-based germ line mutation monitoring that have been shown to produce indistinguishable results (Yauk et al., 2002). Power calculations based on the Poisson approximation shows that a 1.6-fold increase in mutation frequency may be robustly detected by profiling just 3 controls and 3 treated male mice, provided that approximately 150 amplifiable molecules were analysed in each animal. SM-PCR analysis involved the optimisation of a concentration of DNA for each sample that enables there to be one haploid genome, and so one amplifiable molecule of ESTR DNA, in each PCR reaction. To achieve this a range of concentrations were initially investigated for each sample and the concentration that yielded ~50% positive reactions was taken as an estimate of the single-molecule concentration. In this way, the likelihood of there being only one amplifiable molecule in a positive reaction is increased, while providing sufficient negative reactions for Poisson estimation of the total number of amplifiable molecules and the corresponding standard error. Positive reactions were gel electrophoresed together on gels long enough to allow sufficient resolution for mutation scoring. These values were finally used to calculate the mutation frequency for a given sample, which would allow for statistical comparison between each group.

Care was also taken to ensure somatic mosaic mutations were excluded from analysis. This takes into consideration the high incidence of mosaicism that occurs during embryogenesis, which would otherwise bias investigation.
2.4 The Dose Response of Mutation Induction at ESTR Loci

Table 2.2 presents a summary of ESTR mutation data for direct exposure to ionising radiation. Mice irradiated with 50 or 100 cGy of acute γ-rays showed significant increases in germ line ESTR mutation frequency (1.95-fold and 2.39-fold, respectively). The mutagenicity of chronic exposure to 100 cGy was far less pronounced, showing only a marginally significant 1.64-fold elevation in mutation frequency. Lower acute doses produced only small increases in mutation induction.

Table 2.2 – ESTR mutation induction in the germ line of irradiated males

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mutations</th>
<th>Frequency ± s.e.</th>
<th>Ratio†</th>
<th>t‡</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20 (516 ± 27)</td>
<td>0.0388 ± 0.0089</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 cGy, acute</td>
<td>22 (465 ± 27)</td>
<td>0.0473 ± 0.0105</td>
<td>1.22</td>
<td>0.62</td>
<td>0.5339</td>
</tr>
<tr>
<td>25 cGy, acute</td>
<td>17 (313 ± 20)</td>
<td>0.0543 ± 0.0136</td>
<td>1.40</td>
<td>0.95</td>
<td>0.3402</td>
</tr>
<tr>
<td>50 cGy, acute</td>
<td>28 (371 ± 23)</td>
<td>0.0755 ± 0.0150</td>
<td>1.95</td>
<td>2.10</td>
<td>0.0359</td>
</tr>
<tr>
<td>100 cGy, acute</td>
<td>47 (508 ± 32)</td>
<td>0.0925 ± 0.0147</td>
<td>2.39</td>
<td>3.13</td>
<td>0.0017</td>
</tr>
<tr>
<td>100 cGy, chronic</td>
<td>32 (503 ± 27)</td>
<td>0.0636 ± 0.0117</td>
<td>1.64</td>
<td>1.69</td>
<td>0.0920</td>
</tr>
</tbody>
</table>

*Number of amplifiable DNA molecules (± s.e.) is given in brackets.
†Ratio to control.
‡Student’s t-test and probability for difference from controls
This data was used to evaluate the dose-response of ESTR mutation induction. Figure 2.1 shows a clear linear progression of ESTR mutation frequency with increasing acute dose, with doses below 50 cGy producing small, but distinct mutagenic effects. Spontaneous, control mutation frequency, \( m_0 = 0.0388 \pm 0.0089 \) and mean mutation induction, \( ind = (6.85 \pm 0.85) \times 10^{-4} \text{ cGy}^{-1} \) were used to estimate the doubling dose for ESTR mutation:

\[
DD = \frac{m_0}{ind} = 57 \pm 15 \text{ cGy}
\]

Figure 2.1 – The dose and dose-rate effects on ESTR mutation induction in the germ line of irradiated males as measured by the SM-PCR technique.
2.5 Discussion

Investigating germ line mutation frequency at the ESTR locus Ms6-hm of directly exposed male mice revealed a linear dose-response across an acute dose range of 10 to 100 cGy, with a 2.39-fold increase in mutation frequency for the largest dose and distinct mutagenicity observable even at lower doses. This fits the long-standing paradigm of direct mutation induction and is in line with the results of the group’s previous studies on radiation-induced tandem repeat instability following pre-meiotic exposure to low-LET ionising radiation (Dubrova et al., 1993; 1998; Dubrova, 2005). A linear response for mutation induction also correlates well with the linear relationship established for the level of total induced DNA damage within a very wide range of doses (Frankenberg-Schwager, 1990). The higher the energy deposited in a cell the higher the potential for DNA damage and so the greater the probability for the manifestation of mutations following low-fidelity repair and replication. However, caution should be maintained when extrapolating the present findings to doses outside of the range investigated. At higher doses, cell death would begin to have a greater impact on the frequency of cells that may carry mutations and survive. Likewise, the very low doses that have been shown to challenge assumptions of a target theory-based dose response (see 2.2.2.1) would be outside the design of the current study with its focus on a range relevant to the maximum doses of common human occupational and accidental exposure.

Furthermore, with a doubling dose for ESTR mutation of 57 ± 15 cGy the present data closely resembles that previously obtained for the BALB/c strain using a pedigree-based approach (88 cGy ± 29 cGy; t = 0.95; P = 0.32) (Dubrova, 2005). This is also consistent with the group’s SM-PCR-based (Glen et al., 2008) and pedigree-based (Vilarino-Guell et al., 2003) studies on the genetic effects of exposure to DNA-
damaging chemical mutagens. Taken together, these studies clearly show SM-PCR to be a reliable and valuable technique for measuring ESTR mutation induction in the mouse germ line.

Another observation is that acute irradiation appears to be more efficient for mutation induction than chronic exposure to an equivalent dose. This is in accordance with the Russell group observations that fewer mutations are produced in the male mouse germ line after low-dose-rate irradiation than after an acute dose (Russell and Kelly, 1982a; b). However, in contrast, a previous pedigree-based study by the present group, which looked at chronic and acute exposure to X- and γ-rays, reported similar increases in ESTR mutation rate in the germ line of CBA/H male mice for both dose-rates (Dubrova et al., 2000a). Further work on this matter would be necessary to address how varying degrees of chronic irradiation compare to acute exposure to low-LET ionizing radiation and how consistent these findings are across different mouse strains.

In any case, mutations that do not prevent cellular proliferation will be passed on directly to descendant cells and those that do not affect sexual reproduction or do not cause life-threatening dysfunction in the offspring, may be inherited by a new generation of individuals. The fact that exposure to ionising radiation can have genetic consequences is clear. However, though this may be the most apparent risk to the exposed and their children, it is growing increasingly clear that it may not be the only risk and, potentially, not even the most extensive.
Chapter 3: Transgenerational Effects

3.1 Non-Targeted, Delayed Effects

Our current understanding of the human health risks associated with exposure to ionising radiation is primarily based on the assumption that nuclear DNA is the sole critical target and that detrimental effects occur exclusively in irradiated cells, as discussed so far (see Chapter 2). However, it is now growing increasingly apparent that the effects of ionising radiation are far more profound than this previously accepted paradigm of targeted mutation induction suggests and that there are a number of associated non-targeted, delayed effects.

3.1.1 Genomic Instability

A clear demonstration of such phenomenon was provided by Ullrich and colleagues (1998), who showed that the rate of occurrence of chromosome aberrations in the non-exposed clonal progeny of irradiated human MCF10A mammary epithelial remained significantly elevated many cell divisions after exposure. This phenomenon is known as genomic instability. It defines a range of radiation-induced changes observed multiple cell divisions after the initial exposure and describes the susceptibility of this clonally expanded population to non-clonal genetic alterations such as chromosomal rearrangements, micronuclei formation, cellular transformation, gene amplifications, gene mutations and reduced plating efficiency (Morgan et al., 1996; Morgan, 2003a; b). In this way, the initial damage is memorised beyond the simple three-tier paradigm for maintaining genomic integrity; repair, cell cycle checkpoints and apoptosis; retaining the potential for ongoing mutagenic activity.

Another aspect of this phenomenon is that the mutations are induced in a region even without the presence of DNA damage and are thus non-targeted (Friedberg et al.,
2006). This is certainly the case for induced mutations at ESTR loci (see Chapter 2.2.1.2), which occur too frequently to be accounted for by the direct action of radiation at the locus (Sadamoto et al., 1994; Dubrova et al., 1998). Studies into the association of chromosomal instability and human microsatellite mutants with screened thymidine kinase gene mutations (Li et al., 1992; 1994; Grosovsky et al., 1996), also showed this to be a genome-wide process.

Initially, genomic instability had predominantly been observed as chromosome aberrations following in vitro, high dose (3-12 Gy), acute exposure to high-LET (Kadhim et al., 1992; 1994; 1995; Morgan et al., 1996; Morgan, 2003a) and, to a lesser extent, low-LET radiation (Mothersill et al., 2000; Holmberg et al., 1993; Marder and Morgan 1993; Kadhim et al., 1995). However, similar findings have also been observed in vivo by examining cells extracted from X- and γ-irradiated adult mice (Ullrich and Davis, 1999; Kligerman et al., 1990; Jagetia, 1993; Morgan, 2003b) and 2 Gy exposure of mouse zygotes produces an increase in novel chromosomal aberrations during second and third mitoses (Weissenborn and Streffer, 1988) and in derived skin fibroblast cultures (Pampfer and Streffer, 1989). Paquette and Little (1994) even showed that when 10T1/2 cells are exposed, transplanting them into syngeneic and non-immunosuppressed C3H mice results in neoplastic cells with a significantly higher frequency of genomic rearrangements than in subclones passaged in vitro.

Herein lies a major aspect to the biological significance of radiation-induced genomic instability: the increased risk of cancer after radiation exposure and its association with instability. Genomic instability is a hallmark of the oncogenic transformation of early cancer cells (Loeb et al., 2003) and it has been proposed that the increased accumulation of multiple genetic alterations within a cell, which
characterises radiation-induced instability, may in turn lead to an elevated prevalence of induced cancers (Little, 2000; Streffer, 2010). Petridou et al. (1996) reported a 2.6-fold increase in the incidence of leukaemia in children exposed \textit{in utero} to radiation following Chernobyl. An increase in the risk of developing thyroid cancer in individuals aged 0-19 years at the time of the accident has also been well documented (Astakhova et al., 1998; Cardis et al., 2006). Radiation-induced susceptibility to cancer has also long been established in mice following X-irradiation with 1-5 Gy (Ullrich and Storer, 1979a; b; Ullrich et al., 1979; Coggle, 1988). Nevertheless, the contribution of genomic instability to radiation-induced carcinogenesis still remains to be established.

Furthermore, the corresponding experimental data for markers of genomic instability in human populations is contradictory, suggesting problems with extrapolating the results of model systems on the radiation risk factors to humans. Nakanishi et al. (2001) detailed microsatellite instability in acute myelocytic leukaemia developed from atomic-bomb survivors and the effect has been reported in children treated for leukaemia (Finette et al., 2000). Even so, others have reported a lack of persistent chromosome aberrations in radiotherapy patients treated for a range of malignant conditions (Tawn et al., 2000).

### 3.1.2 Bystander Effects

Another non-targeted, delayed consequence of ionising radiation is the bystander effect. The term describes a group of effects whereby the non-exposed bystander cells experience similar mutagenic changes to those typically observed in irradiated cells. This may either be a result of cytoplasmic irradiation (Wu et al., 1999; Shao et al., 2004) or signals from nearby irradiated cells through secreted factors and/or gap...
junction communication pathways (Little et al., 2002). Similarly, bystander effects also occur when the tissue culture medium of irradiated cells is transferred to separate, unexposed cells (Mitchell et al., 2004).

Again, bystander effects have been primarily demonstrated with in vitro models of exposure to high-LET α–particles (Little et al., 2002) and low-LET X- or γ-rays (Seymour and Mothersill, 2000). Interestingly, unlike the genomic instability described above, it also appears to be a common phenomenon at doses lower than 0.5 Sv. Yang and colleagues (2005) reported an increase in micronuclei formation, accumulation of γH2AX foci and induction of the DNA damage sensor p21Waf1 in bystander human fibroblasts following X-irradiation of adjacent cells with doses as low as 0.1 Gy. There is also evidence for a threshold of low-LET irradiation with as low as 2 mGy of γ-rays, below which increased bystander cell survival is observed (Liu et al., 2006).

Even so, these effects are consistently shown to be inhibited by treatment with reactive oxygen species (ROS) inhibitors (Lehnert et al., 1997; Yang et al., 2005), but these authors also reported decreased clonogenic survival of bystander cells despite treatment with superoxidase dismutase (SOD). Taken together, this would suggest that ROS and other factors are released into the cellular environment in response to radiation exposure. Furthermore, subsequent work demonstrated that bystander effects could be suppressed by interruption of intercellular gap junction communication (Azzam et al., 1998), suggesting their involvement in signal propagation.

In vivo evidence for the effect has been shown in animal models (Mancuso et al., 2008; Bertucci et al., 2009) and in 3D human tissue models (Belyakov et al., 2005; Sedelnikova et al., 2007), up to distances of 1 mm from targeted irradiation. It remains to be seen to what extent this may contribute to the carcinogenic risks of low dose...
irradiation in humans (Blyth and Sykes, 2011), but Mancuso and colleagues (2008) have demonstrated malignant tumours in the shielded cerebellum following X-irradiation to the rest of the body.

An interesting link has also been made between radiation-induced genomic instability and bystander effects. Lorimore et al. (1998) interposed a shielding grid between an α-particle source and clonogenic haematopoietic stem cells, such that the surviving population consisted predominantly of unexposed cells, and obtained the same frequency of chromosome aberrations in descendant clones as was induced without the grid. This suggests that a bystander mechanism could largely facilitate the spread of genomic instability. The same group made a similar connection in vivo, whereby mixtures of irradiated and nonirradiated haematopoietic cells, distinguished by a cytogenetic marker, were transplanted into CBA/H mice (Watson et al., 2000). Cytogenetic analysis of the repopulated bone marrow in the 12 months that followed demonstrated chromosome instability in the progeny of both cell populations, implying that bystander effects can be both a cause and a consequence of radiation-induced genomic instability. A reciprocal study, where an individual was accidentally exposed to high-dose, high-LET radiation and received a successful bone marrow transplant, found chromosomal aberrations in donor cells; suggesting that evidence for the association between the two effects is not limited to experimental models (Chiba et al., 2002).

3.1.3 Clastogenic Factors

A related phenomenon is borne in the blood plasma of irradiated individuals and suggests the presence of a long-range, bystander-type effect in response to exposure. Plasma from X-irradiated patients has been shown to induce chromosomal aberrations
in peripheral blood lymphocytes from unexposed individuals after co-culture (Littlefield et al., 1969). The effect is clearly detectable in rodent models (Faguet et al., 1984) and suggests that irradiated cells secrete clastogenic factors that circulate the body in the blood. Tamminga and colleagues (2008b) demonstrated the long distance potential of the effect in vivo and how it may affect the germ line. Male rats were subjected to a localized 20 Gy X-ray exposure to the hippocampal area of the skull, while the rest of the body was completely protected by medical grade shielding. They found a significant accumulation of unrepaired DNA lesions and a global loss of DNA methylation in the mature sperm cells.

Interestingly, clastogenic activity has also been described in the blood plasma of survivors of the atomic bombings in Japan, three decades after the incident (Pant and Kamada, 1977). Several groups even consistently demonstrated the transferable mutagenic capacity of plasma serum obtained from those who had worked as liquidators following the Chernobyl disaster (Emerit et al., 1994; 1997; Marozik et al., 2007), persisting up to 20 years since irradiation (Marozik et al., 2012). Together, this would establish the regular presence of clastogenic effects in human populations with a relatively low level of exposure to low-LET ionising radiation.
3.2 Transgenerational Instability

Since all of an individual’s cells are derived from the same clonally expanded lineage, that of the zygote, every cell will inherit DNA from the constituent germ cell of each parent. In this way, genomic instability, once triggered, may also be transmitted to the offspring derived from the germ line of a directly exposed individual. This would manifest itself as an increase in novel mutations in the unexposed offspring, detectable by similar end-points as for genomic instability in directly exposed individuals. Traditional germ line mutation induction studies analyse the frequency of mutations inherited by the derived offspring (see Chapter 2.2). Investigating the transmission of genomic instability to these progeny requires a comprehensive assessment of the de novo mutations that occur in them and may be inherited by a second generation. In theory, this transgenerational mutagenicity would also imply an increase in the susceptibility of the offspring to carcinogenesis. However, evidence for this is far from conclusive and the mechanism of how instability extends to the next generation is unclear.

3.2.1 Human Data

Transgenerational instability has been difficult to observe in exposed human populations. This is primarily due to large discrepancies in the nature of the irradiation that studied populations have been exposed to and trouble in identifying appropriate control groups. Furthermore, investigating such individuals is often problematic due to the difficulties found in obtaining reliable, accurate dose/dose-rate information (see Chapter 1.2).

There is some evidence for the presence of the phenomenon in children born to families living in the Beskaragai district of Kazakhstan after the Semipalatinsk nuclear
weapons tests (Dubrova et al., 2002a). The germ line of individuals exposed to an estimated dose greater than 1 Sv saw a statistically significantly 1.8-fold elevation in tandem repeat mutation induction, which dropped to a 1.5-fold increase in that of the next generation. Furthermore, several recent studies have shown that children of fathers exposed to post-Chernobyl radiation have an elevated frequency of chromosome aberrations and thus manifest instability (Pilins’ka et al., 2005; Suskov et al., 2008; Aghajanyan and Suskov, 2009; Aghajanyan et al., 2011). An increase in the frequency of stillbirths among the children of male workers at the Sellafield nuclear reprocessing plant was also detailed (Parker et al., 1999). However, no transgenerational effects have been described in the children of A-bomb survivors in Hiroshima and Nagasaki (Schull, 2003). Tawn et al. (2005) also failed to detect any significant changes in chromosome aberration frequency among the offspring of childhood cancer radiotherapy survivors, but then reported clear G2 chromosomal radiosensitivity in the children of patients from the same Danish cohort (Curwen et al., 2005).

Studying radiation-induced, human transgenerational predisposition to cancer has been just as controversial. A widely publicized study by Gardner et al. (1990) reported that the children of Sellafield radiation workers had an extremely high chance of developing leukaemia and non-Hodgkin’s lymphoma, a result that has provoked re-examinations with no real consensus (Draper et al., 1997; Lord, 1999; Roman et al., 1999; Dickinson and Parker, 2002). Several early epidemiological studies also claimed to have observed an increased frequency of leukaemia in the children of fathers exposed to low-dose diagnostic X-rays (Graham et al., 1966; Shiono et al., 1980; Shu et al., 1988). Nonetheless, this relationship has again been ruled out in the children of survivors of atomic bomb radiation in Japan (Yoshimoto et al., 1990).
3.2.2 Animal Data

Just as for genomic instability, its transgenerational manifestation has been prolifically modelled in recent years in animal models using high-dose, acute exposure to radiation. An early study reported a decreased in vitro fertilisation rate for spermatozoa from the offspring of male mice exposed to 1 Gy of $\gamma$-rays (Burruel et al., 1997). However, the genetic interpretation of this data is problematic and so subsequent studies have tended to focus on transgenerational chromosome instabilities and mutation induction. Several groups have demonstrated significant increases in the frequencies of chromosome aberrations in multiple somatic tissues of the offspring of male rats exposed to 2 Gy or higher of acute X-rays or $\gamma$-rays (Vorobtsova, 2000; Kropacova et al., 2002; Slovinska et al., 2004; Sanova et al., 2005). Similar events were described by Fomenko et al. (2001) as an increase in micronucleus frequency in the bone marrow of offspring from exposed male mice.

Such instability in the somatic cells of these offspring would also suggest its presence in their germ line. This has been described since Luning and colleagues (1976) first identified an increase in dominant lethal mutations in the F$_1$ germ line measured by the frequency of embryonic death among the second-generation offspring. The increase was similar to that of the parental germ line of mice exposed to $^{239}$Pu salt injections. Since then, several groups have also reported an elevation in mutations that lead to F$_2$ congenital malformations (Lyon and Renshaw, 1988; Pils et al., 1999) and reduced embryonic cell viability (Wiley et al., 1997) in the germ line of the offspring of low-LET irradiated mice.

In recent years, Dubrova and his group extended their successful utility of ESTR loci as a powerful and efficient means of monitoring germ line mutation induction (see Chapter 2.2.1.2) to transgenerational studies. In this way, an early study investigated
genomic instability in the germ line of two generations of offspring following paternal irradiation with 0.4 Gy of high-LET fission neutrons or 1-2 Gy of low-LET X-rays (Barber et al., 2002). An elevation in mutation rate was found in the germ line of both generations of offspring derived from fathers exposed to either quality of radiation. The extent of instability in these animals was essentially the same, emphasizing the long-term nature of the effect and confirming its transmission. Importantly, this was also found to be consistent across three different strains of inbred mice: C57BL/6J, CBA/H and BALB/c, ensuring that the phenomenon is not restricted to a specific strain. With the development of single-molecule PCR, the group has since demonstrated elevated ESTR mutation frequencies in both the germ line and somatic tissues (bone marrow, spleen and brain) of the offspring of X-irradiated male mice (Barber et al., 2006; 2009).

The study of transgenerational changes in somatic tissues has also been described using protein-coding genes, confirming the genome wide nature of instability. Luke and colleagues (1997) used transgenic mice models, employing a λ shuttle vector to allow mutations in the lacI gene to be analysed. They reported an increased mutation frequency in bone marrow cells of the offspring of γ–irradiated mice. Several authors also found evidence for the phenomenon by analysing the frequency of somatic reversions of the pink-eyed unstable mutation (p\textsuperscript{un}) (Carls and Schiestl, 1999; Shiraishi et al., 2002). This is a highly unstable duplication mutation of the pink-eyed dilution gene, which causes a loss of eye colour. Reversions of the locus via homologous recombination restores colour to these cells, which can be detected as a pigmented cluster in the retinal epithelium. Using two reciprocal crosses of male and female mice homozygous for either p\textsuperscript{un} or a stable allele, Shiraishi and colleagues (2002) reported a significant increase in somatic reversions at both the irradiated, paternally derived p\textsuperscript{un}
alleles and the unexposed, maternally derived $p^{un}$ alleles in the offspring mice. The increase in mutation frequency did not differ between the $p^{un}$ alleles of each paternally-irradiated cross, clearly demonstrating a paternally-inherited instability that also affects DNA inherited from the unexposed mothers. Work by Shimada et al. (2001; 2004; 2005) has described a similar scenario at pigmentation loci in the Medaka fish and differences in ESTR locus size between different mouse strains has also allowed reciprocal crosses to be utilized in revealing a transgenerational instability in the offspring of irradiated male mice that spreads to the unexposed maternal chromosomes (Niwa and Kominami, 2001).

Compelling data supporting a genome wide destabilisation was also provided by Barber et al. (2006) after investigating mutation frequencies at the hypoanthine phosphoribosyltransferase ($hprt$) locus on the X chromosome. Over three times as many mutants were found in spleens of the male offspring of irradiated fathers compared to those of controls. Considering that this was again at unexposed maternal alleles, these results show consistency with the studies described above.

However, connecting these effects to a transgenerational predisposition to cancer has again been contentious. Since genomic instability, producing chromosomal aberrations and gene mutations, is characteristic of most cancers (Loeb et al., 2003), the transgenerational studies discussed above would surely predict an elevated incidence of cancer. With mouse models, Nomura (1982) reported a very high frequency of heritable tumours, particularly of the lungs, in the offspring of exposed ICR males, which behaved as if they were dominant mutations with a reported 40 % penetrance. The increase was dose dependant between 0.36-5.04 Gy of X-rays, with post-meiotic sperm exposure more sensitive than irradiated spermatogonia at the lower doses and a doubling dose of 1.5 Gy for lung and ovarian cancer had been reported.
Nevertheless, later studies failed to reproduce these results using the same experimental protocol (Cattanach et al., 1995; 1999).

More consistency has been found in studies that consider the added effect of treating the offspring with carcinogenic and promoting agents that may naturally exist in the diet and environment (Nomura, 2003). Treating the offspring of high-dose, acutely irradiated male mice with 12-O-tetradecanoylphorbol-13-acetate significantly increases their incidence of skin cancer relative to treated mice descended from unexposed parents (Vorobtsova et al., 1993). Lord et al. (1998a; b) reported a similar effect for the incidence of leukaemia and lymphoma upon treating the offspring with ethynitrosourea. Furthermore, Nomura (1983) showed the same response for lung cancer induction after postnatal treatment with urethene, as well as a 2.4-fold increase in penetrance compared to that reported earlier for irradiation only. However, it should be noted that postnatal urethene exposure of CBA/J mice following paternal irradiation did not reproduce these findings (Mohr et al., 1999).

Taken together, it appears that there are inherent strain differences, based on varying pre-existing genetic predispositions to various cancers, which may be enhanced following irradiation. In this way, it is likely that germ line exposure to ionising radiation is very weakly carcinogenic to mice of the next generation by itself. Instead, there may be a cumulative effect whereby the genomic instability inherited by the offspring greatly sensitises them to tumour promoting agents.
3.3 Potential Mechanisms

Collectively, these studies have provided conclusive evidence for the existence of radiation-induced genomic instability and its propagation to unexposed cells and offspring. However, further analysis of the impact of these non-targeted, delayed effects is currently limited, mainly because the mechanisms underlying them remain poorly understood. Neither is it clear whether or not all of the endpoints of this instability are governed by a single, common mechanism. Nevertheless, there are many clues as to the nature of these processes, which shall be discussed here.

3.3.1 The Initial Trigger

Virtually identical findings were observed in the offspring of mice irradiated with 1 Sv or more of either fission neutrons or X-rays (Barber et al., 2002). This is in line with other non-targeted, delayed effects of ionising radiation, as described above, which have been detected following exposure to either radiation quality, and suggests that a wide range of primary DNA lesions is capable of triggering the same response. Recent work has broadened this to the damage produced by several chemical mutagens. Transgenerational instability at ESTR loci was demonstrated after paternal treatment with the alkylating agent, ethylnitrosourea (Dubrova et al., 2008). Furthermore, clinically relevant doses of the anticancer drugs cyclophosphomide, mitomycin C and procarbazine, which each produce a variety of DNA damage, were also found to be capable of the effect (Glen and Dubrova, 2012). Together, this would suggest that the stimulus responsible for triggering these non-targeted, delayed effects is related to generalized DNA damage and the ability of the target cells to detect and respond to it.
There are around 1,000 genes involved in maintaining mammalian genome stability (Wang et al., 2004). With a maximum spontaneous mutation rate of $10^{-6}$ per locus (Nachman and Crowell, 2000) elevated ~3-fold by exposure to 1 Gy of X-rays (Dubrova et al., 1998), a detrimental, dominant mutation induced at any of these genes would at most affect 0.3% of the progeny of irradiated cells/parents. However, the large-scale changes in these progeny, documented by the present group and others, are manifested by an extremely high frequency of ‘sensitive’ individuals (up to 100%). Therefore, the loss of genomic integrity cannot be the result of conventional, Mendelian inheritance of targeted mutation to a DNA damage response gene. Combined with the consistent observation that genomic instability persists over a long period of time after the initial exposure, this has given strong conviction to the argument that the means of transmission is epigenetic (Morgan, 2003a; b; Dubrova, 2006).

### 3.3.2 Memorising the Insult

Considering the finding that transgenerational instability increases the mutation rate at loci on both irradiated paternal chromosomes and those inherited from the unexposed mothers (Shiraishi et al., 2002; Barber et al., 2002; 2006; Niwa and Kominami, 2001), it appears that the sperm that delivers the former contains a property that goes on to destabilise the genome of the whole zygote. In this way, the various non-targeted, delayed effects of ionising radiation may have a common underlying process of propagation.

As discussed, bystander-type effects are facilitated by intercellular signaling via secretion of ROS and other factors (Yang et al., 2005; Lyng et al., 2002) and cell-to-cell gap junctions (Azzam et al., 1998; Mitchell et al., 2004). The exact nature of these
signals is not known, but the up-regulation of stress-induced cytokines is often shown to be implicated. Dickey et al. (2009) reported that transforming growth factors (TGF-α and TGF-β) and nitric oxide (NO) in media from irradiated cells elevated the frequency of double-strand breaks when used to treat unexposed cells to that of bystander cells. Others have demonstrated that FasL, Tnfα, NO and superoxide were generated by bone marrow cells irradiated in vivo and induced DNA damage and apoptosis in nonirradiated cells (Burr et al., 2010). Similarly, Natarajan et al. (2007) showed that treating cells with TNFα was enough to initiate chromosomal instability in progeny cells after several generations. The diffusion of these cytokine factors triggers membrane signalling and an influx of calcium in bystander cells (Lyng et al., 2011), which in turn drives the release of more ROS from mitochondria and membrane-bound NADPH oxidase (Yang et al., 2007).

ROS may also play a role in producing this genomic instability in the clonal descendants of irradiated and bystander cells. Several studies have shown that persistent dysregulation of oxygen metabolism is often a feature of cells experiencing radiation-induced instability. Rugo and Schiestl (2004) demonstrated that X-irradiation of human lymphoblasts produces an increase in ROS and changes in signal transduction proteins that can be observed after 55 population doublings. A dose-dependant increase in persistent oxy-radical activity was also detected in irradiated bone marrow cells, 7 days after exposure (Clutton et al., 1996). These findings are likely due to mitochondrial dysfunction. Oxidative stress has been reported to induce chromosome aberrations in the clonally expanded population of GM10115 cells treated with H$_2$O$_2$, accompanied by an increase in the number of mitochondria (Limoli and Giedzinski, 2003). Miller et al. (2008) observed similar events in the unstable clones of irradiated cells with a dysregulation of respiration and decreased levels of
mitochondrial antioxidant enzymes. 10 Gy X-irradiation of these cells has also been shown to increase oxygen consumption and activity of mitochondrial electron transport chain (ETC) complex II in descendant cells (Dayal et al., 2009). This was found to increase the steady-state levels of H$_2$O$_2$, resulting in elevated gene amplification and mutation frequencies. Together with the observation that hypoxic conditions (2% oxygen) can inhibit radiation-induced genomic instability (Roy et al., 2000), these data strongly suggest that ROS plays a role in propagating non-targeted, delayed effects of ionising radiation.

With A$_s$ spermatogonia developing in the testes, connected to each other by intercellular bridges (De Rooij, 1998), it is likely that stress signals may propagate between sperm and that these processes have a role in enhancing the observed mutagenicity of ionising radiation on the directly exposed germ line (see Chapter 2.2). However, a high content of free radicals is unlikely to be responsible for transgenerational instability because the cytoplasmic component of mature sperm cells is negligible relative to that of the mammalian ovum. Such a small cell couldn’t possibly contain enough free radicals or other dissolved factors to effect a change in their concentration in the fertilised ovum. Furthermore, such short-lived particles would likely be incapable of prolonging the phenomenon over many cell divisions.

It is more likely that this effect is fixed across multiple loci in the germ cells of the exposed individual. A plausible candidate for facilitating this phenomenon may be DNA methylation of cytosine residues. Methylation occurs predominantly at CpG dinucleotides, which are methylated 60-90% of the time and are mainly distributed in repeat elements and promoter regions of many genes in clusters called CpG islands (Weber and Schubeler, 2007). Highly methylated regions are usually associated with inactive chromatin and repressed gene expression mediated by methyl-CpG-binding
proteins that attract transcriptional co-repressors (Fujita et al., 2003; Kondo et al., 2005). In this way DNA methylation is implicated in a variety of functions including control of tissue specific gene expression (Nagase and Ghosh, 2008), silencing repeat elements (Kato et al., 2007; Huang et al., 2004) and maintaining genomic structural stability (Chen et al., 1998a; Klose and Bird, 2006). Furthermore, it has been established that DNA methylation marks at many loci are resistant to epigenetic reprogramming events during spermatogenesis (Roemer et al., 1997; Constancia et al., 1998) and in the developing embryo (Hajkova et al., 2002; Lane et al., 2003) across many cell divisions (Holliday, 1987).

Therefore, DNA methylation may be ideal to mediate the long-term effects of exposure to ionising radiation. To investigate this, numerous studies into genomic instability have measured changes in methylation levels and those of the proteins that modulate methylation patterns and silencing. Global hypomethylation has been reported following low-LET irradiation of cell lines (Kalinich et al., 1989) and in vivo at 8 hours post-exposure in the livers of exposed mice (Tawa et al., 1998). However, Tawa and colleagues detected no such changes in the spleens and brains of these mice. Later work with C57BL mice showed a dose-dependent loss of global methylation in the livers of exposed females and spleens of exposed males and females following X-irradiation with 0.5-5 Gy (Raiche et al., 2004). This was accompanied by decreased levels of the DNA methyltransferases that establish methylation patterns: the maintenance enzyme DNMT1 and the de novo methyltransferases DNMT3a and DNMT3b. Levels of the methyl-binding-proteins MeCP2 and MBD2 were also diminished and there was an increase in detected double-strand breaks. Others have reported global hypomethylation in the bone marrow of mice with radiation-induced leukaemia (Giotopoulos et al., 2006). Moreover, a recent study demonstrated that X-
irradiation of male and female mice results in hypermethylation of the tumour-suppressor gene \( p16 \) that is both tissue- and sex-specific (Kovalchuk et al., 2004). Taken together, the results of these studies are consistent in proposing that sex- and tissue-specific factors determine the ability of ionising radiation to cause global hypomethylation paralleled by local hypermethylation at some gene promoters. This may be associated with the presence of DNA lesions and altered activity of the proteins that modulate DNA methylation patterns and chromatin inactivation. In this way, radiation-induced changes in methylation patterns closely mimic those that commonly manifest in cancer (Feinberg and Vogelstein, 1983b; Esteller et al., 2001; Rhee et al., 2002). Global hypomethylation may facilitate carcinogenesis by enabling retro-transposon activity (Hedges and Deininger, 2007), expression of oncogenes (Wolff et al., 2010) and destabilising chromosome structure (Gaudet et al., 2003). Conversely, promoter hypermethylation may silence tumour-suppressor genes (Rhee et al., 2002) and mismatch repair genes (Herman et al., 1998; Wheeler et al., 1999). Furthermore, point mutations may be produced by spontaneous deamination of methylated cytosines (Ketterling et al., 1994).

Kaup et al. (2006) were the first to demonstrate that irradiation results in the induction of heritable methylation changes in mammalian cells up to 20 population doublings post-irradiation. This was predominantly detected as hypermethylation at repeat elements and was associated with genomic instability in the form of apoptosis and chromosomal aberrations. Similar effects have been described in 3D human tissue models of bystander effects (Sedelnikova et al., 2003). In this context, investigators reported an increase in double-strand breaks, micronuclei formation and apoptosis in cells up to 2.5 mm away from irradiated targets, associated with global hypomethylation. Koturbash and colleagues (2006b) reported similar disturbances in
vivo in bystander cells at least 7 mm from the irradiated plane. They also later found that localised, 20 Gy cranial X-irradiation results in global hypomethylation in bystander spleen cells 160 mm away, 7 months after exposure (Koturbash et al., 2007). This persistent epigenetic change also accompanied down regulation of DNMT3a and MeCP2 and reactivation of the LINE1 retrotransposon. Given that free radical induced damage can dramatically change methylation patterns (Weitzman et al., 1994; Cerda and Weitzman, 1997; Bhusari et al., 2010), it may be possible that the persistent generation of ROS following irradiation (Wright, 2007) mediates bystander effects and genomic instability by inducing DNA methylation changes. Indeed recent in vitro studies have shown that radiation-induced genomic instability is completely absent in hypoxic conditions (Roy et al., 2000) or following targeted disruption of methyltransferase enzymes (Rugo et al., 2010).

This epigenetically regulated bystander-type effect in distant tissue in vivo has also been demonstrated in the shielded testes after high-dose, acute irradiation to the skull (Tamminga et al., 2008b). Remarkably, this was accompanied with the detection of global hypomethylation in the bone marrow, thymus and spleen, but not liver, of the unexposed progeny. This would imply the contribution of bystander/clastogenic factors to the transgenerational response, associated with DNA damage and epigenetic dysregulation. These findings are also supported by Koturbash and colleagues (2006a), who reported global hypomethylation, reduced levels of methyltransferases and MeCP2 and an increase in DNA strand breaks in thymuses of the progeny of X-irradiated male mice. Therefore, it is very likely that DNA methylation has a major role in preserving the memory of the original insult of ionising radiation and continuously transmitting a signal of instability to unexposed descendant cells and offspring.
However, DNA methylation is not the only means of altering chromatin structure and subsequent gene expression. A variety of covalent modifications can be made to the histones proteins around which DNA is wrapped, including methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, citullination, ribosylation. In combination these alterations form the profoundly complex ‘histone code’ (Jenuwein and Allis, 2001), which works to regulate chromatin structure in processes such as gene regulation, DNA repair, replication and meiosis (Weidman et al., 2007; Song et al., 2011). Even so, as of yet, a functional understanding is only known for a few of these modifications.

Acetylation of histone tails clears their positive charge, thus decreasing their affinity for negatively charged DNA and activating the surrounding chromatin (Jenuwein and Allis, 2001). Conversely, histone deacetylation represses transcription. Interestingly, UV-irradiation has been shown to produce short-term acetylation of H3K9 at the promoters of genes such as IL-8 (Pollack et al., 2009), which has been implicated in the transmission of bystander effects (Laiakis et al., 2008).

Histone methylation is more complicated. Methylation of H3K9 represses gene expression by compacting the chromatin structure (Cheung and Lau, 2005). A recent study by Falk and colleagues (2008) reported that the chromatin structure around double-strand breaks was relaxed immediately following γ-irradiation, but quickly became condensed with increased methylation at H3K9 by 40 minutes. On the other hand, methylation of H3K4/27 relaxes chromatin packaging and activates transcription. Furthermore, a common hallmark of human cancer is a loss of tri-methylation at H4K20, associated with relaxed chromatin and transcriptional activation (Fraga et al., 2005; Tryndyak et al., 2006). Indeed X-ray exposure has been shown to reduce tri-methylation of H4K20 in the murine thymus with a similar
relaxation of chromatin structure (Pogribny et al., 2005). Furthermore, DNA hypomethylation is often seen to accompany the observations of these studies. Cytosine methylation itself also regulates chromatin structure through interactions with histone modifications. Methyl-binding domains such as MeCP2 bind to hypermethylated regions, which in turn recruit histone methyltransferases and deacetylases to compact the surrounding chromatin (Gronbaek et al., 2007).

Another histone modification that has been well studied is the phosphorylation of serine 139 on histone H2AX to form γH2AX. This alteration does not directly influence gene expression, but instead accumulates as foci at double-strand DNA breaks as part of the earliest stages of DNA repair (Rogakou et al., 1998; Sedelnikova et al., 2003) and is important for maintaining genomic stability (Celeste et al., 2002). γH2AX also facilitates repair by recruiting histone acetyltransferases to relax the chromatin surrounding the break (Celeste et al., 2003). γH2AX phosphorylation is frequently utilised as a measure of double-strand breaks following exposure to ionising radiation (Barber et al., 2006; Bonner et al., 2008; Redon et al., 2009).

However, the effects of radiation on core histone modifications, as a propagating mechanism of genomic instability is virtually unknown. Furthermore, most histones are generally replaced by arginine-rich protamines in spermatozoa (Meistrich, 1989; Kimmins and Sassone-Corsi, 2005), making it unclear as to how such modifications would be transmitted to the zygote. Nonetheless, a recent study by Brykczyńska et al. (2010) demonstrated that histones are present in mature sperm at regions covering developmental regulator genes, meaning they may have a role in epigenetic inheritance.

A third potential epigenetic means of transmitting the ‘memory’ of the initial insult to progeny cells/individuals may be through post-transcriptional regulators of
gene expression. Important agents of this process are small non-coding RNAs, particularly microRNAs (miRNAs). These are well conserved small RNAs (18-23 nt), produced following Dicer processing (Lee et al., 2004; Macrae et al., 2006) of primary-miRNA transcripts (Cai et al., 2004; Lee et al., 2004). When one strand of such a miRNA is loaded into an RNA-induced silencing complex (RISC) it binds to the 3′-untranslated region of messenger RNAs, with full or partial complementation, marking them for degradation or reduced translation respectively (Schwarz et al., 2003; Khvorova et al., 2003). In this way, a single miRNA may affect, to some degree, the translation of thousands of transcripts (Selback et al., 2008). It has also been shown that small RNAs may be involved in mediating changes to chromatin structure (Bernstein and Allis, 2005). Subsequently, this RNA interference is involved in a wide range of processes, including differentiation, proliferation, development and apoptosis (Bartel, 2004; Kim and Nam, 2006). Disruption of miRNA expression patterns is a common feature of cancer, where miRNAs with altered levels can be seen as oncogenic or tumour-suppressors. Furthermore, considering reports that sperm can contain small RNA molecules that trigger heritable changes in embryonic and adult development (Rassoulzadegan et al., 2006; 2007; Grandjean et al., 2009), despite general transcriptional repression during the later stages of spermatogenesis (Kimmins and Sassone-Corsi, 2005), these factors may potentially contribute to transgenerational mutagenesis.

However, an understanding of how ionising radiation impacts small RNA and their involvement in the subsequent DNA damage responses is not known. Chaudhry (2009) demonstrated that γ-irradiation of Jurkat and TK6 cells caused a dysregulation of many miRNAs, including increased expression of those that silence the cMyc oncogene. Also affected were miRNA in the let-7 family, which target the Ras
oncogene. Let-7 miRNAs were also reported to be among those whose expression was altered following irradiation of human fibroblasts (Simone et al., 2009). Furthermore, these radiation-induced changes in miRNA expression profiles were shown to be highly similar to those following exposure to oxidative stress and actually accompanied an increase in ROS production. A recent study also found significantly altered expression of let-7 family members in 3D human tissue models, which persisted up to 7 days post-irradiation (Dickey et al., 2011). These studies suggest that ionising radiation may induce microRNAome changes that provide a protective response for the cell.

This is supported by data from several in vivo studies by Kovalchuk and colleagues. It was shown that X-irradiation resulted in sex-specific changes to miRNA expression profiles in spleen and thymus tissues (Ilnytskyy et al., 2008). Notable among those upregulated was the expression of miRNA-34a, a tumour-suppressor that targets oncogenes such as NOTCH1, MYC, E2F3 and cyclin D1. Decreased levels of miR-7 were also detected, which would allow expression of the DNA methylation regulator LSH. This may indicate protection against radiation-induced hypomethylation and genome instability. Similarly, a concurrent study demonstrated that the germ line could be affected by such processes (Tamminga et al., 2008a). Expression of a large number of miRNAs was altered following exposure to radiation, including a damage-induced increase in miR-709 levels. This microRNA targets BORIS, a gene exclusively expressed in the testes where it directs reprogramming during spermatogenesis. Therefore, the radiation-induced silencing of BORIS via RNA interference may also be implicated in preventing DNA hypomethylation. More recently the group investigated the effects of exposure to 1 Gy of X-rays on microRNA expression profiles in various regions of the brain (Koturbash et al., 2011).
The resultant dysregulation was found to be tissue-, time- and sex-specific. Particularly striking was the response found in the frontal cortex where reduced expression of miR-29 in exposed females meant an increase in levels of DNMT3a that prevented the global DNA hypomethylation found in the irradiated males.

However, an earlier study using *Drosophila*, demonstrated radiation-induced upregulation of the miRNA bantam in larval cells, which suppressed Hid-mediated apoptosis (Jaklevic *et al.*, 2008). The increased survival of these cells may indicate that a greater level of instability and detrimental mutations is transmitted to descendant cells. Similarly, several *in vitro* studies have reported radiation-induced miRNA dysregulation that can have more destructive consequences. Cha and colleagues (2009b) showed that 1 or 10 Gy γ-irradiation altered the expression of 73 and 33 miRNA, respectively, more than 2-fold in the IM9 human B lymphoblastic cell line as detected by microarray analysis and confirmed by quantitative real-time PCR. The predicted targets of these miRNAs were predominantly genes involved in the regulation of apoptosis, the cell cycle and DNA repair. Subsequently, they confirmed these findings in a dose-dependant manner for a range of γ-irradiation between 1 and 40 Gy (Cha *et al.*, 2009a). Another recent *in vitro* report described how miR-24 targets γH2AX and is upregulated in terminally differentiated blood cells (Lal *et al.*, 2009). Given the key role of H2AX in the repair of double-strand breaks, these cells were found to be DNA repair deficient and thus hypersensitive to γ-irradiation.

Interestingly, the Kovalchuk laboratory also revealed that changes in miRNA expression profiles might be implicated in genomic instability in bystander cells and in the offspring of irradiated parents. In the rodent model where they detailed hypomethylation of the LINE1 retrotransposon and elevated DNA damage in bystander spleen tissue up to 7 months after cranial irradiation, these authors also
observed an upregulation of miR-194 (Koturbash et al., 2007). This microRNA targets DNMT3a and so may potentially mediate the other effects. Furthermore, Chaudhry and Omaruddin (2012) recently proposed that miRNA modulation is involved in the radiation-induced effects in directly exposed and bystander cells, but is differentially regulated between the two. For example, the let-7 family of miRNAs were upregulated in irradiated cells, but most of them remained unexpressed in bystander cells. Even so, Kovalchuk and colleagues (2010) have utilised the 3D human tissue model to show that DNA hypomethylation and cell cycle dysregulation in bystander cells accompanies changes in miRNA expression profiles that may cause them. Increased levels of the miR-17 family that target the tumour-suppressors E2F1 and RB1 were detected, suggesting aberrant proliferation. Also upregulated were the miR-29 group that silence expression of MCL1 and DNMT3a. This would affect apoptosis and produce the observed DNA hypomethylation. The miR-29 family were also found to be overly expressed in the germ line of male mice exposed to 2.5 Gy of $\gamma$-rays (Filkowski et al., 2010). This led to hypomethylation of LINE1 and SINE B2 retrotransposons. Remarkably hypomethylation of these repetitive elements was also detected in the thymus tissue of the offspring of these mice. The authors associated this with a transgenerational increase in expression of miR-468 and decreased levels of its target LSH. Together these studies strongly suggest that changes in the microRNAome may also be implicated in the non-targeted, delayed, deleterious effects of ionising radiation.

Even so, in order for these changes in gene expression to produce transgenerational instability they would have to be lasting. Here an important issue arises: when would the changes that facilitate them occur and for how long after exposure would they endure? In the animal systems that model transgenerational
instability, the time between paternal irradiation and when the offspring are conceived accurately corresponds to the stage of spermatogenesis the germ cells that sire them were exposed (see Table 2.2). Several groups have shown that mature sperm cells, irradiated less than a week before mating, can initiate genomic instability in the derived offspring (Vorobtsova et al., 1993; Vorobtsova, 2000; Niwa and Kominami, 2001; Shiraishi et al., 2002; Hatch et al., 2007). Post-meiotic spermatids, irradiated 3 weeks before conception, were also shown to trigger the effect (Barber et al., 2002). The ability to detect and repair DNA damage is progressively lost during the final stages of spermatogenesis, with all major repair pathways inactivated by the time sperm are mature (Olsen et al., 2005; Ahmed et al., 2007). This has a significant impact on the amount of DNA damage that is passed to the zygote (Marchetti & Wyrobek, 2008). For the first round of mitosis the maternal and paternal genomes reside in separate pronuclei (McGaughey & Chang, 1969). However, the fertilized egg has been shown to detect and repair lesions in the male genome (Derijck et al., 2006) and sperm with radiation-induced damage activates cell-cycle checkpoints, suppressing DNA synthesis in both pronuclei (Shimura et al., 2002). Therefore, it is likely that mutation induction following low-fidelity responses to damage in sperm irradiated late in maturation, and any potential epigenetic changes that accompany this, takes place after fertilisation.

Interestingly, transgenerational instability has also been reported following exposure of in utero primordial stem cells (Barber et al., 2009) and spermatogonia irradiated 6-8 weeks before conception (Dubrova et al., 2000b; Barber et al., 2002; 2006). These cells are fully capable of detecting and repairing any damage induced by ionising radiation (Olsen et al., 2005). In this situation, it appears that the insult of radiation may result in changes to the epigenome that persist for a considerable period
of time in the developing sperm, before going on to propagate instability in the zygote. However, it should be noted that, although Dubrova and colleagues found transgenerational instability was detectable in both the male and female offspring of irradiated fathers (Barber et al., 2002), neither in utero nor adult maternal exposure was capable of triggering a response in their offspring, despite an increase in direct germ line mutation induction (Barber et al., 2009; Abouzeid Ali et al., 2012). This would suggest that irradiated mothers do not pass the epigenetic signal of instability to their children. These authors speculated that the active demethylation during reprogramming of the paternal genome (Morgan et al., 2005) might somehow preserve more radiation-induced epigenetic modifications than the passive process that occurs in the maternal pronucleus.

### 3.3.3 Transgenerational DNA Damage

Investigating the continuous mutagenic effect of this unknown signal Balentova and colleagues (2008) reported an enhancement of the proliferative activity of F₁ embryos as measured by an increase in DNA and RNA concentration and a reduction in histone content following paternal, 3 Gy acute γ-irradiation. This activation of cellular proliferation and transcription with a loss of nucleosome integrity has been shown to indicate persistent DNA damage that escapes repair (Fiszer-Szafarz and Szafarz, 1984; Klimová and Mišírová, 2002). Alteration in proliferative activity was also described in the rostral migratory stream in the brain of neonatal and young progeny of exposed males (Balentova et al., 2007). Being one of the few sites of neurogenesis in the adult brain, the rostral migratory stream is particularly sensitive to radiation-induced mutagenesis.
This idea that genomic instability is characterised by persistent DNA damage in the offspring of irradiated cells/individuals is also supported by the work of Barber et al. (2006). The authors used single cell gel electrophoresis (the comet assay) and histochemical analysis of the phosphorylated histone $\gamma$H2AX to measure the presence of single-strand breaks in the bone marrow and double-strand DNA breaks in the spleen, respectively, of the offspring of X-irradiated mice. A more than 2-fold increase relative to levels in control mice was demonstrated for both types of endogenous damage. This was confirmed in two different strains and would explain the detection of genomic instability by both chromosome aberrations (double-strand breaks) and gene mutations (single-strand breaks). Such a high level of deleterious breaks would prevent replication and be highly detrimental in tissues with such high mitotic indices. Therefore, these studies would suggest that the damage produced by genomic instability is an ongoing process.

Recent work by Adiga et al. (2009) directly monitored this correlation of genomic instability with DNA damage throughout the development of offspring by mice exposed to 2.5-10 Gy of testicular $\gamma$-irradiation. An increase in micronuclei frequency was detectable in the F$_1$ embryos, but only from 3.5 days post-fertilisation. This stage was previously shown to correspond to the delayed formation of $\gamma$H2AX foci (Adiga et al., 2007) and the authors deduced that micronuclei must have been induced by newly generated double-strand breaks.

One proposed explanation for the high levels of endogenous DNA damage in these individuals would be that they experience oxidative stress (Morgan, 2003a; b). The free radicals that abound in such a state would produce a variety of DNA lesions, including characteristic nucleotide modifications. However, Barber et al. (2006) have provided strong evidence to suggest that these processes are not implicated. Using the
FPG modification of the comet assay, the number of oxidatively damaged bases in the bone marrow cells of offspring derived from irradiated mice was measured and found to be comparable to that of controls.

It may also be possible that the DNA repair ability of the offspring of irradiated males is compromised, albeit epigenetically. However, the same study showed this to also be unlikely (Barber et al., 2006). To this end, bone marrow from the offspring of exposed males was irradiated *ex vivo* and the presence of single-strand breaks and alkali labile sites was then monitored over 1 hour. The efficiency of repair was found to closely match that of controls for both the CBA/Ca and BALB/c strains of mice.

This is also in accordance with the work of Little and colleagues, who showed that the spectrum of delayed mutations at the *hprt* locus in the clonal population derived from irradiated cells significantly differed from that of directly induced mutations and instead closely matched those of spontaneous mutations (Little et al., 1997; Little, 1998). A compromised ability to repair damage would surely affect the structure of mutations that arise from genomic instability. Therefore, it may be possible that delayed mutations arise in a similar manner to that of spontaneous ones.

One such process that may be implicated in both spontaneous and delayed mutations is through forms of replication delay. It has been shown that radiation-induced chromosome instability *in vitro* may be attributed to long-term delays in chromosome replication (Breger et al., 2004). Several studies by Dubrova and colleagues have also reported an association between murine non-targeted ESTR mutation and replication slippage (Yauk et al., 2002; Barber et al., 2004; Dubrova, 2005; Hardwick et al., 2009). Similarly, delayed replication due to disrupted cell cycle control has been described in early human cancer cells associated with allelic imbalance at loci prone to double-strand break formation (Bartkova et al., 2005;
Gorgoulis et al., 2005). Therefore, replicative stress may be a likely candidate for producing novel damage and mutations in the offspring of irradiated parents.
3.4 Experimental Design

The studies detailed above describe a range of non-targeted, delayed effects of exposure to ionising radiation and how they are propagated to the unexposed offspring of irradiated and bystander cells in an epigenetic fashion, continuously inducing damage and mutations through a process likely to revolve around replication stress. DNA damage is the apparent trigger of this response, but how it does so is not understood. An important progression from these studies to elaborate on the nature of the transgenerational effect and to determine the actual mechanisms of the underlying instability is to investigate the initial cellular events triggering instability in the non-exposed progeny of irradiated cells/parents. As has been discussed, the majority of experimental data on radiation-induced genomic instability has been obtained by studying the progeny of cells/parents acutely exposed to quite high doses of ionising radiation (> 1 Sv). Therefore, it is not known what involvement these non-targeted, delayed effects have in the biological risk posed by lower doses. Furthermore, the significance of the accumulated model data to human risk assessment is highly questionable since chronic low-dose irradiation represents the main source of human occupational and accidental exposure (see Chapter 1.2). Consequently, it remains to be seen whether low-dose acute or low-dose-rate parental irradiation, relevant to human exposure, can induce genomic instability in the next generation. The present study seeks to address these important issues.

Prior to tissue isolation for direct mutation-induction analysis (see Chapter 2.3), irradiated male, inbred BALB/c mice were mated to control BALB/c females 12 weeks after exposure, ensuring that the litters were derived from irradiated A_{s} spermatogonia (Searle, 1974). Although there is no consensus, exposed spermatogonia (> 6 weeks before reaching the ejaculate) may provide the most consistent means of
demonstrating the transgenerational effects of ionising radiation (Dubrova et al., 2000b; Barber et al., 2002; 2006; 2009). Furthermore, stem cells at the older range of spermatogonia (> 10 weeks before reaching the ejaculate) are known to have a lower sensitivity to cell killing by ionising radiation (Searle, 1974). As fewer cells are removed from the germ line, a more accurate understanding of the transmission of genomic instability through these cells may be obtained.

The mice were exposed to acute irradiation at 6 weeks old with 10, 25, 50 or 100 cGy of low-LET $\gamma$-rays (see Chapter 4.2.1) to consider the range of low to intermediate doses that characterise incidents of human exposure to ionizing radiation, as discussed. Another group was exposed to 100 cGy at a very low-dose rate to reflect more common, chronic exposure (UNSCEAR, 2000).

At 8 weeks old the male progeny of these mice were culled for their caudal epididymides and brain tissue to analyse transgenerational processes occurring in germ line and somatic cells, respectively. DNA was then extracted from each tissue and purified, before the frequencies of $Ms6$-hm ESTR mutation induction in them were measured using single-molecule PCR, to directly determine the genomic stability of the progeny (see Chapter 4). Brain tissue was chosen for analysis because of its relatively low mitotic index in adult individuals and the observation that direct exposure produces no significant ESTR mutation induction in this tissue (Yauk et al., 2002). In this way, any observed instability in the brain DNA of offspring from exposed mice would emphasize the transmission of genomic instability and its mutagenic action during embryogenesis when a high number of neural stem cells are still present.
3.5 The Dose Response of Transgenerational Instability

Upon analyzing DNA samples extracted from sperm and brain of the offspring of irradiated males, ESTR mutation frequencies were calculated and compared with those of controls in Table 3.1. Investigating the transgenerational effects of paternal irradiation revealed that the frequency of ESTR mutation induction was significantly elevated, for both tissues, in the offspring of the 50 and 100 cGy acutely $\gamma$-irradiated groups. Furthermore, the extent of transgenerational instability in the offspring of either irradiated group did not significantly differ from each other ($P=0.45$ and $P=0.52$ for sperm and brain, respectively). In contrast, exposure to lower, acute doses (10 and 25 cGy) failed to destabilize the $F_1$ genomes. Notably, as did low dose-rate, chronic exposure to 100 cGy ($P=0.7359$ and $P=0.9417$ for sperm and brain, respectively). Figure 3.1 illustrates these findings, where a clear threshold of acute dose resulting in transgenerational instability can be found lying between 25 and 50 cGy, alongside the apparent inability of low dose rate exposure to induce instability.
<table>
<thead>
<tr>
<th>Group, tissue</th>
<th>No. mutations</th>
<th>Frequency ± s.e.</th>
<th>Ratio†</th>
<th>t‡</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- sperm</td>
<td>20 (516 ± 27)</td>
<td>0.0388 ± 0.0089</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>- brain</td>
<td>15 (492 ± 27)</td>
<td>0.0305 ± 0.0080</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F₁</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 cGy, acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- sperm</td>
<td>16 (408 ± 25)</td>
<td>0.0392 ± 0.0101</td>
<td>1.01</td>
<td>0.03</td>
<td>0.9760</td>
</tr>
<tr>
<td>- brain</td>
<td>12 (463 ± 27)</td>
<td>0.0259 ± 0.0076</td>
<td>0.85</td>
<td>0.41</td>
<td>0.6803</td>
</tr>
<tr>
<td>25 cGy, acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- sperm</td>
<td>14 (416 ± 25)</td>
<td>0.0337 ± 0.0092</td>
<td>0.87</td>
<td>0.40</td>
<td>0.6905</td>
</tr>
<tr>
<td>- brain</td>
<td>10 (369 ± 23)</td>
<td>0.0271 ± 0.0087</td>
<td>0.89</td>
<td>0.28</td>
<td>0.7756</td>
</tr>
<tr>
<td>50 cGy, acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- sperm</td>
<td>29 (377 ± 23)</td>
<td>0.0769 ± 0.0151</td>
<td>1.98</td>
<td>2.18</td>
<td>0.0294</td>
</tr>
<tr>
<td>- brain</td>
<td>25 (375 ± 23)</td>
<td>0.0667 ± 0.0139</td>
<td>2.19</td>
<td>2.25</td>
<td>0.0248</td>
</tr>
<tr>
<td>100 cGy, acute</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- sperm</td>
<td>26 (271 ± 18)</td>
<td>0.0959 ± 0.0199</td>
<td>2.48</td>
<td>2.62</td>
<td>0.0088</td>
</tr>
<tr>
<td>- brain</td>
<td>24 (296 ± 20)</td>
<td>0.0811 ± 0.0174</td>
<td>2.66</td>
<td>2.64</td>
<td>0.0085</td>
</tr>
<tr>
<td>100 cGy, chronic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- sperm</td>
<td>16 (367 ± 23)</td>
<td>0.0436 ± 0.0112</td>
<td>1.12</td>
<td>0.34</td>
<td>0.7359</td>
</tr>
<tr>
<td>- brain</td>
<td>17 (543 ± 30)</td>
<td>0.0313 ± 0.0078</td>
<td>1.03</td>
<td>0.07</td>
<td>0.9417</td>
</tr>
</tbody>
</table>

Table 3.1 - ESTR mutation frequencies in the offspring of irradiated males

*Number of amplifiable DNA molecules (± s.e.) is given in brackets.
†Ratio to control.
‡Student’s t-test and probability for difference from controls
Figure 3.1 – The dose and dose-rate effects on transgenerational instability in the offspring of irradiated male mice as measured by the SM-PCR technique. Mutation frequencies and standard errors are plotted as ratios to the frequency of ESTR mutation in the offspring of control male mice.
3.6 Discussion

3.6.1 Main Findings

This aspect of the study was designed to investigate the manifestation of transgenerational instability in the offspring of those exposed male mice for which a clear, linear dose response was observed for germ line mutation induction. This involved varying acute dosage in a range relevant to human exposure, 10-100 cGy, and also testing the effect of low dose-rate irradiation on the phenomenon.

As of yet, transgenerational instability has only been consistently demonstrated in the offspring of male mice exposed to at least 1 Sv of acute ionizing radiation, equivalent to 1 Gy (100 cGy) of X-rays or \( \gamma \)-rays (Dubrova et al., 2000b; Shiraishi et al., 2002; Hatch et al., 2007; Barber et al., 2002; 2006; 2009). Here, 50 cGy of acute \( \gamma \)-rays have also been shown to be capable of inducing the effect, with the magnitude of increased transgenerational mutation induction roughly similar between the two doses. However, acute exposure to 10-25 cGy produced no observable instability in the F\(_1\) offspring, despite its shown mutagenic capacity. This was also seen to be true for low dose-rate irradiation. Even though such a chronic dose clearly causes mutation induction in directly exposed mice there is no elevation in mutation frequency in their offspring. Taken together, this strongly implies the existence of a threshold of radiation dose, below which these effects do not occur. As opposed to the proportional response of mutation induction to radiation dose for direct exposure, the occurrence of transgenerational instability depends on which side of the threshold the dose lies. The present data reports this threshold of paternal exposure to acute \( \gamma \)-irradiation to be between 25-50 cGy and it should be noted that Zyuzikov et al. (2011) and Rithidech et al. (2012) also recently reported the inability of low-LET irradiation at doses below
100 cGy to manifest long-term chromosomal instability in the bone marrow of acutely exposed mice.

This all provides important clues regarding the elusive underlying mechanisms directing genomic instability. The implication of such a threshold determining the long-term stability of descendant cells/organisms and the need for the dose to be acute raises the possibility that a stress-like response triggers the effect in the germ line of exposed parents. Previous studies have shown that the instability signal is dependant on the amount of generalized DNA damage and not a particular mutagen producing a specific subset of DNA lesions. Indeed, high-dose paternal exposure to ethynitrosourea, an alkylating agent, and several anticancer drugs, that produce an array of damage, have also been shown to affect the integrity of F\textsubscript{1} genomic stability as measured at ESTR loci (Dubrova et al., 2008; Glen and Dubrova, 2012).

For ionizing radiation, a generally linear increase in the level of total induced-DNA damage has been established across a vast range of doses (Frankenberg-Schwager, 1990). Therefore, it would appear that the threshold reported here is characterized by a saturation of the exposed cell’s DNA repair processes by a certain amount of damage in a short time interval. This would certainly be endorsed by the current data, which shows that F\textsubscript{1} genomes remain stable after very-low-dose-rate paternal exposure to 100 cGy, but reports transgenerational instability in the offspring of male mice exposed to only half that accumulated dose, 50 cGy, when it is delivered acutely. The duration of paternal exposure differs dramatically: 2 weeks and 2 minutes, respectively. Therefore, it would appear that the amount of DNA damage induced over a given period of time is the most deterministic factor for whether the phenomenon occurs or not. This would reflect an inability of the exposed cell to cope with a certain amount of DNA damage inflicted during an acute exposure, saturating
the cell’s capacity for effective DNA repair. In the cell’s homeostatic attempt at coping with this intensity of damage production, repair systems are saturated, beyond which DNA-damage-tolerance mechanisms may down-regulate genomic maintenance to allow damaged cells to survive and proliferate (Friedberg, Walker and Siede, 2006).

However, in turn, this may require that the burden of the original insult be taken up by the epigenome; as revealed by recent microarray analysis comparing the gene expression profiles of mammalian cells exposed to either high or low dose X-radiation. Ding et al. (2005) showed that the former predominantly facilitates changes in the expression of genes that regulate apoptosis and cell proliferation, whereas low dose irradiation is more likely to affect cell signalling, development and DNA damage responses in this way. Recent work by Neumaier and colleagues (2012) has suggested that this translates to a significantly higher increase in DNA repair centre formation after X-irradiation with 10 cGy, compared to higher exposures in excess of 100 cGy. Highly similar patterns have also been found in 3D human tissue models (Mezentsev and Amundson, 2011), where TP53 was thought to dominate the response to high dose irradiation, but where the transcription factor HNF4A is most prominent for the low-dose response. The disparity of an essentially non-overlapping gene expression-based response to high or low doses of X-rays may help to explain the non-linear correlation of non-targeted effects of ionising radiation to varying dose. Furthermore, if some of these changes in response to acute irradiation become permanent, descendant cells may inherit them. If this occurs in the germ line of exposed parents and the epigenetic alterations manage to escape the reprogramming that succeeds fertilisation, these changes may be passed to the next generation and provide the basis for a signal of instability.
The present study also provides a plausible explanation for the persistent controversy regarding the experimental evidence for these effects in humans. A plethora of studies have set out to investigate whether or not genomic stability is at risk in the children of individuals exposed to ionising radiation and yet a consensus remains elusive for two main reasons. The first is that the doses/dose-rates of paternal exposures analysed in these studies differ substantially, from each other and between model systems and human epidemiological studies in general. Moreover, the accuracy of information on the latter is often complicated by factors such as added internal exposure, non-uniform long-term dose-rates and varying radiation quality; confounding understanding as to what an individual was actually exposed, especially in the case of accidental or hostile exposure, where dosimetry is mostly monitored retrospectively. Secondly, the vast majority of human exposure occurs at relatively low to intermediate doses and dose-rates, for which most end-points of genomic instability are generally not powerful enough to detect a clear response; and those that are raise questions of the relevance of their evaluation.

Here, an acute dose range has been investigated that well reflects the higher doses to which humans are exposed, survive and are capable of then having children. 10 cGy is the maximum lateral side scatter dose that surrounding, normal tissue would generally receive in a single procedure of radiotherapy (UNSCEAR, 2000). This is because all current courses of external beam radiotherapy are designed to directly and efficiently target tumours whilst avoiding and shielding normal tissues, greatly minimizing the dose to the latter. According to the present data, it would appear highly unlikely that such doses could manifest genomic instability in these individuals and their children. If these results hold true, they may also explain the inconsistencies in observing transgenerational instability in the children of survivors of various incidents.
of exposure. An average exposure of 30 cGy has been estimated for survivors of the atomic bombings and best estimates of maximum dose due to fallout, as determined by the official DS02, are under 50 cGy for both Hiroshima and Nagasaki (Young and Kerr, 2005). As are the majority of doses that survivors, who went on to father children, have received from accidental external exposure (see Chapter 1.2). Examples of such individuals exposed to radiation sufficient enough to breach the apparent threshold, such as the most highly exposed groups at Semipalatinsk (Simon et al., 2003) or Chernobyl (NEA, 2002), are rare. Therefore, it would appear that transgenerational instability is likely to be an uncommon phenomenon in the world of human exposure to ionising radiation. The regular presence of these non-targeted effects in humans becomes even more uncertain if the apparent stability of F₁ genomes following 100 cGy maternal X-irradiation, (Abouzeid Ali, Barber and Dubrova, 2012), is taken into account.

3.6.2 Considerations and Future Work

Even so, there are a few issues to consider with regards to this work that may provide a course for further study. The total absorbed dose to the shielded germ line after the completion of a fractionated course of radiotherapy can reach as high as 100 cGy (UNSCEAR, 2000) and so may have the potential to trigger transgenerational instability. As noted, numerous studies have shown that the mutagenicity of fractionated low-LET exposure is much lower than that for acute irradiation and at a similar level following low dose-rate chronic exposure (Russell and Kelly, 1982a; b; Lyon et al., 1972; Tucker et al., 1998; Brenner, 1999). Nevertheless, though it would appear that the present data on low dose-rate exposure vindicates such a treatment course from triggering instability, it remains that the transgenerational effects of a
fractionated daily irradiation have not been thoroughly investigated. This would be an important direction of further study.

Furthermore, the data of the present study was obtained for the BALB/c inbred strain of mice. Although past work has confirmed transgenerational instability in the offspring of three very different mouse strains (Barber et al., 2002), the relative level of destabilisation varied significantly between them, with BALB/c mice exhibiting the most dramatic changes. This correlates well with the different survival rates of the strains in response to high-dose, acute X-irradiation (Roderick, 1963). However, given this inherent radiosensitivity in the BALB/c strain, due to a fixed mutation affecting the NHEJ repair pathway (Yu et al., 2001), analysing dose and dose-rate effects on instability in other strains would provide a useful comparison. This is especially appealing considering the high incidence of genetic variants affecting DNA repair capacity in humans (Mohrenweiser et al., 2003), which may profoundly influence epidemiological investigations in exposed populations, particularly with regards to identifying relevant control groups.

Another issue that should be clarified is how the efficiency of the transgenerational effects discussed here is affected by high-LET sources of parental irradiation. The present study focused on the more common low-LET of X- and γ-rays, but a similar understanding of the effects of sources such as α–particles, fission neutrons and heavy ions is not well characterised. Transgenerational instability has been demonstrated following chronic irradiation with fission neutrons (Barber et al., 2002) and their low dose-rate exposure has even been shown to be more mutagenic than acute X-irradiation (Dubrova et al., 2000a). With its much faster rate of energy deposition high-LET radiation has a relative biological effectiveness that, depending on total energy, can be 2 to 20 times that of low-LET (ICRP, 2003). This is thought to
be due to the complex distribution of ionising events produced by the former, which result in double-strand DNA breaks that are clustered and more densely scattered than those caused by latter (Teoule, 1987; Roots et al., 1990). A recent study by Pang and colleagues (2011) showed that short DNA fragments (< 42 bp) formed by this clustered damage interact inefficiently with DNA repair proteins, inhibiting not only their own repair processing, but also that of larger fragments (> 300 bp) and caused genomic instability following cellular transfection with them. This strongly supports the findings of the present work in that a high intensity of DNA damage saturates the repair response and provides further clues into the mechanisms of how this may affect genomic stability. However, a quantitative understanding of this connection with regards to radiation dose is completely unknown.

Furthermore, due to the growing interest in hadron particle radiation therapy, investigating the dose/dose-rate effects of such sources is becoming more and more relevant. In the context of radiotherapy, this intense mutagenicity may be tempered by use of the Bragg peak effect where charged particles can be arranged to release relatively little energy as they enter the skin at high speed, deliver their full impact at the tumour and come to rest with virtually no damage beyond (DeLaney and Kooy, 2007). Also, due to their relatively large mass, a beam of such particles does not broaden much and may deliver minimal scatter dose to the surrounding, normal tissue. In any case, projects similar to the present study will be essential in understanding the nature of the long-term effects to high-LET sources of radiation.

In recent years, there has also been a growing body of evidence to suggest that various environmental factors can epigenetically influence the health of several generations of offspring. The soft, ‘Lamarckian’ inheritance of mutagenic signals, described here following high doses of ionising radiation, has also been described
following treatments with a variety of mutagenic compounds, including benz(a)pyrene (Csaba and Inczefi-Gonda, 1998; Turusov et al., 1990), orthoaminoasotoluol (Popova, 1989) and dioxin-like compounds (Ikeda et al., 2005). Epidemiological studies have linked paternal tobacco smoking with transgenerational instability (Pembrey et al., 2006; Laubenthal et al., 2012) and exposure to particulate air pollution has even been shown to cause germ line ESTR mutation induction in mice (Somers et al., 2002) associated with DNA damage and persistent global hypermethylation (Yauk et al., 2008). Further research will be vital in clarifying the effectiveness of these damaging agents in disrupting the genomic integrity of the offspring of exposed individuals and the associated epigenetic processes.

However, most remarkable in this context are the results of studies that observe detrimental transgenerational responses following non-mutagenic treatment of the parents. In utero exposure to endocrine disruptors has been reported to increase the frequency of male infertility in several subsequent generations of rats, associated with altered sperm DNA methylation patterns (Anway et al., 2005). Others have shown that parental deficiencies in gestational nutrition can cause transgenerational increases in the incidence of type 2 diabetes (Portha, 2005) and growth defects (Cesani et al., 2003). Such data clearly implies the transmission of epigenetic signals with grave consequences for future generations. However, to what extent these effects occur in relation to parental treatment and the underlying mechanisms are yet to be fully understood.
3.6.3 Conclusion

In summary, the present study has shown that direct acute exposure to low to intermediate doses of low-LET ionising radiation produces a linear dose response for germ line mutation induction, with a clear reduction in the mutagenic capability of a chronic irradiation. However, further investigation revealed that transgenerational genomic instability is instead determined by a relatively high threshold of acute dose. This uncovers a stress-like response to a level of DNA damage that saturates the repair capabilities of the exposed cells, advancing our understanding of the long-term genetic effects that may potentially affect humans. It is growing increasingly clear that these phenomena are propagated by epigenetic signals that challenge our knowledge of the type of information inherited by subsequent generations.

Even so, it would appear that many types of relatively high-dose paternal exposure in humans, including radiotherapy regimens, are not a threat to the genomic stability of children of irradiated fathers. Additionally, transgenerational instability does not pose a challenge to current protective, effective dose limits as defined by the various ionising radiation regulatory bodies. For the UK, the Health and Safety Executive sets a special case, adult occupational dose limit of 100 mSv in 5 years with no more than 50 mSv in a single year, subject to strict conditions (HSE, 2008). This is reduced to 6 mSv in a single year for trainees and 1 mSv for any other person.
Chapter 4 - Materials & Methods

4.1 Materials

4.1.1 Mice

Inbred BALB/c mice chosen for the study were irradiated at the Panorama Facility, Obninsk, Russia. Absorbed dose was measured using 27012 and DKS-101 dosimeters. Mice were housed and DNA samples were extracted at the N.I. Vavilov Institute of General Genetics, Moscow, Russia.

4.1.2 Chemical and Molecular Reagents

Chemical reagents were supplied by Cambrex Bio Science (Rockland, USA), Fisher Scientific (Loughborough, UK), Flowgen (Ashby de la Zouch, UK), FMC Bioproducts (Rochland, USA) and Sigma-Aldrich Company Ltd. (Poole, UK).

Molecular biology reagents were purchased from ABgene (Epsom, UK), Healthcare UK Ltd (Chalfont, UK), Invitrogen (Paisley, UK), New England Biolabs (Hitchin, UK), Promega (Southampton, UK), Roche Molecular Biochemicals (USA), Sigma-Aldrich Company Ltd (Poole, UK) and United States Biochemical Corp (USB) (Cleveland, USA).

4.1.3 Radiochemical Reagents

$\alpha$-$^{32}$P-dCTP used to radioactively label probes was obtained from PerkinElmer (Austria).
4.1.4 Specialised Equipment

Equipment was provided by Bio-Rad (Hemel Hempstead, UK), Eppendorf (Hamburg, Germany), Fisher Scientific (Loughborough, UK), Genetic Research Instrumentation (GRI) (Braintree, UK), Heraeus Instruments (Hanau, Germany), Thermo Scientific (Ashford, UK), Ultra Violet Products Life Sciences (Cambridge, UK), Qiagen (Crawley, UK) and NanoDrop Technologies (Ringmer, UK).

4.1.5 Enzymes

The restriction endonuclease MseI was provided by New England Biolabs (NEB) (Hitchin, UK). Thermo-stable DNA polymerases (Taq & Tgo of the Expand High Fidelity PCR system) were obtained from Roche Ltd. (Hertfordshire, U.K.). Synthetic Klenow enzyme, derived from DNA polymerase 1 of *E. coli* was supplied by United States Biochemical Corp (USB) (Cleveland, USA).

4.1.6 Molecular Weight Markers

DNA fragment sizes were measured against 1 kb (Invitrogen, Paisley, UK) and 200 bp (Promega, Southampton, UK) DNA ladders.

4.1.7 Oligonucleotides

Primers for the amplification of sample DNA and probes were supplied by Sigma-Aldrich (Poole, U.K.).

4.1.8 Standard Solutions

10x Tris-borate/EDTA (TBE) buffer, depurinating solution, denaturing solution, neutralising solution and 20x sodium chloride-sodium citrate (SSC) were produced by
the Media Kitchen (Department of Genetics, University of Leicester) as described by Sambrook (Sambrook and Russell, 2001).

### 4.1.9 Computer Software

Results from single-molecule PCR optimisations and Poisson analysis were recorded using Microsoft Office Excel 2007. Analysis of optimisation data was performed using software written in BASIC by Professor Y.E. Dubrova. Analysis of mutation frequencies was accomplished using the programs STATISTICA (Version 6) and SYSTAT (Version 10). Text and figures were produced using Microsoft Office Word 2010 and Adobe Photoshop.
4.2 Methods

4.2.1 Irradiation Scheme and Mouse Model

6-week-old male mice were exposed to gamma rays using a $^{137}$Cs source, with proper dosimetry around each cage to regulate the accumulated dose. In this way, absorbed dose-rates of 4.26 cGy min$^{-1}$ (10 cGy acute), 50 cGy min$^{-1}$ (25, 50 and 100 cGy acute), and 0.005 cGy min$^{-1}$ (100 cGy chronic) were administered to investigate the 5 exposure profiles. Irradiated males were mated to control females 12 weeks after exposure for transgenerational studies. Caudal epididymides were then collected from the F$_0$ males upon mating. The male F$_1$ offspring of control and irradiated parents were sacrificed at 8 weeks old and culled for caudal epididymides and brain tissue. Sperm and brain DNA was then extracted from each tissue, respectively, and purified.

Animal irradiation was performed and DNA provided by Professor Alexander Rubanovich and Dr Andrey Myazin of the N.I. Vavilov Institute of General Genetics, Moscow, Russia. All animal procedures were approved by the institute’s Ethical Committee.
4.2.2 Single-Molecule PCR Mutation Frequency Analysis

4.2.2.1 DNA Digestion and Precipitation

15.5 µl of DNA was digested in 1x NEB buffer 2, 20 ng of BSA and 25 U of MseI, an enzyme that cleaves outside the Ms6-hm ESTR array to render the DNA fully soluble. This mixture was placed in a 37 °C water bath for 2 hours. DNA was then fully precipitated in 2-3 volumes of 100 % ethanol and 10 % of 3 M NaAc (pH 5.2) at -80 °C for at least 2 hours. The tubes were centrifuged in an Eppendorf Centrifuge 5804 for 20 minutes at 13,000 rpm. Upon rotating the tubes by half a turn they were spun for another 20 minutes. The resultant pellet was then washed in 80 % ethanol and re-suspended in 50 µl of ultra pure water.

4.2.2.2 DNA Quantification

An estimation of the digested DNA concentration was made using an ND-1000 Spectrophotometer (NanoDrop Technologies). Each sample was then diluted appropriately to a concentration of 10 ng/µl with ultra pure water in a separate Eppendorf tube.

4.2.2.3 Single-Molecule Optimisation

Stock DNA of 10 ng/µl was diluted to evaluate the single-molecule concentration that enables there to be one haploid genome, and so one amplifiable molecule of ESTR DNA, in each positive reaction. To achieve this DNA was diluted to 100, 50, 20, 10, 5 and 2 pg/µl using appropriate volumes of dilution buffer. 1 µl samples of DNA in 8 reactions per dilution were then amplified and analysed by Southern blotting hybridisation (as per Chapter 4.2.3). The single-molecule concentration was
then estimated by ascertaining the concentration where ~50 % of 8 reactions yielded a positive result (Figure 4.1).

Figure 4.1 – SM-PCR optimisation. Sample DNA is diluted to a range of concentrations, 2-5 pg/µl, to estimate the single-molecule concentration at which one ESTR molecule may be amplified. This is approximated by the concentration that produces a positive result for 50 % of reactions, 5 pg/µl for the present example.
4.2.2.4 Poisson Analysis with Single-Molecule Concentration

The concentration obtained by optimisation was used to generate positive reactions for subsequent mutation scoring. 1 µl samples of DNA, diluted to single-molecule concentration, were then amplified in 96 reactions and analysed by Southern blotting hybridisation (as per Chapter 4.2.3). Positive and negative reactions were then noted (Figure 4.2). Mutation scoring required that 90-150 individual alleles be obtained and so aliquots of each DNA sample were used for analysis until a sufficient number of positive reactions were attained, adjusting the single-molecule concentration estimate as appropriate.

Figure 4.2 – SM-PCR Poisson analysis. The estimated single-molecule concentration is used to amplify the ESTR arrays in 96 reactions per plate such that single alleles may be identified from positive reactions for use in subsequent mutation scoring.
The Poisson distribution was then used to calculate the average number of amplifiable molecules per reaction by considering the number of negative reactions:

\[ P.(k = 0) = e^{-\lambda} \text{ or } \lambda = -\ln(P.) \]

- \( P. \) - frequency of negative reactions
- \( k \) - total number of reactions
- \( \lambda \) - mean number of amplifiable molecules

Multiplying this value by the total number of reactions provided an estimate of the total number of amplifiable molecules.

4.2.2.5 Mutation Scoring

All of the reactions that ostensibly yielded a single amplified allele were then electrophoresed together on gels sufficiently long to provide adequate resolution for mutation scoring after Southern blot processing (as per Chapter 4.2.3).

Mutant Ms6-hm alleles were identified with 1 mm change in length from that of progenitor bands (of approximately 2.5 kb) representing a mutation of 2 repeats difference (Figure 4.3). Fragments higher or lower than the progenitor allele corresponded to a gain or loss of repeats, respectively.
Figure 4.3 – Mutation detection. ESTR PCR products are electrophoresed together with a 200 bp DNA step ladder and inspected for mutant alleles. $Ms6-hm$ mutants are identified among progenitor alleles and indicated by red marker.

4.2.2.6 Statistical Analysis

The frequency of ESTR mutation in each tissue was finally estimated by dividing the number of scored mutants by the total number of amplifiable DNA molecules. Standard errors were calculated using a modified algorithm proposed by Chakraborty (Zheng et al., 2000).
4.2.3 Techniques Used for Ms6-hm Amplification and Autoradiograph Preparation

4.2.3.1 Polymerase Chain Reaction

Ms6-hm ESTR arrays were amplified (Saiki et al., 1988) in 10 µl reactions using 1x buffer, 200 µM dNTPs, 0.4 µM HM1.1F (5’ - AGA GTT TCT AGT TGC TGT GA - 3’) and HM1.1R (5’ - ATG CCT TAG AAC TGA CTC TC - 3’) primers, 1M betaine, ultra-pure water and 1 U of enzyme mixture of the Expand High Fidelity PCR system. The reactions were performed using 0.2 ml thin-walled PCR tube strips of 8. After being firmly sealed, these were transferred to a PCT-225 DNA Engine Tetrad (Bio-Rad). This was programmed using heated lids to denature the DNA for 3 minutes at 96 °C and then run 29 cycles of 96 °C for 20 seconds, 58 °C for 30 seconds and 68 °C for 3 minutes. The program terminated with additional 68 °C incubation for 10 minutes.

4.2.3.2 Short Agarose Gel Electrophoresis

To observe the DNA amplicons, for SM optimization and Poisson analysis, 5 µl of loading dye (1x TAE, 2.5 % Ficoll 400 and 0.02 % Bromophenol blue) was added to each PCR product, which were then loaded (5 µl each) onto 1 % agarose gels prepared on 25 x 24 cm gel trays with 4 rows of 26 teeth gel combs. 200 ng of 1 kb DNA ladder was also loaded on each row. The trays were held in electrophoresis tanks, made in-house, using 0.5x TBE (22.5 mM Tris-borate pH 8.3 and 0.5 mM EDTA) as running buffer. These were then electrophoresed at 200 V for 1½ hours; until the blue loading dye had migrated to the next row of wells.
4.2.3.3 Long Gel Electrophoresis

PCR products for mutation scoring were loaded onto 1 % agarose gels cast on 40 x 20cm gel trays with a single 27 teeth gel comb. 1x TBE buffer (44.5 mM Tris-borate pH 8.3, 1 mM EDTA and 0.5 µg/ml ethidium bromide) was used in gel preparation and as a running buffer. In addition to 200 ng of 200 bp ladder (loaded into 3 wells), 1 µg of 1 kb ladder was also loaded into the first well to later determine how far the DNA had migrated. The trays were held in long electrophoresis tanks, made in-house, which needed to be balanced. These were then electrophoresed at 200 V for ~24 hours; until the 1.6 kb band of the 1 kb ladder had migrated to the bottom edge of the gel. This ensured consistent resolution for mutation scoring between autoradiographs.

4.2.3.4 Southern Blotting

Excess agarose was removed and the gels inverted into plastic trays to be rinsed with blotting solutions. They were rinsed twice in depurinating solution (0.25 M HCl), twice in denaturing solution (0.5 M NaOH and 1 M NaCl) and another two times in neutralising solution (0.5 M Tris and 3 M NaCl). DNA was then transferred onto a sheet of MAGNA nylon transfer membrane (MSI, Osmonics Laboratory Products), pre-soaked in 2x SSC (0.3 M NaCl and 0.03 M Na-citrate), by the capillary transfer method (Southern, 1975) using 20x SSC. 3MM Whatmann filter papers were used to help induce uptake and Saran wrap was used to ensure efficiency. After 5 hours (at least) the nylon membrane was rinsed in 2x SSC. This was then dried for 15 minutes in an 80 °C oven. The transferred DNA was then covalently cross-linked to the membrane by exposure to $7 \times 10^4$ J/cm$^2$ using a UV crosslinker (Amersham Biosciences).
4.2.3.5 Probe Labelling and Recovery

Double stranded *Ms6-hm* probe DNA was produced via PCR using the primers HMA (5’ - GGGCA GGGCA GGGCA GGGCA GG - 3’) and HMB (5’ - TGCCC TGCCC TGCCC - 3’). 1 kb ladder DNA (10 ng/µl) was used for ladder probe for short gel membranes and 200 bp ladder DNA (10 ng/µl) for long gel membranes. 20 ng of probe DNA was made up to 30 µl with ultra-pure water, denatured and labelled by random primer incorporation (Feinberg and Vogelstein, 1983a; 1984). This involves the use of randomly generated hexamers and the *E. coli*-derived DNA polymerase Klenow fragment to incorporate α-32P-dCTP (1000 Ci/mmol, PerkinElmer, Austria) into newly synthesised DNA. The reactions were incubated at 37 °C for 5 hours, upon which the probe was precipitated with 90 µg of salmon sperm DNA, 30 µl of 2 M NaAc (pH 5.6) and 425 µl of 100 % ethanol. The remaining pellet was then washed in 80 % ethanol and resuspended in 600 µl of ultra-pure water. 100 µl of the appropriate labelled ladder was added to the labelled *Ms6-hm* probe which was then denatured in a heating block prior to hybridisation.

4.2.3.6 Hybridisation

The nylon transfer membrane was pre-washed with hybridisation buffer (0.5 M Na2PO4 pH 7.2, 7 % SDS and 1 mM EDTA), modified from Church and Gilbert (1984), in a pre-warmed Mini 10 hybridisation oven (Thermo Scientific) at 65 °C for at least 20 minutes. Labelled probe DNA was then hybridised with the membrane-bound DNA for 5 hours or overnight. Phosphate wash solution (0.04 M Na-Phosphate and 0.5 % SDS) was then used to wash the nylon membrane for another 10 minutes. Further 10 minute washes were performed using high stringency wash solution (0.1x
SSC and 0.01 % SDS) until the radioactivity detected from the poured off solution was below 50 counts/minute.

4.2.3.7 Autoradiography

The nylon membrane was then wrapped in Saran wrap and taped DNA side up into an autoradiograph cassette (GRI) containing an intensifying screen. A Fuji Rx100 X-ray film (GRI) was inserted into the cassette which was then stored in a -80 °C freezer for 3 days before being developed. The developing process was stopped when sufficient band clarity was achieved and the image was then fixed using fixer solution, after which the film appeared transparent.
References


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