ROLE OF EPIGENETIC CHANGES IN DIRECT AND INDIRECT RADIATION EFFECTS

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Abstract

For over 100 years, cancer radiation therapy has provided patients with increased survival rates. Despite this success, radiation exposure poses a threat to the progeny of exposed parents. It causes transgenerational genome instability that is linked to transgenerational carcinogenesis. The exact mechanisms in which this instability occurs have yet to be discovered. Current evidence points to their epigenetic nature, specifically changes in DNA methylation.

Using mouse and rat models, this thesis investigated the transgenerational effects of radiation in the offspring from parents who received whole body or localized exposure to ionizing radiation (IR). Both types of exposure resulted in significant global DNA hypomethylation in the somatic tissues of the progeny. These changes were paralleled by the significantly decreased levels of methyltransferases and methyl-CpG-binding protein.

In summary, our results suggest that both localized and whole body parental exposures to IR result in transgenerational epigenetic instability within the unexposed offspring.

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[^{3}H]-dCTP:	Tritiated cytidine triphosphate
BER:	Base excision repair
C57BL/6:	C57 black six
COBRA:	Combined bisulphate restriction analysis
DNMT:	DNA methyltransferase
DSB:	Double strand break
ESTR:	Expanded simple tandem repeat
Gy:	Gray
HR:	Homologous recombination
IAP-LTR:	Intracisternal A particle long terminal repeat
IR:	Ionizing radiation
LINE1:	Long interspersed nuclear element
MBD:	Methyl-CpG-binding domain
MeCP2:	Methyl-CpG-Binding protein
miRNA:	Micro RNA
NHEJ:	Nonhomologous end joining
PAGE:	Polyacrylamide gel electrophoresis
PCNA:	Proliferating cell nuclear antigen
PCR:	Polymerase chain reaction
POL B:	DNA polymerase β
PVDF:	Polyvinylidene Fluoride
rDNA:	Ribosomal DNA
RISC:	RNA-induced silencing complex
SINE:	Short interspersed nuclear element
UTR:	Untranslated region

CHAPTER 1

General Introduction

Direct effects of radiation exposure

The carcinogenic effects of ionizing radiation (IR) were established very soon after its discovery, when the first radiation-induced tumor was reported in 1902 (Little 2000, 2003). In recent years, extensive studies have concentrated on discerning the cellular and molecular mechanisms of radiation induced carcinogenisis in mammalian cells. A complete understanding of the IR-induced carcinogenesis process is essential. Paradoxically, IR is also one of the primary methods used for fighting human malignancies.

The cellular processes that IR can affect include: gene expression changes, disruption of mitochondrial processes, cell cycle arrest and apoptotic cell death (Amundson and Fornace, 2003; Andreev et al., 2006; Criswell et al., 2003; Fei and El-Deiry, 2003; Iliakis et al., 2003; Jeggo and Lobrich, 2006; Powell and Kachnic, 2003; Rodemann and Blaese, 2007; Valerie et al. 2007). IR is also a potent DNA damaging agent, capable of producing DNA damage such as cross linking, nucleotide base damage, and double strand breaks (Huang, 2003; Little, 2000; Ward, 1995). The mis-repair of radiation-induced DNA double-strand breaks and other lesions is believed to be the principal pathway for the induction of mutation, chromosome and gene alterations responsible for the lethal, mutagenic, and carcinogenic effects of IR (Ward, 1995). An accumulation of DNA damage caused by IR can lead to carcinogenesis (Little, 2000).

Indirect radiation effects: Radiation-induced genome instability and bystander effects

Until recently, the central belief of radiation biology stated that the effects of IR were restricted only to the cells which were directly subjected to irradiation (Little, 2000). Yet this paradigm has been challenged by numerous observations in which cells that were not directly exposed presented the responses very typical of the directly irradiated cells. These responses have been demonstrated in cells that are descendents of the directly irradiated cells, and were termed "radiation-induced genome instability".

The radiation-induced genome instability phenomenon is defined as an increase in the acquisition rate of genomic alterations, such as late cell death, chromosome destabilization, sister chromatid exchange, gene mutation, and aneupliody in the progeny of irradiated cells for many consecutive cell divisions (Little, 2006; Morgan, 2003a), all of which may be causative factors in cancer development. Therefore, the presence of genome instability is thought to be a precursor to cancer formation (Goldberg 2003; Little, 2003).

Effects of radiation can also be seen in the naïve cells that either received certain irradiation 'distress' signals from, or were in contact with, the directly exposed cells. Such an exposure-communication process is termed the "radiation-induced bystander effect" (Morgan, 2003a; Morgan, 2003b; Morgan and Sowa, 2005; Mothersill and Seymour, 2003; Mothersill and Seymour, 2004; Mothersill and Seymour, 2006).

Like radiation induced genome instability, bystander effects encompass a wide range of genetic alterations, including gross genome rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, and gene mutations and

amplifications (Azzam and Little, 2004; Azzam et al., 2001, 2003, 2004; Gaugler et al., 2007; Hamada et al., 2007; Hu et al., 2006; Little, 2006; Lorimore et al., 1998, 2001, 2005; Maguire et al., 2005, 2007; Morgan, 2003a, 2003b; Mothersill et al., 2001; Nagasawa and Little, 2002; Smilenov et al., 2006; Suzuki et al., 2003; Yang et al., 2007; Zhou et al., 2001, 2002, 2005). They also influence gene expression, cellular proliferation, senescence, and cell death (Belyakov et al., 2002, 2003; Chaudhry, 2006 ; Sedelnikova et al., 2007).

Besides the cell cultures, the bystander effects have also been observed in three dimensional tissue models including spheroids (Persaud et al., 2005, 2007), and in the reconstructed human tissue models (Belyakov et al., 2005; Sedelnikova et al., 2007). Therefore, bystander effects are generally accepted as an omnipresent result of radiation exposure (Mothersill and Seymour, 2004).

Bystander effects also manifest in the whole-organism context. Compared to the bystander effect data based on the cell culture, the conclusive data on the somatic bystander effects *in vivo* are relatively scarce (Goldberg and Lehnert, 2002; Hall, 2003; Koturbash et al., 2006). Radiation exposure can lead to the release of soluble 'clastogenic' factors into circulating blood, that were shown to be capable of damaging chromosomes in the cultured cells (Emerit et al., 1994,1995; Goh and Summer, 1968; Hollowell and Littlefield, 1968; Marozik et al., 2007; Pant and Kamada, 1977). Such clastogenic activity was found both in plasma of patients receiving high dose radiotherapy and individuals accidentally exposed to radiation (Emerit et al., 1994, 1995; Goh and Summer, 1968; Hollowell and Littlefield, 1968; Marozik et al., 2007; Pant and Kamada, 1977). Bystander effects were shown to be significant within an exposed organ. When the

lung base was irradiated, significant molecular and cellular damage was observed in the shielded lung apex (Khan et al., 1998, 2003). Also, a marked increase of micronuclei was noted in the unexposed shielded lung when one lung, either right or left, was exposed (Khan et al., 1998; Khan et al., 2003). Similar within-the-organ bystander effects were observed during the partial liver irradiation (Brooks et al., 1974).

The Kovalchuk laboratory recently confirmed the existence of *in vivo* bystander effects using a mouse skin model, whereby half of the animal body was exposed to radiation, while the other half was protected by a medical grade shield (Koturbash et al., 2006). The data showed that radiation exposure to one half of the body leads to extensive cellular and molecular changes in the unexposed bystander half of the body at least 0.7 cm from the irradiated tissue 4 days after exposure (Koturbash et al., 2006). Importantly, the molecular changes in bystander tissues were not due to the insufficient shielding or radiation scattering (Koturbash et al., 2006).

Using the same model, the existence of the IR-induced bystander effects was confirmed in mouse spleen (Koturbash et al., 2007a). Additionally, bystander effects were also seen in the rat model, whereby cellular and molecular changes were noted in the bystander spleen tissue seven months after the localized cranial irradiation (Koturbash et al., 2007b).

The frequency of induction and long-term persistence of the bystander responses pointed to their possible epigenetic background (Lorimore et al., 2003; Morgan, 2003a; Morgan, 2003b; Nagar et al., 2003; Wright and Coates, 2006). Indeed, the epigenetic nature of the bystander effect was recently confirmed by the Mothersill, Bonner and

Kovalchuk laboratories using the cell culture, 3D human tissue and whole-animal models (Sedelnikova et l., 2007; Kaup et al., 2006; Koturbash et al., 2006, 2007b).

Transgenerational radiation-induced effects

Irradiation effects can span several generations. Many studies have shown that parental irradiation can lead to detrimental transgenerational effects within the offspring. The response of the progeny to parental irradiation has manifested as both phenotypic and genotypic alterations. Phenotypic effects include decreased fertility and teratogenic effects (Dasenbrock et al., 2005; Luning et al., 1976; Mohr et al., 1999; Nomura, 1988, 2003; Nomura et al., 2004; Pils et al., 1999; Tomatis, 1994). Genotypic alterations appear as increased mutation rates and elevated frequencies of chromosome aberrations, micronuclei formation, among several other possible expressions of transgenerational genome instability (Barber and Dubrova, 2006; Barber et al., 2002; Barber et al., 2006; Carls and Schiestl, 1999; Dasenbrock et al., 2005; Dubrova, 2003a, 2003b; Mohr et al., 1999; Morgan, 2003b; Niwa, 2003; Nomura et al., 2004; Nomura, 2003; Shiraishi et al., 2002; Singer et al., 2006; Slovinska et al., 2004; Tawn, 2005; Tomatis, 1994)

Most importantly, the exact molecular mechanisms of the radiation-induced genome instability and transgenerational effects have yet to be discovered. A variety of studies have suggested epigenetics as the mode of transmission for transgenerational effects (Dubrova and Plum, 2002; Dubrova et al., 2000; Morgan, 2003a; Morgan, 2003b; Vance et al., 2002, Wiley et al., 1997; Zhu et al., 2006).

Wiley and colleagues used a mouse preimplantation embryo chimera assay to demonstrate heritable effects of paternal irradiation on embryonic cell proliferation that

persisted for two generations with no decrease in the incidence or severity of the effect between generations (Wiley et al., 1997). These observations led the authors to hypothesize that a non-Mendelian mode of inheritance was involved. Later Vance and Wiley (1999) showed that by blocking gap junction-mediated intercellular communication they could 'rescue' the irradiated embryo, allowing normal cell proliferation in the irradiated embryonic component of the embryo chimera. These data demonstrated that the decreased cell proliferation rate observed in the embryo from the irradiated parent occurred as a result of gap junction-mediated signaling between the irradiated embryo and the control embryo. They also demonstrated that offspring from F_0 parental irradiation exhibited pronounced biochemical alterations (Baulch et al., 2001; Baulch and Raabe, 2005; Vance et al., 2002). Specifically, four generations of offspring from the irradiated sires exhibited changes in the protein kinase C, mitogen-activated protein kinase, Tpr53 and $p21^{waf1}$ levels. These changes were not consistent in magnitude or direction of change within the offspring of a given generation, nor were they consistent between generations (Baulch and Raabe, 2005). The authors again hypothesized that the expression of these genes might be altered through an epigenetic process (Baulch and Raabe, 2005).

Furthermore, high frequency persistent heritable genetic effects on embryonic and spermatogenic cell proliferation rates that spanned over 2-3 generations of animals following paternal irradiation also suggested an epigenetic pattern of regulation of these transgeneration effects (Baulch et al., 2002).

The possible mechanism that may underlie the observed persistent transgenerational gene expression phenotype was termed 'epigenomic instability'

(Baulch and Raabe, 2005). Recent studies using the neutral pH sperm comet assay have also demonstrated the effects of IR on sperm DNA electrophoretic mobility of irradiated male mice 7 weeks post irradiation (Baulch et al., 2007). The same assay also demonstrated an unconventional, heritable DNA damage effect, or changes in chromatin conformation, in non-irradiated offspring of irradiated male mice (Baulch et al., 2007). These findings further support the proposed 'epigenomic instability' theory (Baulch and Raabe 2005).

Dubrova et al. (2000) and Dubrova and Plumb (2002) used expanded simple tandem repeats (ESTRs) as a tool to analyse mutation rates in offspring of radiation exposed fathers. The offspring derived from ten independent sperm cells of the irradiated father all showed uniform, increased levels of mutation rates. Both studies speculated that due to the random structural damage to DNA caused by irradiation, it was very unlikely that the same genes were affected in the different sperm cells. They concluded that radiation- induced genomic instability is transferred to offspring in an epigenetic manner, such as alterations in DNA methylation. To further support their speculation, the increased mutation rates in the germline of the progeny following paternal irradiation may suggest that mechanisms of delayed radiation-induced genome instability are acting. However, the minimal cytoplasm of the sperm could not transfer radiation-induced DNA damaging species such as free radicals. This suggests that the "signal" is attributed to altered DNA methylation patterns which may influence DNA repair systems (Dubrova et al., 2000).

The analysis of repeat elements in the genome has not only been applied to the transgeneration mouse model, but has also been utilized to study transgeneration effects

in human populations including the atomic bomb survivors, individuals affected by the Chernobyl accident, the Chernobyl clean up workers, and those living around the Semipalatisk nuclear test site (Dubrova et al., 1996; Dubrova et al., 1997; Dubrova et al., 2002).

As with other studies of transgeneration effects, the high magnitude non-Mendelian inheritance of increased ESTR mutation rates suggested the epigenetic deregulation as a possible causative factor (Barber et al., 2006).

The recent study by Zhu and colleagues (2006) directly monitored the methylation status of the paternal H19 allele differentially methylated region following paternal whole body X-ray exposure and subsequent transfer from sperm to offspring. In mice, the H19 allele is repressed via paternal imprinting and is expressed from the hypomethylated maternal allele (Ferguson-Smith et al., 1993). It is important to note that methylation imprints are thought to be established in the gametes and maintained during embryonic development despite global demethylation (Olek and Walter, 1997). Upon sequencing the imprinting control region of H19 in the sperm of the irradiated father and the liver from his progeny (born to an unirradiated mother), the authors observed a similar pattern of hypomethylation confirming that radiation exposure is capable of interfering with imprinting patterns in the parent and these patterns can be inherited and maintained by the offspring (Zhu et al., 2006).

In summary, the current data strongly support altered epigenetics, specifically DNA methylation patterns, as a key component in the transgenerational radiation induced genomic/epigenomic instability.

The majority of the past research carried out on transgenerational radiation effects have focused on the role of whole body exposure, yet such exposure is relatively rare. In contrast, localized body-part exposures occur very frequently during processes such as radiation diagnostics and therapy. There is a high potential for offspring to be subject to genome instability and deleterious changes as a result of parental exposure, and this issue needs to be addressed. This is specifically important as more people of child-bearing age are diagnosed with cancer, and subsequently undergo radiotherapy. The potential risk to their progeny is as yet unknown, and therefore this issue needs to be addressed.

Epigenetic changes

Epigenetic changes are meiotically heritable and mitotically stable alterations in gene expression that include DNA methylation, RNA-associated silencing and histone modification. Cytosine DNA methylation is the most widely studied epigenetic mechanism (Baylin and Ohm, 2006; Baylin, 2005; Ehrlich, 2002; Jaenisch and Bird, 2003; Rountree et al., 2001) as it is crucially important for many cellular processes, including the normal development, proliferation, and maintenance of genome stability in a given organism (Baylin and Ohm, 2006; Baylin, 2005; Ehrlich, 2002; Jaenisch and Bird, 2003; Jirtle and Skinner, 2007; Robertson, 2002). In mammals, DNA methylation occurs predominantly in the context of CG dinucleotides that are methylated to 60–80% (Hermann et al., 2004; Weber and Schuebeler, 2007). DNA is methylated at cytosines by DNA methyltransferase enzymes to form 5-methylcytosines (Hermann et al., 2004; Weber and Schuebeler, 2007). In mammals, three DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are primarily responsible for establishing and maintaining DNA

methylation patterns at CpG sites (Goll and Bestor, 2005; Robertson, 2002; Rountree et al., 2001). DNMT1 prefers hemimethylated DNA over unmethylated DNA up to 40-fold *in vitro* (Karpinets and Foy, 2005). During replication, DNMT1 (*in vivo*) is the major enzyme involved in the maintenance of the pattern of DNA methylation (Liang et al., 2002). It is localized to the fork where it could directly modify nascent (Goll and Bestor, 2005; Jirtle and Skinner, 2007; Weber and Schuebeler, 2007).

Dnmt3a and Dnmt3b are *de novo* methyltransferases targeting unmethylated and hemimethylated sites (Goll and Bestor, 2005; Okano et al., 1999; Weber and Schuebeler, 2007). Deregulation of these proteins may lead to altered methylation patterns (Goll and Bestor, 2005; Jirtle and Skinner, 2007; Weber and Schuebeler, 2007).

DNA methylation is known to be associated with the inactive chromatin state and in most cases with repressed gene expression activity. In mammals, the association of DNA methylation with transcriptional repression is thought to be mediated by the MBD (methyl CpG-binding domain) family of proteins (Klose and Bird, 2006). The MBD proteins, including MeCP2, MBD1, MBD2, and MBD3, interact selectively with methylated DNA and play a pivotal role in methylation-mediated chromatin remodeling and gene silencing (Bowen et al., 2004; Hendrich and Tweedie, 2003; Jaenisch and Bird, 2003; Robertson and Wolffe, 2000; Robertson, 2002). Moreover, methylated cytosines themselves can physically prevent the proper binding of transcription factors to promoter regions (Weber and Schuebeler, 2007).

Altered global DNA methylation patterns are a well-known characteristic of tumor cells (Weidman et al., 2007). The DNA methylation profile of cancer cells is frequently characterized by global genome hypomethylation as well as concurrent

hypermethylation of selected CpG islands within gene promoters (Baylin, 2005; Baylin and Ohm, 2006; Jaenisch and Bird, 2003, Weidman et al., 2007; Kovalchuk and Baulch, 2007). The global loss of DNA methylation has been linked to the activation of transposable elements, elevated chromosome breakage, aneuploidy, increased mutation rates and thus to the phenomenon of global genomic instability (Robertson and Wolffe, 2000; Robertson, 2002; Weber and Schuebeler, 2007; Weidman et al., 2007; Kovalchuk and Baulch, 2007).

Undoubtedly, changes in DNA methylation are not isolated events, and they occur in the context of the global chromatin deregulation and altered histone modification levels (Jaenisch and Bird, 2003; Weidman et al., 2007; Kovalchuk and Baulch, 2007). Histone modifications, including acetylation, methylation, phosphorylation and ubiquitination, are important in transcriptional regulation (Jenuwein and Allis, 2001; Weidman et al., 2007; Kovalchuk and Baulch, 2007). Many histone modifications are stably maintained during the process of cell division. The acetylated histone tails lose their positive charge, reducing the affinity to the negatively charged DNA, and leading to relaxed chromatin packaging. Therefore, histone acetylation is linked to transcriptional activation, while histone deacetylation generally represses transcription (Jenuwein and Allis, 2001; Kovalchuk and Baulch, 2007). Histone methylation can result in different transcriptional consequences depending upon the residue affected (Cheung and Lau, 2005; Saha et al., 2006; Kovalchuk and Baulch, 2007). Methylation of lysine 9 of histone H3 is associated with chromatin compaction and gene silencing, while methylation of lysines 4 and 27 of histone H3 results in transcription activation and chromatin relaxation. Additionally, histone residues can be mono-, di- and tri-methylated, adding an

enormous complexity to the yet unexplored histone code (Cheung and Lau, 2005; He et al., 2007; Saha et al., 2006; Weidman et al., 2007; Kovalchuk and Baulch, 2007). It was recently shown that tumors undergo a massive loss of tri-methylation at lysine 20 of histone H4 (Kovalchuk and Baulch, 2007; Fraga et al., 2005; Tryndyak et al., 2006a; Tryndyak et al., 2006b). This loss occurs along with DNA hypomethylation and is linked to chromatin relaxation and aberrant expression. It was suggested that this histone demethylation may be a universal marker for malignant transformation (Kovalchuk and Baulch, 2007; Fraga et al., 2005; Sanders et al., 2004).

Phosphorylation is another important histone modification (He et al., 2007; Kovalchuk and Baulch, 2007). One of the best studied modifications is phosphorylation of histone H2AX. H2AX is a member of the H2A histone family. It becomes phosphorylated at serine 139 (γH2AX), possibly as one of the earliest cellular responses to double strand breaks (DSBs) (Pilch et al., 2003; Rogakou et al., 1998; Sedelnikova et al., 2003). γH2AX accumulates at DSBs, forming γH2AX foci, and a direct correlation has been found between H2AX phosphorylation and the number of DSBs resulting from radiation (Celeste et al., 2003a). γH2AX is crucially important for the repair of DNA strand breaks and for the maintenance of genome stability (Celeste et al., 2003b; Kovalchuk and Baulch, 2007).

Finally, epigenetic control can also be mediated by small regulatory RNAs (Bernstein and Allis, 2005; Kovalchuk and Baulch, 2007). Among them, microRNAs (miRNAs) are of a special interest. MicroRNAs are abundant, small, single-stranded noncoding RNAs that regulate gene expression. These molecules are conserved across species (Hwang and Mendell, 2006; Sevignani et al., 2006). To control the translation of

the target mRNAs, miRNAs associate with the RNA-induced silencing complex (RISC) proteins and bind to the 3'UTR of mRNAs, thus serving as translational suppressors that regulate the protein synthesis (Hutvagner et al., 2002). Regulatory miRNAs impact cellular differentiation, proliferation, apoptosis and, possibly, even predisposition to cancer (Fabbri et al., 2007). Aberrant levels of miRNAs have been reported in a variety of human cancers (Volinia et al., 2006; Wiemer, 2007). Furthermore, it was recently suggested that small RNAs may be involved in the regulation of chromatin packaging (Bernstein and Allis, 2005; Grewal and Moazed, 2003; Kovalchuk and Baulch, 2007).

Radiation-induced epigenetic changes

Direct radiation exposure strongly influences epigenetic effectors. A variety of DNA damaging agents including ionizing radiation are known to affect genome stability by altering DNA methylation patterns (Kalinich et al., 1989; Minamoto et al., 1999; Tawa et al., 1998; Kovalchuk and Baulch, 2007). Most of the studies on the radiation-induced epigenetic changes are being conducted by the Kovalchuk laboratory. Radiation exposure has been shown to result in the hypermethylation of p16 promoter in a sex- and tissue-specific manner (Kovalchuk et al., 2004a). It was also noted that radiation exposure leads to the profound dose-dependent, and sex- and tissue-specific global DNA hypomethylation (Kovalchuk et al., 2004a; Pogribny et al., 2004a). DNA hypomethylation observed after radiation exposure has been shown to be related to DNA repair (Pogribny et al., 2004a). It was also correlated with the radiation-induced alterations in the expression of DNA methyltransferases, especially *de novo* methyltransferases DNMT3a and DNMT3b (Pogribny et al., 2004b). The radiation-induced global genome DNA hypomethylation appears to be linked to genome instability

in the radiation carcinogenesis target tissue (bone marrow, thymus or spleen) (Kovalchuk et al., 2004b; Pogribny et al., 2004b; Pogribny et al., 2004c). DNA methylation is closely connected with other components of chromatin structure (Kovalchuk and Baulch, 2007). Although much attention has been given to radiation-induced changes in DNA methylation, histones are key players as well, and have been largely overlooked. Amongst histone modifications, phosphorylation of histone H2AX (a variant histone H2A) upon radiation exposure is the most widely studied. Furthermore, analysis of histone H2AX phosphorylation is widely used to assess the extent of radiation damage to cellular DNA (Sedelnikova et al., 2002; Sedelnikova et al., 2003; Sedelnikova et al., 2004; Kovalchuk and Baulch, 2007).

In sum, from the existing literature we have learned that:

- Radiation exposure induces genome instability in the directly exposed cells and their progeny. It also destabilizes the unexposed neighboring naïve cells - the "bystander effect"
- Radiation exposure leads to significant and persistent epigenetic changes in the directly exposed tissue. The changes include alterations in the global DNA methylation, expression of methyltransferases and methyl-binding proteins, and alterations to histone methylation patterns in the exposed animals
- Parental irradiation poses a threat to the progeny and leads to transgenerational genome instability which manifests as increased mutation, chromosome aberration, and cancer incidence in the unexposed progeny

Epigenetic mechanisms may be involved in the molecular etiology of the aforementioned transgenerational phenomena, yet their exact roles have to be defined. Therefore, our aim was define the exact roles of epigenetic changes in the radiation-induced transgenerational effects.

PRESENT STUDY: OBJECTIVES AND HYPOTHESES

The main goal of the current thesis was to investigate whether or not parental exposure to IR influences the epigenetic parameters in the unexposed progeny (transgenerational epigenetic effects).

Guiding Hypothesis:

Based on the existing evidence **we hypothesize** that epigenetic reprogramming is a key phenomenon implicated in the molecular etiology of transgenerational radiation-induced genome instability. We hypothesize that the progeny of the exposed parents (receiving localized and whole body exposures) will exhibit altered epigenetic status in the key radiation target organs.

The following experiments were conducted to **achieve the goal and test the proposed hypotheses:**

Experiment 1: To Analyze the global epigenetic changes in the unexposed progeny of animals subjected to whole body irradiation.

Experiment 2: To Dissect the DNA methylation changes in the repetitive and transposable elements loci of the unexposed progeny of animals subjected to whole body irradiation.

Experiment 3: To Study the epigenetic changes in the progeny of animals subjected to localized cranial irradiation.

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CHAPTER 2

Epigenetic Dysregulation Underlies the Transgenerational Genome Instability in the Progeny of Exposed Parents

Abstract

While modern cancer radiation therapy has led to increased patient survival rates, the risk of radiation treatment-related complications is becoming a growing problem. Amongst various complications, radiation also poses a threat to the progeny of exposed parents. It causes transgenerational genome instability that is linked to transgenerational carcinogenesis. While the occurrence of transgenerational genome instability that manifests as elevated delayed and non-targeted mutation has been well documented, the mechanisms by which it arises remain obscure.

We hypothesized that epigenetic alterations may play a pivotal role in the molecular etiology of transgenerational genome instability. We thus studied the levels of cytosine DNA methylation in somatic tissues of unexposed offspring upon maternal, paternal or combined parental exposure. We show a significant loss of global cytosine DNA methylation in the thymus tissue of the offspring upon paternal exposure. The loss of DNA methylation was paralleled by a significant decrease in the levels of maintenance (DNMT1) and *de novo* methyltransferases DNMT3a and 3b and methyl-CpG-binding protein MeCP2. Along with profound changes in DNA methylation, we noted a significant accumulation of DNA strand breaks in thymus, which is a radiation carcinogenesis target organ. The observed changes were indicative of a profound epigenetic dysregulation in the offspring, which in turn could lead to genome destabilization and, possibly, serve as precursor for transgenerational carcinogenesis.

Introduction

One of the documented, yet not very well understood risk factors for cancer in children is preconception parental exposure to genotoxic stress, particularly to ionizing radiation (IR) (Anderson et al., 2004; Dubrova, 2003a; Nomura, 2003; Tomatis, 1994). Studies have shown that IR exposure has an indirect effect on genome stability, which is transmitted through the germline of exposed parents to their progeny leading to genome destabilization (Dubrova, 2003a; Dubrova, 2003b; Morgan, 2003a). The resulting transgenerational genome instability was suggested to be a driving force of the radiation-induced transgenerational carcinogenesis (Dubrova, 2003a; Morgan, 2003a; Morgan, 2003b; Nomura, 2003; Tomatis, 1994).

To date, a plethora of evidence has accumulated, and increased our knowledge as to the nature of transgenerational changes in the somatic tissues of the progeny of exposed parents, particularly exposed fathers (Barber et al., 2002; Dubrova, 2003a, 2003b; Dubrova et al., 2002; Morgan, 2003a, 2003b; Watson, 1991). Significant progress has been made in the analysis of the transgenerational changes in somatic mutation rates (Barber et al., 2002; Carls and Schiestel, 1999; Dubrova, 2003a; Morgan, 2003; Shiriashi et al., 2002; Dubrova et al., 2000; Watson, 1991). Incidence of cancers was shown to be significantly elevated in the progeny of irradiated male mice (Cheng et al., 2004; Dubrova, 2003b; Hoyes et al., 2001; Lord and Hoyes, 1999; Morgan, 2003). Furthermore, increased cancer predisposition was noted in the progeny of nuclear industry workers (Dubrova, 2003b; Egger et al., 2004; Watson, 1991; Gardner et al., 1990). Notwithstanding, the exact molecular mechanisms leading to radiation-induced transgenerational genome instability and carcinogenesis remain elusive. The occurrence

of genome instability and elevated mutation rates was attributed to some yet unknown mechanism, which was suggested to be of epigenetic nature.

In recent years the role of epigenetics in the etiology of diseases including cancer has been increasingly recognized. Epigenetic changes are meiotically and mitotically heritable alterations in gene expression that include DNA methylation, RNA-associated silencing and histone modification. Cytosine DNA methylation was the first epigenetic alteration identified and is the most widely studied epigenetic mechanism (Goll and Bestor, 2005; Hermann et al., 2004; Pogribny et al., 2005). It is crucially important for the normal development, cell proliferation, and proper maintenance of genome stability of a given organism (Goll and Bestor, 2005; Pogribny et al., 2005; Robertson and Wolffe, 2000).

Altered Global DNA methylation patterns are a well-known characteristic of cancer cells (Cheng et al., 2004; Gaudet et al., 2003; Robertson and Wolffe, 2000; Rountree et al., 2001; Shiraishi et al., 2002). DNA methylation profile of cancer cells is frequently characterized by the global genome hypomethylation as well as concurrent hypermethylation of the selected CpG islands within the gene promoters (Raiche et al., 2004). The global loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality identified in cancer cells (Feinberg, 2004; Gaudet et al., 2003; Jaenisch and Bird, 2003) and has been linked to the activation of transposable elements, elevated chromosome breakage, aneuploidy, increased mutation rates and the phenomenon of global genomic instability (Cheng et al., 2004; Gaudet et al., 2003; Pogribny et al., 2005; Robertson and Wolffe, 2000; Shiraishi et al., 2002; Sobol et al., 2003).

We hypothesized that epigenetic alterations may play a pivotal role underlying genome instability in the progeny of exposed parents. We thus studied the levels of DNA

methylation in spleen, thymus, and liver tissue of unexposed progeny upon either maternal or paternal exposure, or exposure to both parents.

Materials and Methods

Model:

In this study we utilized an *in vivo* murine model to analyze the role of epigenetic alterations in transgenerational radiation effects. The murine model is widely used, well characterized, and generally accepted for studies of radiation-induced changes (Boulton et al., 2002; Pogribny et al., 2005; Utsuyama and Hirokawa, 2003; Wakabayashi et al., 2003).

It has also been successfully applied to study transgenerational induction of genome instability (Barber et al., 2006a; Barber et al., 2006b; Barber et al., 2004, 2002; Carls and Schiestl, 1999; Yauk et al., 2002).

Handling and care of animals was in strict accordance with recommendations of the Canadian Council for Animal Care and Use (1993).

In this study, sexually mature male and female C57BL/6 mice were randomly assigned to unexposed or exposed groups. Exposed groups received 2.5 Gy of total body irradiation, 90kV, 5mA. Control groups were sham treated. Seven days after exposure four bredding pairs were randomly assigned to 4 mating groups. These were as follows: (*1*) animals with maternal exposure (exposed females mated to unexposed males); (*2*) animals with paternal exposure (unexposed females mated to exposed males); (*3*) animals with combined parental exposure; and (*4*) sham-treated animals serving as control group. No significant litter size differences were noted between the groups. The progeny were sacrificed at the age of 15 days.

DNA methylation analysis:

To monitor changes in DNA methylation, we employed the well-established, very sensitive, reliable and reproducible cytosine extension assay, which measures the proportion of unmethylated CCGG sites in genomic DNA (Pogribny et al., 1999; Fujiwara & Ito, 2002; Kovalchuk et al., 2004; Pogribny et al., 2004, 2005). The method is based on the usage of methylation-sensitive restriction endonuclease *Hpa*II. This enzyme has relatively frequent recognition sequences at CpG sites throughout the genome. The enzyme leaves a 5' guanine overhang after DNA cleavage that can be used for subsequent single nucleotide extension with labeled [^{3}H]-dCTP. The extent of [^{3}H]-dCTP incorporation opposite the exposed guanine is directly proportional to the number of cleaved and thus unmethylated CpG sites and inversely proportional to the levels of methylation. The higher the methylation, the lower the incorporation of [^{3}H]-dCTP (Pogribny et al.,, 1999; Fujiwara & Ito, 2002; Kovalchuk et al., 2004; Pogribny et al., 2004; Koturbash et al., 2005; Pogribny et al., 2005).

Genomic DNA was isolated from mouse spleen, thymus and liver tissue by using Qiagen DNAeasy^{TM} Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. In brief, 1.0 μg of genomic DNA was digested with 20 U of methylation-sensitive *Hpa*II restriction endonuclease (New England Biolabs, Beverly, MA) for 16-18 h at 37^{o}C. A second DNA aliquot (0.5 μg) of undigested DNA served as background control. The single nucleotide extension reaction was performed in a 25 μl reaction mixture containing 1.0 μg DNA, 1X PCR buffer II, 1.0 mM $MgCl_2$, 0.25 U AmpliTaq DNA polymerase, and 0.1 μl of [^{3}H]dCTP (57.4 Ci/mmol), and incubated at 56^{o}C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed three times with 0.5 M

Na-phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. The [^{3}H]dCTP incorporation into DNA was expressed as mean disintegrations per minute (dpm) per μg of DNA after subtraction of the dpm incorporation in undigested samples (background).

DNA strand break measurement:

The levels of radiation-induced damage were studied by accumulation of phosphorylated histone H2AX (γH2AX) foci. Studied tissues of control and experimental animals were touch-printed onto positively charged slides and processed for γH2AX immunohistochemistry using anti-γH2AX primary antibodies, as described (Sedelnikova et al., 2003; Pogribny et al., 2005). The γH2AX foci were be counted by eye in a blinded fashion by two independent investigators. At least 400 cells from each studied tissue of each animal were examined (Sedelnikova et al., 2003; Pogribny et al., 2005).

Western immunobloting:

Western immunoblotting for DNMT1, DNMT3a, DNMT3b, MeCP2, RAD51, KU70, POL B and PCNA was conducted using spleen, thymus, and liver tissue. Tissue samples were sonicated in 0.4-0.8 ml of ice-chilled 1% sodium dodecyl sulphate (SDS) and boiled for 10 min. Small aliquots (10 μl) of homogenate were reserved for protein determination using protein assay reagents from BioRad (Hercules, CA). Equal amounts of proteins (25 μg) were separated by SDS-polyacrylamide electrophoresis (PAGE) in slab gels of 8 or 12% polyacrylamide, made in triplicate, and transferred to PVDF membranes (Amersham, Baie d'Urfé, Québec). Membranes were incubated with antibodies

against DNMT1 (1: 1000, Abcam, Cambridge, MA), DNMT3a, DNMT3b (1:500, mouse monoclonals, Abgent, San Diego, CA), MeCP2 (1:1000, Abcam), RAD51, KU70 (1:1000, BD Biosciences), POLB (1:1000, Biomeda, Foster City, CA), and PCNA (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham) and the ECL Plus immunoblotting detection system (Amersham). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Unaltered PVDF membranes were stained with Coomassie Blue (BioRad, Hercules, CA) and the intensity of the Mr 50,000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software and normalized to the Mr 50,000 protein.

Statistical analysis:

The statistical procedures used are described by Sokal and Rohlf (1995). For the determination of the significance of the difference between the means the Student's t-test was used with post hoc Bonferroni correction for multiple comparisons. P values < 0.0125 were considered significant. Statistical treatment and plotting of the results were performed using the Excel for Windows XP, and JMP 5.0 software.

Results

Experimental animals, sexually mature C57BL/6 mice, were exposed to 2.5 Gy of X rays and mated 7 days after exposure. To test whether changes in genome DNA methylation were observed in the somatic tissue of the offspring, global cytosine methylation was measured in the spleen, thymus, and liver of the offspring of all four mating groups using a sensitive HpaII-based cytosine extension assay (Pogribny et al., 1999; Pogribny et al., 2005). We noted a DNA hypomethylation trend in the thymus tissue of the progeny upon paternal exposure, yet the trend was not statistically significant. On the other hand, combined parental exposure led to statistically significant (p= 0.001049) loss of DNA methylation in the thymus of offspring as compared with controls (Figure 2-1).

Having observed the loss of global genome methylation in thymus tissue of the progeny of exposed animals, we decided to elucidate the possible mechanism(s) of this phenomenon. The DNA methyltransferases DNMT1, DNMT3a, and DNMT3b are the three main functional enzymes that are responsible for setting and maintaining DNA methylation patterns in mammalian cells (Goll and Bestor, 2005; Hermann et al., 2004). We evaluated the effects of parental exposure on the expression of DNMT1, DNMT3a, and DNMT3b in thymus, spleen, and liver tissue of the progeny (Figure 2-2). DNMT1, DNMT3a, and DNMT3b expression was decreased in the progeny of exposed males or those progeny with combined parental exposure. In contrast to the thymus tissue, no DNMT expression changes were seen in either spleen or liver tissues.

The decrease in global cytosine DNA methylation, seen in thymus of the progeny upon combined parental irradiation, was paralleled by a decrease in the level of

methylbinding protein MeCP2, a transcriptional repressor that selectively recognizes methylated DNA and that plays a central role in chromatin remodeling (Robertson and Wolffe, 2000) (Figure 2-2). MeCP2 levels were also decreased in paternal exposure group.

Transgenerational genome instability effects in mammals have manifested as increased chromosomal aberrations, minisatellite instability, and homologous recombination (Carls and Schiestel, 1999; Dubrova, 2003a; Morgan, 2003a), all of which can be induced by DNA double strand breaks (DSBs). We thereafter decided to measure the levels of DSBs in the offspring of exposed parents. As a measure of DSBs we studied the presence of phosporylated S139 (γH2AX). γH2AX is one of the earliest cellular responses to DSBs and is a well-accepted marker for DSB analysis (Sedelnikova et al., 2003).

We observed a significant ~2-fold elevation in the levels of γH2AX foci in the thymus tissue of the progeny of exposed males (p= 0.00369) and in both exposed parents (p= 1.57902E-07) as compared with unexposed animals (Figure 2-3). Accumulation of γH2AX foci in this study was not linked to changes in cellular proliferation as parental exposure did not lead to any changes in PCNA expression (Figure 2-4). Interestingly, recent studies suggested that accumulation of γH2AX is an important epigenetic alteration and may be an early sign of predisposition to carcinogenesis (Gorgoulis et al., 2005). The main function of γH2AX in the cell, however, is to serve as a DNA damage indicator and a recruiter of DNA repair proteins. Accumulation of γH2AX may be indicative of persistence of unrepaired DNA breaks, possibly because of ineffective repair of background DNA damage levels.

We next asked if DNA repair mechanisms responsible for maintenance of DNA strand breaks are affected. Mammalian cells employ homologous recombination (HR) and nonhomologous end joining (NHEJ) as major pathways to repair DSBs (Hoeijmakers, 2001). We thus assayed the levels of HR (Rad51) or NHEJ (Ku70) related proteins (Hoeijmakers, 2001) in the somatic tissues of the progeny of exposed parents. No changes were noted in the expression of Ku70. The levels of Rad51 were downregulated in the thymus tissue of progeny upon paternal and combined parental exposure (Figure 2-4). Thus, persistent DNA strand breaks could be a result of compromised HR repair that was ineffective because of suppressed Rad51 levels.

The elevated levels of DSBs may result from corrupt processing of the internal oxidative damage. Base oxidation is usually fixed via a base excision repair (BER) mechanism. BER intermediates, abasic sites, and single strand breaks can be converted into DSBs. PolB plays a pivotal role in BER mechanism, filling a nucleotide into a BER-generated abasic site (Hoeijmakers, 2001; Sobol et al., 2003). We assayed the levels of PolB and noted a downregulation in the thymus tissue of the unexposed offspring upon paternal and combined parental exposure (Figure 2-4). As before, the decreased levels of PolB were noted only in thymus tissue, correlating with the elevated levels of DSBs and with DNA hypomethylation.

Importantly, the levels of proliferating cells nuclear antigen (PCNA) remained unchanged in spleen, thymus and liver of the progeny of exposed parents (Figure 2-4). Therefore, the aforementioned changes in DNA methylation and protein expression were not due to increased cellular proliferation.

No sex-related differences in the genetic and epigenetic changes were noted between the male and female offspring of exposed parents (data not shown).

Discussion

In the present study, we demonstrated that pre-conception paternal and combined parental whole-body radiation exposure led to major epigenetic changes in the thymus tissue of the unexposed progeny. Our findings revealed significant changes in DNA methylation levels, expression of DNA methyltransferases and methyl-binding proteins as well as accumulation of unrepaired DNA damage and altered expression of DNA repair enzymes.

The main findings of the present study are: (i) parental exposure is a potent inducer of DNA damage and epigenetic changes in thymus tissue of the unexposed progeny; (ii) specifically, exposure regimes where the male parents were pre-conceptionally irradiated resulted in a strong and significant genome hypomethylation, which was paralleled by decreased expression of DNMT1, 3a and 3b as well as a pronounced reduction in the expression of methyl-binding protein MeCP2 in the thymus tissue of the progeny; (iii) male parent preconception exposure led to the accumulation and persistence of DNA damage as monitored by γH2AX foci accumulation; (iv) all the aforementioned changes were specific solely to the radiation-induced transgeneration carcinogenesis target organ, thymus.

Most importantly, we have proven that parental radiation exposure led to very pronounced and significant loss of global DNA methylation, paralleled by the decreased expression of DNA methyltransferases and methyl-binding proteins. This is an extremely important finding, which in turn may help to explain the genome instability in the

progeny of exposed parents, which was reported to manifest as elevated levels of chromosome aberrations and satellite instability (Barber et al, 2002; Dubrova, 2003a; Dubrova 2003b; Dubrova et al., 2002; Dubrova et al., 2000; Morgan 2003a; Morgan 2003b).

Mammalian genomes heavily depend upon properly set patterns of methylated cytosines for their function (Rollins et al., 2006). A recent study revealed high levels of DNA methylation at repetitive satellite DNA sequences, retro- and DNA transposons (Rollins et al., 2006). It was also shown that altered levels of DNMT1, DNMT3a, or DNMT3b significantly reduced DNA methylation levels in the previously mentioned repetitive "parasite" sequence elements and genome instability (Martens et al., 2005).

Down regulation of methyl-CpG binding protein MeCP2 was shown to predispose the cells to elevated levels of mutation and retrotransposon reactivation and to result in genome instability, leading to carcinogenesis (Yu et al., 2001).

In the light of these findings we can speculate that global loss of DNA methylation preferentially occurs in the retrotransposons and satellite DNA, thus underlying the genome instability. Importantly, such a speculation allows us to explain the fact that transgeneration genome instability in the progeny of exposed parents was reported to manifest specifically as satellite DNA instability (Barber et al., 2002; Dubrova, 2003a; Dubrova 2003b; Dubrova et al., 2002; Dubrova et al., 2000; Morgan 2003a; Morgan, 2003b). Yet, the exact nature of the DNA sequences affected by DNA methylation loss in the progeny has to be studied in the future. The changes were observed in radiation carcinogenesis of a target organ, the thymus, factors that predispose individuals to a high risk of cancer and were linked to male parent exposure. The paternal

genome is extremely sensitive to genotoxic agent exposure (Anway et al., 2005; Barber and Dubrova, 2006; Dubrova, 2003a; Morgan, 2003a). Our data agree with the previous reports on the induction of transgenerational genome instability upon paternal exposure (Barber and Dubrova; 2006 Dubrova, 2003a; Morgan, 2003a) and on paternal chemical exposure–induced transgenerational alterations in DNA methylation (Anway et al., 2005). Mammalian genomes undergo marked demethylation after fertilization to establish correct parent-of-origin developmental programs (Anway et al., 2005; Barber and Dubrova, 2006). Paternal genome demethylation is much more pronounced than maternal (Anway et al., 2005, Mayer et al., 2000). Radiation-induced damage to sperm cells, which is usually repaired in the first hours after fertilization, may thus affect the fertilized egg and interfere with epigenetic reprogramming (Barber and Dubrova, 2006). The repair events may be accompanied by suppression of DNA synthesis, alterations in DNA repair gene expression, DNA methylation, and histone modifications (Barber and Dubrova, 2006). These alterations in the fertilized egg may result in subsequent changes in the embryo. Both parents' exposure may result in even more dramatic changes in the fertilized egg because of the damage to the egg cell DNA and cytoplasmic components. The mechanisms of apparent synergistic effects of combined parental exposure still have to be determined. Notably, the studied progeny were conceived 7 days after parental exposure. The results thus reveal the deleterious changes in the mature sperm cells. The effect of IR on spermatogonia and further epigenetic alterations in the progeny, still have to be analyzed.

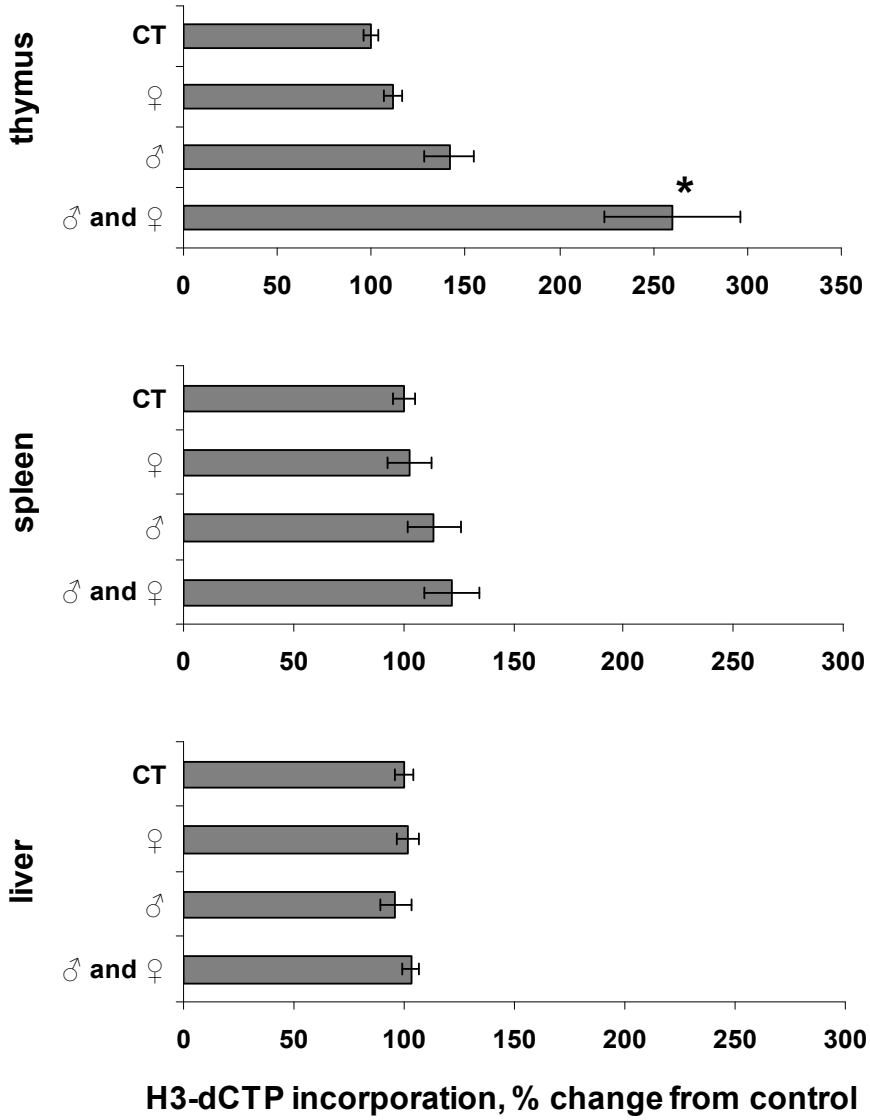

Figure 2-1. Parental exposure leads to global genome hypomethylation in thymus of the offspring. Levels of global genome DNA methylation. The extent of [3H]dCTP incorporation is inversely proportional to the levels of methylation. Mean values ± SEM. Significant differences from the control animals are shown: * $p < 0.0125$ according to post-hoc Bonferroni correction. Ct – progeny of the control cohort; ♀ - progeny of the maternal exposure group; ♂ - progeny of the paternal exposure group; ♂+♀ - progeny of the combined parental exposure group (n=20 for all groups).

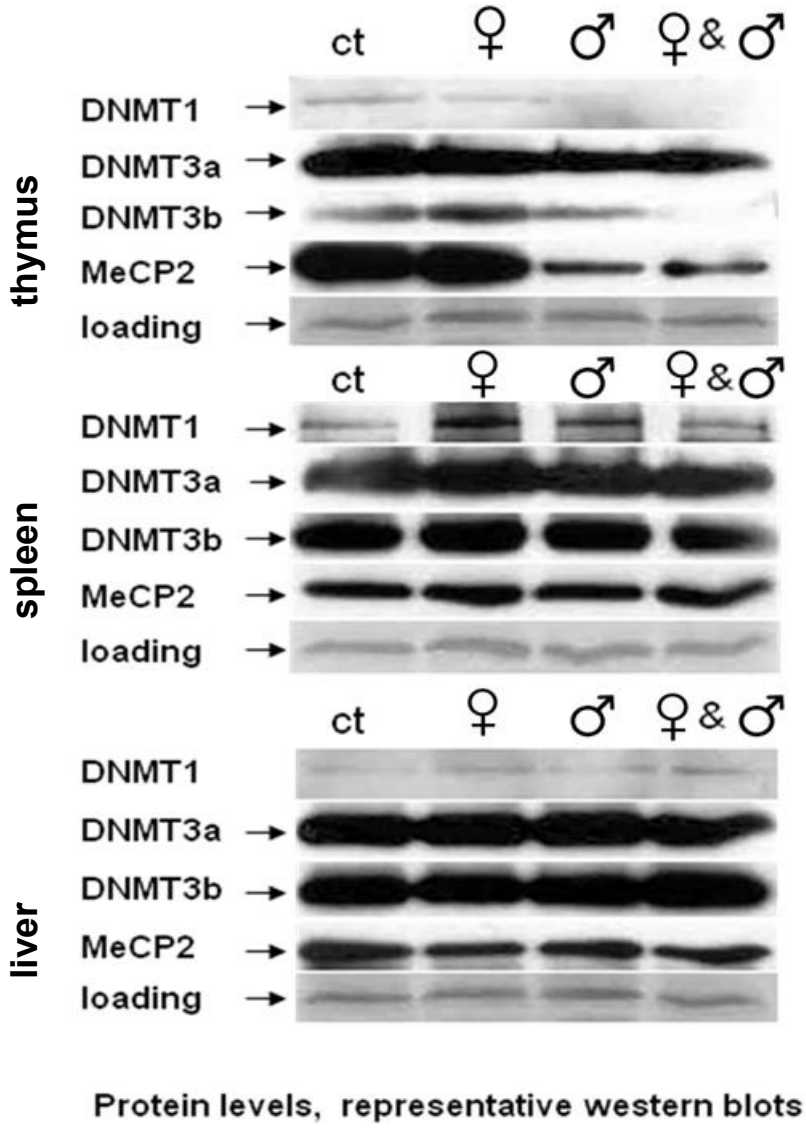

Figure 2-2. Parental exposure profoundly alters the expression of DNA (cytosine-5) methyltransferases and methyl-binding proteins in thymus of the offspring. Representative western blots. Each experiment included pooled lysates from 5 animals for each cohort, with equal representation of each animal. Western blots were repeated at least 4 times to ensure the reproducibility and robustness of the results. Ct – progeny of the control cohort; ♀ - progeny of the maternal exposure group; ♂ - progeny of the paternal exposure group; ♂+♀ - progeny of the combined parental exposure group.

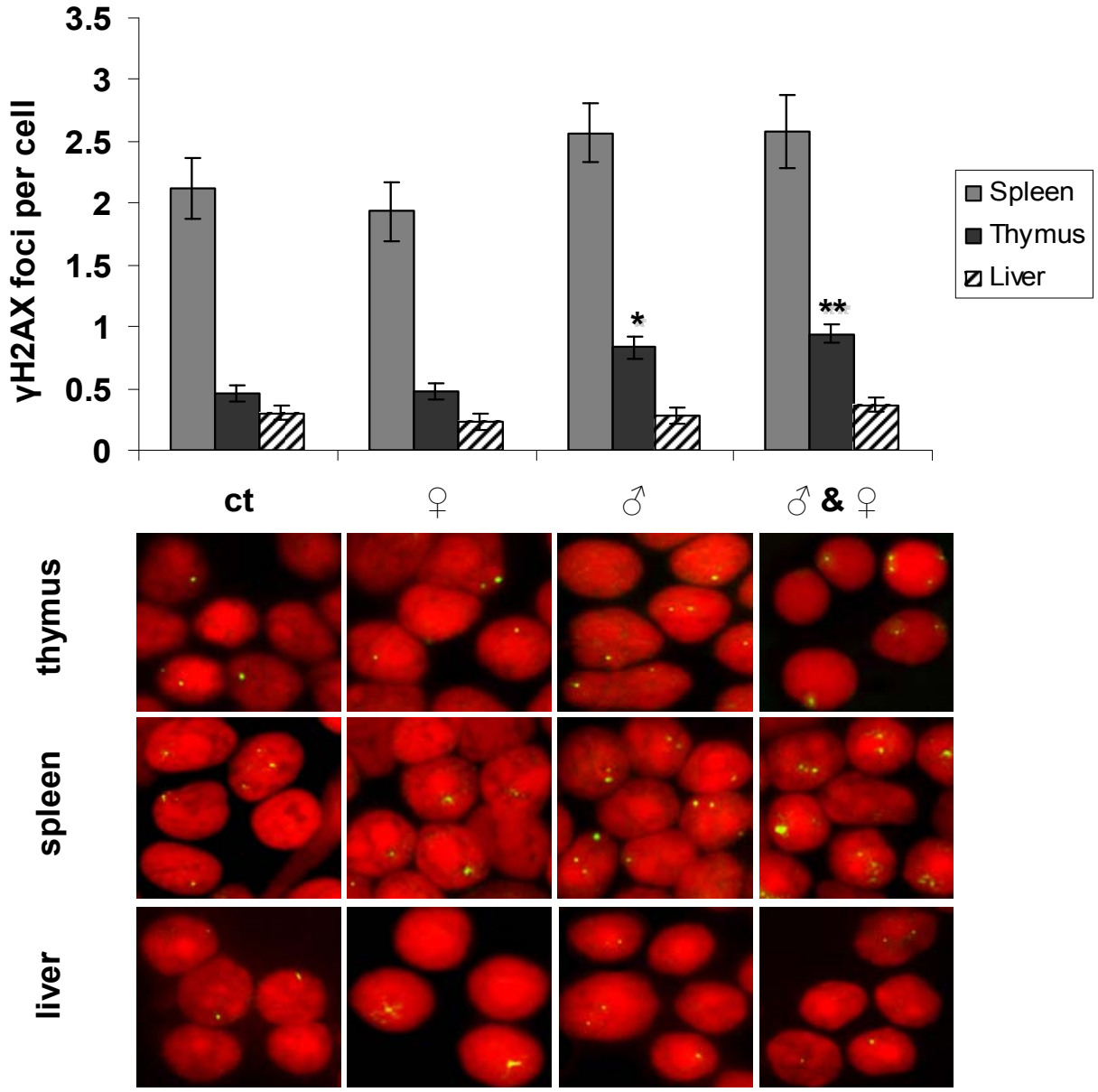

Figure 2-3. Significant accumulation of γH2AX foci in thymus of the progeny of exposed parents. Mean values± SEM. Significant differences from the control animals are shown: * $p < 0.0125$, ** $p<0.0001$ according to post-hoc Bonferroni correction (n=400).

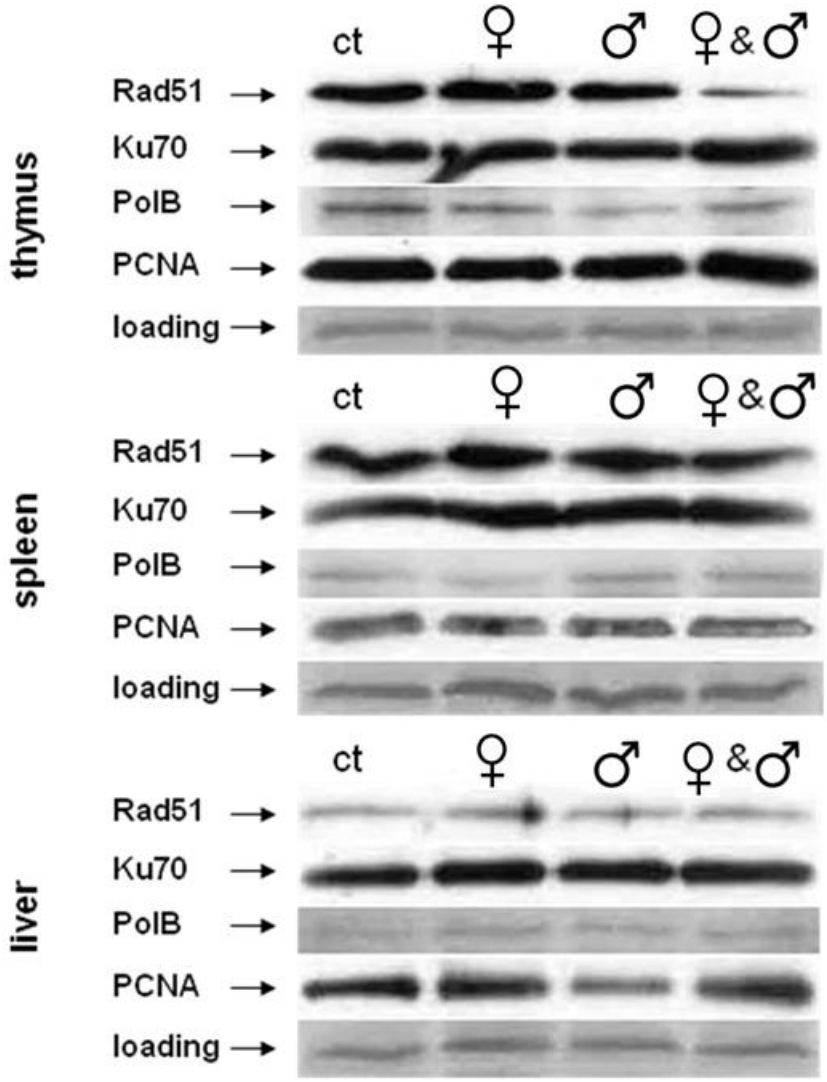

Figure 2-4. Altered expression of DNA repair proteins in the progeny of exposed parents. Representative western blots from 4 independent experiments. Each experiment included pooled lysates from 5 animals for each cohort, with equal representation of each animal. Western blots were repeated at least 4 times to ensure the reproducibility and robustness of the results. Ct – progeny of the control cohort; ♀ - progeny of the maternal exposure group; ♂ - progeny of the paternal exposure group; ♂+♀ - progeny of the combined parental exposure group.

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CHAPTER 3

Dissecting the locus-specificity of the DNA methylation changes in the progeny of exposed parents

Abstract

It is well known that exposure to ionizing radiation can lead to epigenetic dysregulation, specifically a global loss of DNA methylation. We have previously shown that paternal IR exposure results in a global hypomethylation in the thymus tissue of unexposed offspring. Yet, the precise location of the observed DNA hypomethylation in the tissues of the unexposed progeny of irradiated parents remains unknown.

In mammals, the majority of DNA methylation occurs on repetitive elements. In an effort to determine the location of DNA hypomethylation in the unexposed progeny of the IR exposed parents, we investigated the methylation levels of repetitive elements such as ribosomal DNA (rDNA), intracisternal A type particle-long terminal repeat (IAP-LTR), and β-tubulin loci.

We analyzed the rDNA, IAP-LTR and β-tubulin methylation patterns in spleen, thymus and liver tissue of the progeny following paternal, maternal and combined parental exposure. Using a modified McrBC-PCR approach, we found that parental IR exposure did not significantly alter the methylation levels of the aforementioned repetitive elements in the offspring. We therefore concluded that the analyzed repetitive sequences may be protected from DNA hypomethylation, as their activation may have a dramatic genome destabilization potential. Further studies should investigate the methylation levels of other repetitive elements.

Introduction

The mammalian genome depends on patterns of methylated cytosines for normal function, but the relationship between genomic methylation patterns and the underlying sequence is unclear (Rollins et al., 2006). Recent studies in mice and humans proved that loss of DNA methylation results in the reactivation of retrotransposons and genome destabilization in both germ and somatic cells (Bourc'his and Bestor, 2004; Jaenisch and Bird, 2003; Kazazian, 2004). The genome sequencing of several model organisms revealed that mammals have a highly complex genome organization, largely resulting from the accumulation of a variety of repetitive elements and non-coding sequences (Lander et al, 2001; Waterston et al, 2002). In mice, repetitive and non-coding DNA elements account for 44 and 52% of the genomic DNA content, respectively (Martens et al., 2005).

For a long time the non-specific repetitive sequences and transposons have been thought of as putative epigenetic elements that can modulate gene expression and impact the chromatin structure and organization (Martens et al., 2005). Furthermore, transposable and repetitive elements pose an inherent burden to genome stability (Kazazian, 2004). The organisms have thereafter developed elaborate ways to immobilize and inactivate the transposable and repetitive elements, thus securing the genome integrity. One such crucial immobilization control mechanism is DNA methylation (Jaenisch and Bird, 2003; Bourc'his and Bestor, 2004). A recent study by Martens et al. (2005) analyzed the distribution of DNA methylation over the mouse genome. The analysis revealed high levels of DNA methylation at IAP-LTRs, moderate levels at major

and minor satellite DNA repeats and LINEs, and low levels at SINEs and DNA transposons.

It was also proven that altered levels of DNA methyltransferases significantly reduced DNA methylation levels in the repetitive "parasite" sequence elements, thus leading to their reactivation, and genome destabilization (Martens et al., 2005).

We have previously shown that radiation exposure results in the global loss of DNA methylation in the progeny of exposed parents. We speculated that global loss of DNA methylation preferentially occurs in the retrotransposons and satellite DNA, thus underlying the genome instability. We therefore checked the methylation status of the repetitive elements usually methylated in the mouse genome. These were rDNA, IAP-LTR, and tubulin loci.

Materials and Methods

Model:

We used the same animal model as described in Chapter 2. In brief, sexually mature male and female C57BL/6 mice were randomly assigned to unexposed or exposed groups. Exposed groups received 2.5 Gy of total body irradiation, 90kV, 5mA. Control groups were sham treated. Seven days after exposure the animals were randomly assigned to 4 mating groups, 4 breeding pairs per mating group. These were as follows: (*1*) animals with maternal exposure (exposed females mated to unexposed males); (*2*) animals with paternal exposure (unexposed females mated to exposed males); (*3*) animals with combined parental exposure; and (*4*) sham-treated animals serving as control group.

No significant litter size differences were noted between the groups. The progeny were sacrificed at the age of 15 days.

DNA extraction:

Genomic DNA was isolated from mouse spleen, thymus and liver tissue by using a Qiagen DNAeasy^{TM} Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

DNA methylation analysis of repetitive elements by the McrBC-PCR assay:

As recommended by Martens et al. (2005), genomic DNA (1ug) was digested with 20U of the restriction enzyme McrBC (New England Biolabs, Beverly, MA) which selectively digests methylated DNA and will not act on unmethylated DNA (Raleigh, 1992; Sutherland et al., 1992). Undigested DNA served as a control. McrBC digestion of methylated DNA prevents PCR amplification and conversely, unmethylated cytosines prevent digestion which can be detected by PCR amplification (Pogribny et al., 2006; Nakayashiki et al., 2004).

Digested DNA was amplified using 0.2ug (0.1ug for rDNA) of template DNA, 20 pmol primers (Table 1.), 10 mM dNTPs, and Phusion ^{TM} Taq Polymerase (Finnzymes) as per manufacturer's instructions in 50 μL of 1x reaction buffer. All reactions were heated to 98^{o}C for 2.5 min, followed by 30 cycles (27 for rDNA) of 30 sec at 98^{o}C, 30 sec at the annealing temperature (Table 1), and 30 sec at 72^{o}C. A final extension of 10 min at 72^{o}C was followed by a hold at 10^{o}C. The PCR products were run on 2% agarose gels (EMD chemicals, Gibbstown NJ), stained with ethidium bromide, photographed, and the band intensity was analyzed by analysed with NIH Image J software version 1.34s. The results

are presented as ratio of PCR product recovery after digestion of DNA with McrBC to undigested DNA.

Statistical analysis:

The statistical procedures are described by Sokal and Rohlf (1995). For the determination of the significance of the difference between the means the Student's t-test was used with post hoc Bonferroni correction for multiple comparisons. P values < 0.0167 were considered significant. Statistical treatment and plotting of the results were performed using the Excel for Windows XP, and JMP 5.0 software.

Results

In several previous studies using a well established mouse model we have seen a pronounced loss of DNA methylation in the somatic tissues of unexposed progeny. Yet, the nature of the loci affected remains to be discerned (Koturbash et al., 2007). With this in mind, we set out to determine the precise nature of epigenetic DNA methylation changes induced by parental radiation exposure in the somatic tissues of unexposed progeny.

A novel approach, modified from McrPCR (Rabinowicz et al., 2003) and McrBC-msAP-PCR (Tryndyak et al., 2006), was used to determine the methylation status of CpG islands of two repetitive domains and one gene locus, those being IAP-LTR elements, rDNA and β-tubulin, respectively.

The primers used in this study (see Table 1), as designed by Martens and colleagues (2005), generate fragments from within the intergenic spacer of the rDNA

repeat, the LTR of the IAP, and coding sequence of the β-tubulin gene. This approach cannot differentiate between different chromosomes, nor can it discriminate between single copies of a given repeat (Martens et al., 2005). It does, however, offer an estimation of the average change in the methylation status of these elements. Furthermore, since the two half-sites recognized by McrBC can be separated by up to 3 kb, with an optimal separation of 55-103 bp (Stewart and Raleigh, 1998), the methylation status of nearby CpG islands could also influence digestion of the target sequence.

Band intensities of McrBC-digested DNA were compared to undigested DNA following PCR amplification. The level of methylation in the tissues of the progeny of exposed parents was compared to those of unexposed parents. Methylation levels of the repetitive elements did not significantly change due to parental IR exposure (Figures 3-1 to 3-3).

Discussion

In the present study, we found that the DNA methylation levels of repetitive elements IAP-LTR, β-tubulin and rDNA did not significantly change in the progeny of IR exposed parents. These results were contrary to our original hypothesis that DNA methylation levels of repetitive elements would decrease within the progeny of IR exposed parents. Our hypothesis was based on the results of a recent study by Martens et al. (2005) which found that altered levels of DNMT1, DNMT3a or 3b significantly reduced DNA methylation levels in the aforementioned repetitive "parasite" sequence elements (Martens et al., 2005). Furthermore, in our case, the methyltransferase expression was reduced leading to the global DNA methylation loss (see chapter 2 and

Koturbash et al., 2006). Yet, in the current experiment the methylation levels of the repetitive elements remained relatively unchanged.

There may be several explanations for this phenomenon. Firstly, this approach does not discriminate between the different chromosomes, thus there may be an increase of DNA methylation in one genome area and a decrease in the other one. Alternatively, the repetitive sequences may be protected from the DNA methylation loss, as their destabilization may have a dramatic genome destabilization potential. Repetitive DNA elements such as major and minor satellites are additional elements which also have a high amount of methylation (Martens et al., 2005). Future analysis of the methylation levels of these elements may provide insight as to where the hypomethylation is occurring.

Table 1. **Primer sequences and PCR conditions for the McrBC-PCR assay**

Gene	Primer Sequence	Tm*	Amplicon (bp)	Sequence Reference
rDNA	Forward: CCTGTGAATTCTCTGAACTC Reverse: CCTAAACTGCTGACAGGGTG	59^{o}C	198	Martens et al., 2005
IAP-LTR	Forward: TTGATAGTTGTGTTTTAAGTGGTAAATAAA Reverse: AAAACACCACAAACCAAAATCTTCTAC	60.5^{o}C	258	Lane et al., 2003; Martens et al., 2005
β-tubulin	Forward: GACAGAGGCAAACTGAGCACC Reverse: CAACGTCAAGACGGCCGTGTG	64.5^{o}C	395	Martens et al., 2005

* Annealing Temperature

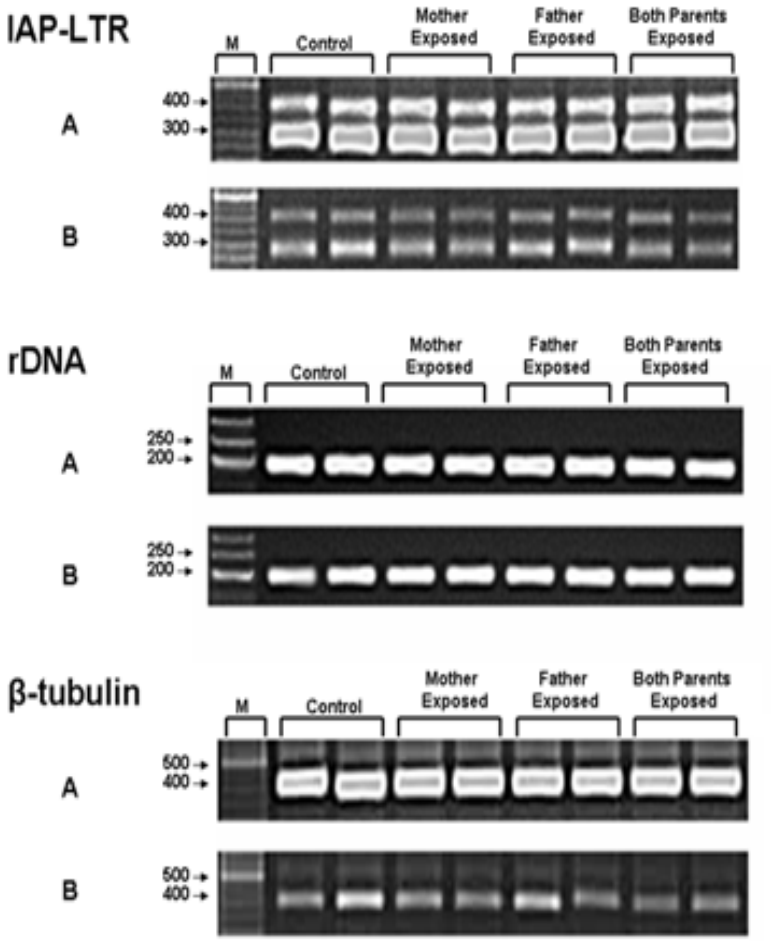

Figure 3-1. Methylation status of IAP-LTR, rDNA and β-tubulin from liver tissue of progeny from irradiated and non-irradiated parents.
Levels of repetitive element methylation were determined by a McrBC-PCR assay (see Materials and Methods). (A) total DNA (B) unmethylated DNA. Each band consists of pooled lysates from 3 animals with equal representation of each animal.

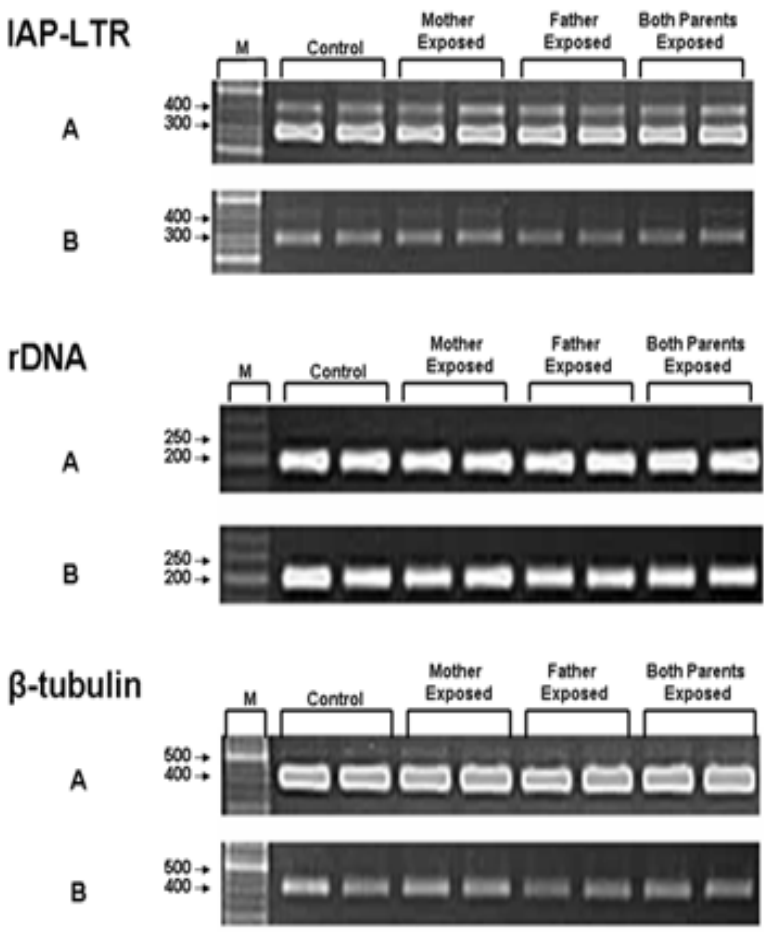

Figure 3-2. Methylation status of IAP-LTR, rDNA and β-tubulin from spleen tissue of progeny from irradiated and non-irradiated parents.
Levels of repetitive element methylation were determined by a McrBC-PCR assay (see Materials and Methods). (A) total DNA (B) unmethylated DNA. Each band consists of pooled lysates from 3 animals with equal representation of each animal.

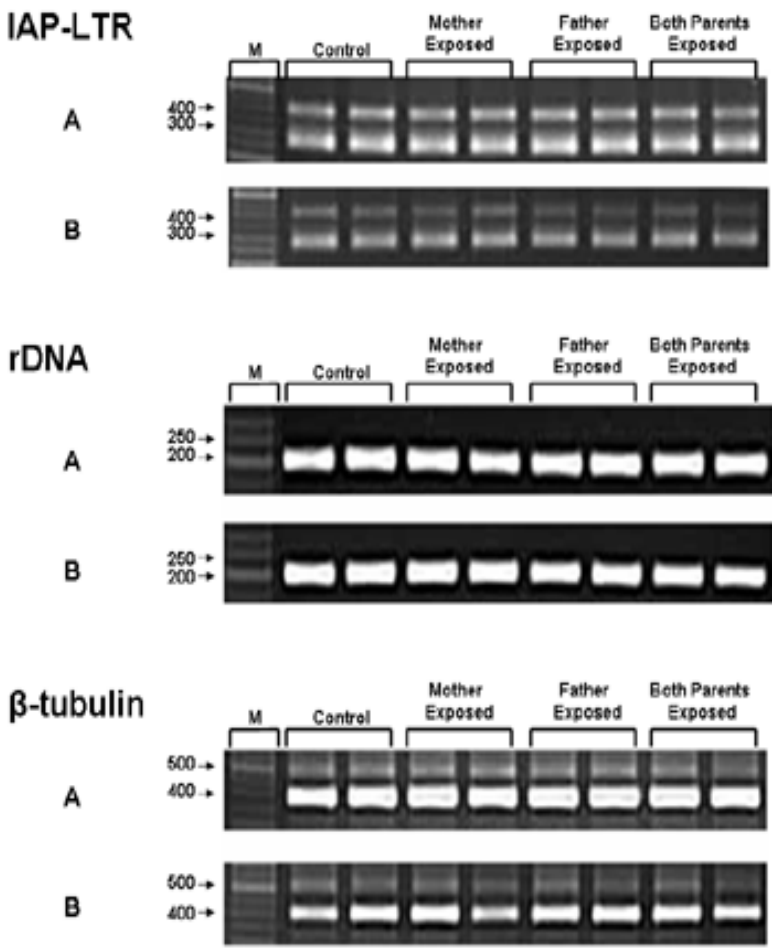

Figure 3-3. Methylation status of IAP-LTR, rDNA and β-tubulin from thymus tissue of progeny from irradiated and non-irradiated parents.
Levels of repetitive element methylation were determined by a McrBC-PCR assay (see Materials and Methods). (A) total DNA (B) unmethylated DNA. Each band consists of pooled lysates from 3 animals with equal representation of each animal.

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CHAPTER 4

Cranial Parental Radiation Exposure Leads to Epigenetic Alterations in the Offspring

Abstract

The majority of human radiation exposures (either therapeutic, diagnostic, or even accidental) are localized and focused. Although we have previously shown that parental whole body irradiation causes epigenetic dysregulation in the offspring, nothing is known on the effects of the localized, radiotherapy-like exposure.

Based on the results of the *in vivo* radiation induced bystander effect studies, one may predict that a localized body part exposure may affect the gonads through a radiation bystander response and thus affect the offspring in a transgenerational manner.

To simulate this scenario, male rats were subjected to the cranial irradiation while the rest of the body was protected by a lead shield. A week after exposure the animals were mated with non-exposed females and the progeny were obtained, analyzed and compared to the progeny of control unexposed rats. Analysis of global cytosine DNA methylation levels revealed significant DNA hypomethylation in the thymus, bone marrow, and spleen tissue of the progeny upon localized paternal exposure. The loss of global DNA methylation was paralleled by a tissue dependent decrease in the expression levels of *de novo* methyltransferases DNMT3a and 3b and methyl-CpG-binding protein MeCP2. Analysis of the repetitive element methylation status yielded varied, tissue dependent results. We concluded that localized, paternal irradiation fosters significant epigenetic dysregulation within the offspring.

Introduction

Currently it is well accepted that the pre-conception whole-body radiation exposure poses a significant threat to the progeny of irradiated parents (Mohr et al., 1999; Nomura et al., 2004; Dasenbrock et al., 2005; Aitken and De Iuliis, 2007).

The whole body exposures cause transgenerational genome instability which manifests as increased levels of mutation, elevated frequencies of chromosome aberrations, micronuclei formation and many other effects (as reviewed by Nomura, 2003; Nomura et al., 2004; Barber and Dubrova, 2006). The radiation-induced genome instability in the progeny of the whole-body exposed parents is linked to epigenetic dysregulation (Baulch and Raabe, 2005; Dubrova et al., 2000; Dubrova and Plum, 2002; Jirtle and Skinner, 2007; Morgan 2003a; Morgan 2003b; Vance et al., 2002; Wiley et al., 1997), specifically DNA methylation changes (Koturbash et al., 2006; Zhu et al., 2006).

DNA methylation is an epigenetic process which maintains genome stability and promotes normal development and proliferation (Okano et al., 1999; Pogribny et al., 2004; Raiche et al., 2004; Robertson and Wolffe, 2000). The global loss of cytosine methylation has been linked to the activation of transposons (Hata and Sakaki, 1997), increased mutation rates (Chen et al., 1998) and cancer (Esteller, 2003; Gaudet et al., 2003; reviewed by Ehrlich, 2002 and 2006).

Using a murine model, we have previously shown (Chapter 2) transgenerational changes in the progeny of parents who received whole body exposure to radiation. In addition to increased DSBs and a decrease in DNA repair proteins, we found significant epigenetic dysregulation (DNA hypomethylation) and decreased methyltransferase expression in a radiation target organ – thymus (Koturbash et al., 2006).

The majority of studies on the transgeneration radiation effects have analyzed the consequences of the parental whole body irradiation; however, such exposure types are relatively rare. In contrast, the localized body-part exposures occur very frequently during radiation diagnostics and therapy. One third of people alive today are likely to get cancer, and more than half will receive some form of radiotherapeutic treatment (American Cancer Society, 2003).

Amongst the radiotherapy procedures, brain irradiation is among the most widespread, since brain tumors are the second most common cancers in the young adults of reproductive age (Lyons and Vora, 2007). The recent advances in radiation therapy have led to the increased treatment success and patient survival rates. Yet, the potential of the localized parental exposures to affect the germline and thus induce genome instability and deleterious changes in the progeny needs to be addressed. This is especially important since the exposed cells may communicate the damage to the distant unexposed cells and tissues resulting in genome instability (Morgan, 2003a; Morgan, 2003b). This phenomenon is widely known as a bystander effect and is very well studied *in vitro* using cell culture models (Mothersill and Seymour, 2004). Recent studies have shown that bystander effects also operate *in vivo*, and that body part or cranial irradiation may exert deleterious changes in the distant protected somatic tissues (Koturbash et al., 2006; Koturbash et al., 2007).

To investigate if paternal cranial irradiation can exert deleterious changes in the protected germline, we studied the transgenerational changes in the progeny of male rats subjected to cranial exposure.

Materials and Methods

Model:

Rats (5 months old male Long Evans animals) were randomly assigned to different treatment groups. Handling and care of animals were in accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures were approved by the University of Lethbridge Animal Welfare Committee. Animals were housed in a virus-free facility and given food and water *ad libitum*.

The cranial-exposed cohort (4 animals) received 20 Gy (5cGy/s) of X-rays (90kV, 5mA) to the hippocampal area of the skull, applied as two doses of 10Gy in two consecutive days. The rest of the animal body was protected by a ~3 mm thick lead shield, the same type as used for the human body protection in diagnostic radiology. The protection of shielded 'bystander' tissue was complete, as verified by careful dosimetry using the RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, Carle Place, NY). Control rats (4 animals) were sham treated. For the sham treatment the animals were placed into the irradiator machine and completely shielded by lead. No radiation leakage through the shield occurred, as verified by dosimetry using the RAD-CHECK™ monitor.

To analyze the effects of cranial exposure on the progeny, 7 days after irradiation control and head-exposed animals were mated with unexposed females. The control group mating resulted in the production of 52 pups. The exposed male mating produced 56 pups. Both sets of progeny were sacrificed 15 days after birth. The bone marrow, thymus, spleen and liver tissues were extracted, immediately frozen and stored at -80°C until the analysis.

DNA extraction:

DNA was extracted from bone marrow, thymus, spleen and liver tissues using a Qiagen DNAeasy kit (Qiagen), according to the manufacturer's instructions.

Global DNA methylation analysis:

To monitor changes in DNA methylation, we employed the well-established, very sensitive, reliable and reproducible cytosine extension assay, which measures the proportion of unmethylated CCGG sites in genomic DNA (Pogribny et al., 1999; Fujiwara & Ito, 2002; Kovalchuk et al., 2004; Pogribny et al., 2004; Pogribny et al., 2005). In brief, 0.5 μg of genomic DNA was digested with 20 U of methylation-sensitive *Hpa*II restriction endonuclease (New England Biolabs, Beverly, MA) for 16-18 h at 37^{o}C. A second DNA aliquot (0.5 μg) of undigested DNA served as background control. The single nucleotide extension reaction was performed in a 25 μl reaction mixture containing 1.0 μg DNA, 1X PCR buffer II, 1.0 mM $MgCl_2$, 0.25 U AmpliTaq DNA polymerase, and 0.1 μl of [^{3}H]dCTP (57.4 Ci/mmol), and incubated at 56^{o}C for 1 h. Samples were applied to DE-81 ion-exchange filters and washed three times with 0.5 M Na-phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for scintillation counting. The [^{3}H]dCTP incorporation into DNA was measured by scintillation counting as mean disintegrations per minute (dpm). Background label incorporation was subtracted from enzyme-digested samples, and results were expressed as a relative [^{3}H]dCTP incorporation/1 μg of DNA and as a percent change from control.

DNA methylation analysis of long interspersed nucleotide elements (LINE1) by the COBRA assay:

The methylation status of LINE1 in bone marrow, thymus, spleen and liver tissues of progeny was measured with the combined bisulfite restriction analysis (COBRA) assay. The COBRA assay is based on a bisulfite conversion of unmethylated cytosine to uracil, PCR amplification and subsequent digestion of the PCR product with RsaI and BstUI restriction endonucleases (Xiong and Laird, 1997 and Pogribny et al., 2006). Cleavage of PCR product with RsaI indicates sites of unmethylated cytosines and BstUI digestion shows sites of methylated cytosines (Pogribny et al., 2006). Briefly, 2ug of genomic DNA was modified with a bisulfite conversion as previously described (Xiong and Laird, 1997). Following bisulfite conversion, the DNA was de-salted with a Wizard DNA Purification Kit (Promega Corp. Madison WI). Once the bisulfite was removed, the converted DNA was neutralized and further purified with ethanol precipitation. The bisulfite-modified DNA was PCR-amplified with Takara Ex Taq (Fisher Scientific, Ottawa, ON) and primers corresponding to the regulatory region of rat LINE1 sequence. The sense primer; 5'-TTGGTGAGTTTGGGATA-3' and the antisense primer; 5'-CTCAAAAATACCCACCTAAC-3' were used to amplify the rat Line 1 regulatory region (Pogribny et al., 2006). The PCR amplification consisted of an initial denaturation of 10min at 95^{o}C, followed by 40 cycles of denaturation for 30s at 94^{o}C, annealing for 30s at 56^{o}C and extension for 30s at 72^{o}C. The PCR products were each digested with 20 units of BstUI or RsaI endonuclease (New England BioLabs, Ipswich, MA) for 16 hours. Following digestion, the products were separated on an ethidium bromide 3% agarose gel (EMD chemicals, Gibbstown NJ), photographed and analysed with NIH Image J software version 1.34s.

DNA methylation analysis of identifier (ID) elements by the McrBC-PCR assay:

As described by Pogribny et al. (2006), the genomic DNA (1ug) was digested with 20U of the restriction enzyme; McrBC (New England Biolabs, Beverly, MA) which selectively digests methylated DNA and will not act on unmethylated DNA (Raleigh, 1992; Sutherland et al., 1992). Undigested DNA served as a control. McrBC digestion of methylated DNA prevents PCR amplification and conversely, unmethylated cytosines prevent digestion which can be detected by PCR amplification (Pogribny et al., 2006; Nakayashiki et al., 2004). Following McrBC treatment, the resulting fragments and control samples were PCR amplified with Takara Ex Taq (Fisher Scientific, Ottawa, ON). Primers corresponding to the rat ID elements were used. The sense primer was 5'-TGGCAGCAAGAGCTAACGTTCG -3' and the antisense primer was 5'-TTCGGAGCTGAGGACCGAA -3'. The PCR conditions consisted of an initial denaturation at 95°C for 10 min, followed by 26 cycles of denaturation at 95°C for 30 s, primer annealing at 61.5°C for 30s, and extension at 72°C for 35s. Following PCR, the products were separated on an ethidium bromide 3% agarose gel (EMD chemicals, Gibbstown NJ), photographed and analysed with NIH Image J software version 1.34s. The results are presented as a ratio of the PCR products of McrBC-digested DNA to undigested DNA.

Western immunoblotting:

Western immunoblotting for DNMT1, DNMT3a, DNMT3b, MeCP2 and PCNA was conducted using bone marrow, spleen, thymus, and liver tissue. Tissue samples were sonicated in 0.4-0.8 ml of ice-chilled 1% sodium dodecyl sulphate (SDS) and boiled for 10 min. Small aliquots (10 µl) of homogenate were reserved for protein determination using protein assay reagents from BioRad (Hercules, CA). Equal amounts of proteins (25 µg) were separated by SDS-polyacrylamide electrophoresis (PAGE) in slab gels of 8 or 12% polyacrylamide, made in triplicates, and transferred to PVDF membranes (Amersham, Baie d'Urfé, Québec). Membranes were incubated with antibodies against DNMT1 (1: 1000, Abcam, Cambridge, MA), DNMT3a, DNMT3b (1:500, mouse monoclonals, Abgent, San Diego, CA), MeCP2 (1:1000, Abcam), and PCNA (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham) and the ECL Plus immunoblotting detection system (Amersham). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Unaltered PVDF membranes were stained with Coomassie Blue (BioRad, Hercules, CA) and the intensity of the Mr 50,000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software and normalized to the Mr 50,000 protein.

Statistical analysis:

The statistical procedures are described by Sokal and Rohlf (1995). For the determination of the significance of the difference between the means the Student's t-test was used. P values < 0.05 were considered significant. Statistical treatment and plotting of the results were performed using the Excel for Windows XP, and JMP 5.0 software.

Results

To simulate the brain irradiation scenario, we developed an animal model whereby the sexually mature Long Evans 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 a medical grade shield. The lead shielding used for these studies was similar to one used for patients exposed to radiation in the clinic. The same shielding was used in the published studies on the bystander effect in mouse skin and in rat spleen (Koturbash et al., 2006; Koturbash et al., 2007).

In order to determine if the localized cranial paternal radiation exposure exerts deleterious effects on the progeny, the control and cranially irradiated animals were mated to unexposed females 7 days after irradiation. Two mating groups were established: (1) control mating groups of mock treated animals and (2) paternal exposure – unexposed females mated to exposed males. All the progeny was delivered after the proper gestation period. The progeny of head-exposed fathers had a slightly lower body weight (5.5% less), the difference was statistically significant ($p<0.05$) (data not shown). Additionally, no visible teratogenic effects were observed.

The progeny of the whole-body irradiated animals and humans were shown to be prone to hematological malignancies (Anderson, 2004; Luke et al., 1997; Lord, 1999; Gardner, 1992). Therefore we decided to focus on the changes in the bone marrow, thymus, spleen and liver, the organs that play an important part in hematological malignancies. Since the radiation-induced genome instability in the progeny of the whole-body exposed parents was linked to epigenetic dysregulation, especially to DNA

methylation changes (Koturbash et al., 2006; Baulch and Raabe, 2005), we have chosen to analyze this epigenetic landmark in the progeny of cranially exposed rats.

DNA methylation is known to maintain genome stability and promote normal development and proliferation (Klose and Bird, 2006; Weber and Schebeler, 2007). The altered DNA methylation is linked to the increased mutation rates and cancer (Weber and Schuebeler, 2007; Ehrlich, 2006), including hematological malignancies (Galm et al., 2006; Toyota and Issa, 2005).

To determine if changes in DNA methylation were observed in the somatic tissue of the offspring, the global cytosine methylation was measured in the bone marrow, thymus, spleen, and liver of the offspring of sham-treated and irradiated animals by the cytosine extension assay. We found that the paternal cranial irradiation led to a significant ($p<0.05$) loss of DNA methylation in the bone marrow, thymus and spleen of the offspring (Figure 4-1). Loss of DNA methylation was the most pronounced in the bone marrow. The methylation levels were unchanged in the liver tissue of the progeny (Figure 4-1). The observed loss of DNA methylation was not a consequence of the elevated levels of DNA damage (data not shown).

Alternatively, the altered DNA methylation may be a consequence of the deregulated expression of DNA methyltransferases (DNMTs). DNMT1, DNMT3a and 3b are the three main functional enzymes that are responsible for setting and maintaining DNA methylation patterns in mammalian cells. DNMT1 is responsible for the maintenance of the existing methylation patterns during cellular division, while DNMT3a and 3b catalyze de novo methylation (Weber and Schubeler, 2007; Goll and Bestor, 2005). We studied the effects of the parental cranial exposure on their expression in the

bone marrow, thymus, spleen and liver tissue of the progeny. In the bone marrow of the progeny following the paternal exposure, where the most pronounced decrease of DNA methylation was seen, we have also observed a significant decrease in the expression of DNMT1 (results not shown), DNMT3a and 3b (Figure 4-2).

The expression of DNMT3b was slightly decreased in the spleen and thymus of the exposed progeny (Figure 4-2). No significant changes were noted in the liver tissue (Figure 4-2).

The marked decrease in DNA methylation and the decrease in the DNMTs' espression levels that were seen in the bone marrow of the progeny upon the paternal exposure were paralleled with a significant decrease in the level of methyl-binding protein MeCP2 (Figure 4-2). This is an important novel finding. MeCP2 is a transcriptional repressor and selectively binds to a methylated DNA. This protein is central to the methylation-mediated gene silencing and chromatin remodeling (Klose and Bird, 2006. The MeCP2 expression was also decreased in the thymus tissue of the progeny of cranial-exposed animals (Figure 4-2). The decreased DNA methylation and altered MeCP2 expression may predispose the tissue to genome instability. On the contrary, in spleen MeCP2 levels were elevated (Figure 4-2). Importantly, the levels of proliferating cells nuclear antigen (PCNA) were slightly decreased in the bone marrow and unchanged in spleen, thymus and liver of the progeny of exposed parents (Figure 4-2). Therefore, the aforementioned changes in DNA methylation and protein expression are not due to the increased cellular proliferation.

It is well known that the majority of cytosine methylation (m^5C) in the human and rat genome occurs in the extremely abundant and CpG rich transposons, including

the long and short interspersed nuclear elements (LINEs and SINEs respectively) (Yoder et al., 1997 and Yu et al., 2001; Kaneda et al., 2004; Martens et al., 2005). In tumor cells the global loss of DNA methylation is largely associated with hypomethylation of transposons. Both LINE1 and rat ID elements make up a significant portion of the rat genome. LINE1 make up approximately 23% of the rat genome (Pogribny et al., 2006), and Rat ID elements (a major family of SINEs (Ono et al., 2001)) are widely distributed throughout the rat genome (130,000-150,000 copies (Kass et al., 1996; Ono et al., 2001). In order to determine if the global DNA hypomethylation seen in the somatic tissues of the progeny of cranially exposed parents was in fact attributed to altered transposon methylation, we measured the methylation status of LINE1 and rat ID elements.
The methylation status of LINE1 in bone marrow, thymus, spleen and liver tissues of progeny was measured with the COBRA assay. The assay is based on the bisulfite conversion of unmethylated cytosine to uracil, PCR amplification and subsequent digestion of the PCR product with RsaI and BstUI restriction endonucleases (Xiong and Laird, 1997; Pogribny et al., 2006). Cleavage of PCR product with RsaI indicates sites of unmethylated cytosines and BstUI digestion shows sites of methylated cytosines (Pogribny et al., 2006).

The results of the COBRA assay indicated that in all progeny tissues, there was no difference in methylation status between progeny of exposed and unexposed fathers. (Results not shown).

In parallel, we analyzed the methylation status of the rat ID elements that belong to the SINE group. We used a methylation sensitive McrBC-PCR assay to determine the methylation status of ID elements in bone marrow, thymus, spleen, and

liver tissues of progeny of locally IR exposed fathers. The McrBC-PCR assay is based on the ability of McrBC to digest heavily methylated DNA and not affect unmethylated DNA (Pogribny et al., 2006). Following McrBC digestion of DNA, PCR was used to amplify the ID elements. Product recovery from PCR allows for the determination of methylation status. Heavily methylated ID elements are indicated by minimal product recovery (maximal digestion) while conversely, an increase in product indicates the presence of unmethylated cytosines.

In comparison to the control samples, a small but significant decrease in PCR product can be seen in the thymus and bone marrow tissues of progeny from exposed fathers (Figure 4-3 and Figure 4-4 respectively). This was indicative of a slight but statistically significant increase in the methylation of the ID elements. Namely, the methylation of ID elements increased by ~9% ($P<0.05$) in the thymus tissue and 6% ($P<0.05$) in the bone marrow tissue. Alternatively, a slight loss of DNA methylation was noted in the ID elements of both liver and spleen (Figure 4-5 and Figure 4-6 respectively.). In liver tissue, a significant ~7% ($P<0.05$) decrease in methylation was noted and a slight, although insignificant, decrease (4%) in methylation was noted in the spleen.

Discussion

The main finding of this study is that the localized paternal cranial irradiation induces significant epigenetic changes in the somatic tissues in the unexposed progeny. The observed changes were the most pronounced in the bone marrow and thymus, the organs that are targets for leukemogenesis and lymphomogenesis. Leukemia and

lymphoma are characterized by significant epigenetic alterations including the loss of global DNA methylation and genome instability (Galm et al., 2006). Thus, the observed changes may indeed predispose the progeny of the head-exposed rats to the development of hematologic malignancies.

According to the rat spermatogenesis timing, in the current study we analyzed the effects of mature sperm cells. Future studies are needed to address the effects of the bystander-induced damage to spermatogonia, spermatocytes and elongating spermatids.

Most importantly, we have found a significant decrease in global DNA methylation in the thymus, spleen, and bone marrow tissues from progeny of the cranially exposed fathers. Additionally, decreased levels of global DNA methylation were paralleled by reduced expression levels of DNA methyltransferases. This correlation is not surprising, as global DNA hypomethylation has been previously correlated with decreased levels of DNMTs in tissues of irradiated mice (Raiche et al., 2004; Pogribny et al., 2005).

The expression level of MeCP2 also paralleled the global DNA hypomethylation in bone marrow and thymus tissues of progeny from exposed fathers. Decreased DNA methylation and altered MeCP2 expression may predispose the tissue to genome instability. Contrarily, in spleen, MeCP2 expression increased with decreased global DNA methylation. This attribute may be a protective mechanism. The mechanisms and biological repercussions of the tissue-specific MeCP2 expression need to be further analyzed.

It has been speculated that epigenetic changes, specifically altered patterns of DNA methylation, may underlie transgenerational genome instability (Dubrova et al.,

2000; Koturbash et al., 2006; Zhu et al., 2006). We have previously shown that the progeny of mice, receiving whole body IR exposures, showed global DNA hypomethylation which was paralleled by decreased expression of DNMTs and methyl binding proteins (Koturbash et al., 2006). Additionally, decreased levels of methylation were correlated with increased DNA damage in the form of DSBs. Here we show that in rats, localized cranial paternal IR exposure leads to altered DNA methylation levels which may reflect an increase in genome instability.

In an effort to determine where global DNA hypomethylation was specifically occurring, we analyzed the methylation levels of repetitive elements, LINE1 and rat ID. LINE1 and ID elements (SINE) make up a significant proportion of the rat genome (Kass et al., 1996; Ono et al., 2001; Pogribny et al., 2006) and are heavily methylated (Yoder et al., 1997; Yu et al., 2001; Kaneda et al., 2004; Martens et al., 2005). Based on the literature, one would predict that LINE1 and SINE methylation levels would reflect the global DNA methylation status. Here we show that this correlation is not the case. Specifically, we found that LINE1 and ID element methylation levels were really minor in all thymus, bone marrow, spleen, and liver tissue of the progeny from exposed fathers, and did not correlate with the global DNA hypomethylation.

The apparent lack of DNA methylation changes in LINES and IDs is an interesting finding. This may be viewed as a certain defense strategy, whereby the methylation patterns over LINES and ID that span the genome are protected in order to insure genome stabilization.

The slight hypermethylation of the rat ID element in thymus and bone marrow tissues indeed suggests a protective mechanism may be occurring to ensure inactivation

of SINEs in these target organs. However, significant global DNA hypomethylation suggests that other areas of DNA are hypomethylated, implying that any protective mechanisms that do occur, do not act on a global basis. The methylation status of the other loci needs to be further analyzed. Liver and spleen both exhibited hypomethylation of the ID element. As these organs are not considered primary radiation carcinogenesis targets, perhaps a protective mechanism did not occur, resulting in subsequent hypomethylation of the ID elements. The observed results were linked to the accumulation of unrepaired DNA damage in the sperm cells of the cranially exposed animals (Tamminga et al., submitted).

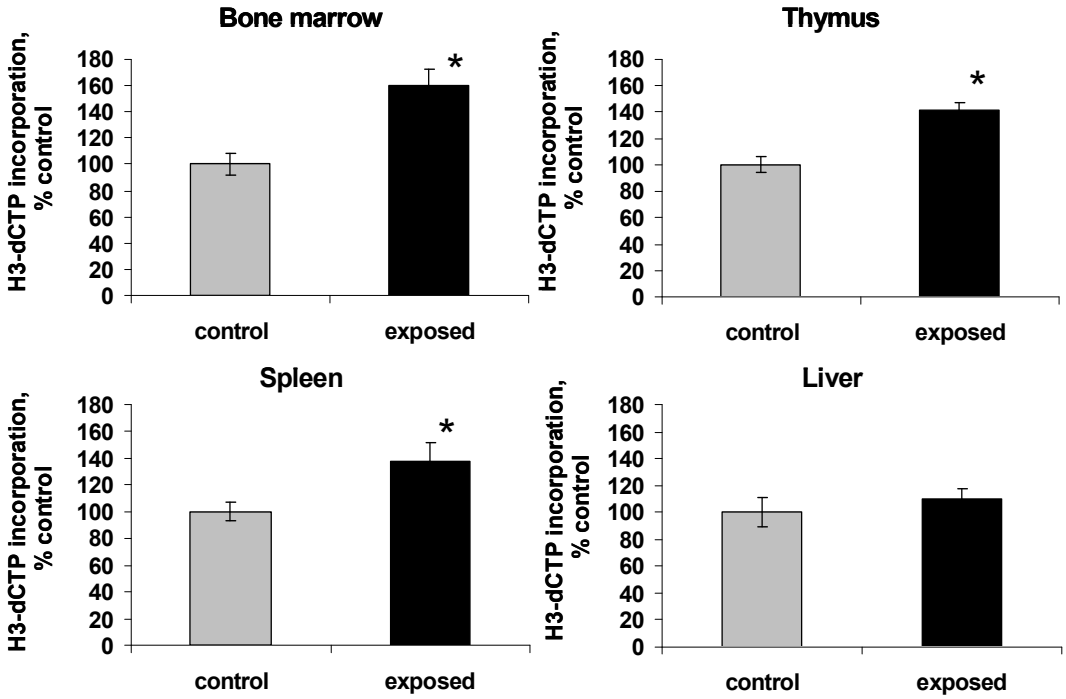

Figure 4-1. Global DNA methylation levels in bone marrow, thymus, spleen, and liver tissues of progeny from exposed and unexposed male rats. The results are presented as mean values ± S.D. Gray bars-progeny of unexposed fathers, n=52; **p*<0.05. Black bars-progeny of exposed fathers, n=56;**p*<0.05.

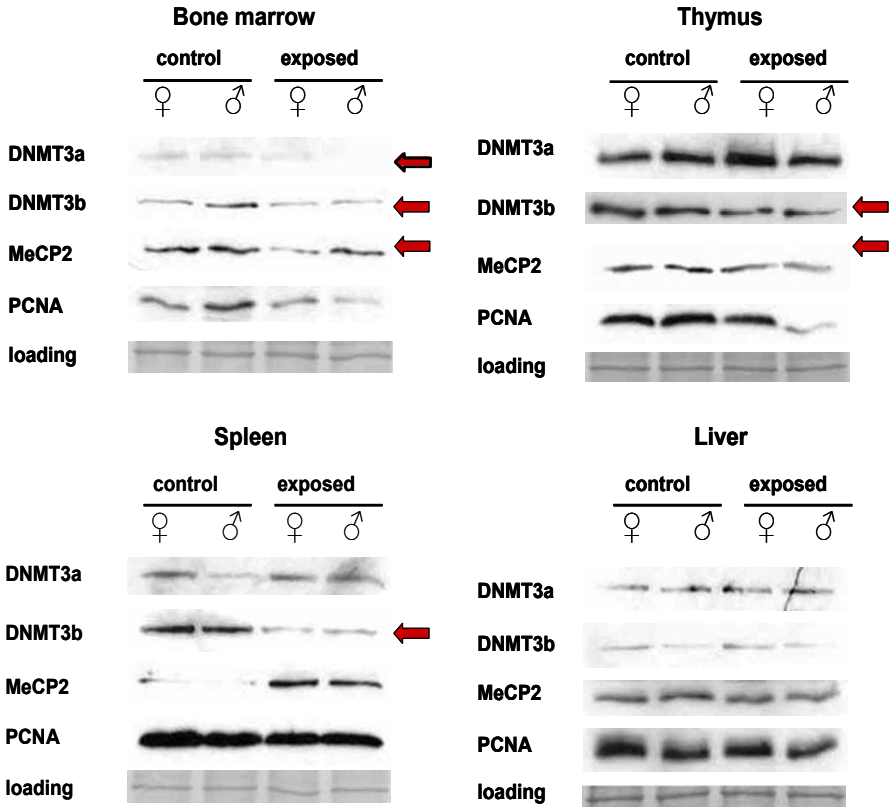

Figure 4-2. Cranial paternal irradiation profoundly alters the expression of DNA methyltransferases and methyl-binding proteins in the bone marrow, thymus and spleen of the unexposed progeny. Tissue lysates were subjected to immunoblotting using antibodies against DNMT3a, DNMT3b, MeCP2 and PCNA. Sample loading was normalized to the protein content. Representative western blots from among 3 independent technical repeats of the experiments are shown; each experiment included pooled lysates from 18 animals for each experimental cohort, with equal representation of each animal.

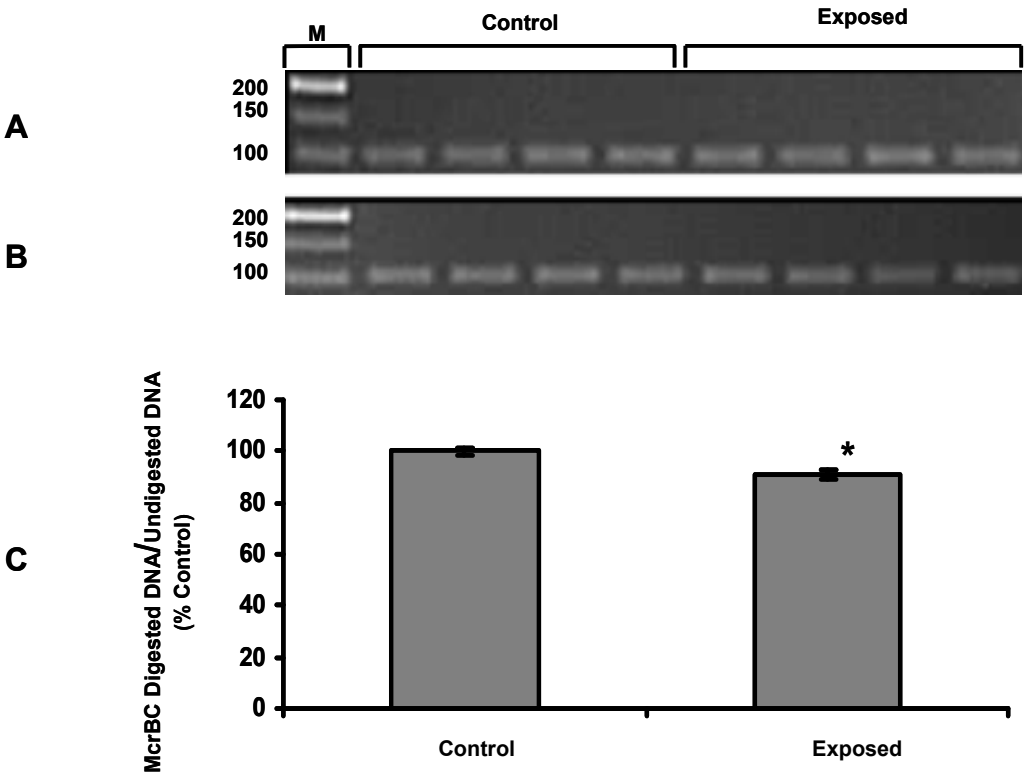

Figure 4-3. ID element methylation status in thymus tissue of progeny from exposed and unexposed male rats. Three bands resulted from PCR amplification (98bp, 330bp and 690bp). The 98bp band was analyzed as it shows 96-100% homology to the ID core domain (Ono et al., 2001; Pogribny et al., 2006). (A) PCR product of unmethylated DNA. (B) PCR product of methylated DNA. (C) ratio between methylated and unmethylated DNA (%control). An increase in McrBC-digested DNA/undigested DNA indicates hypomethylation. The results are presented as mean values ± SD, n=4 (however, each n value represents 3 animals pooled together); *p<0.05.

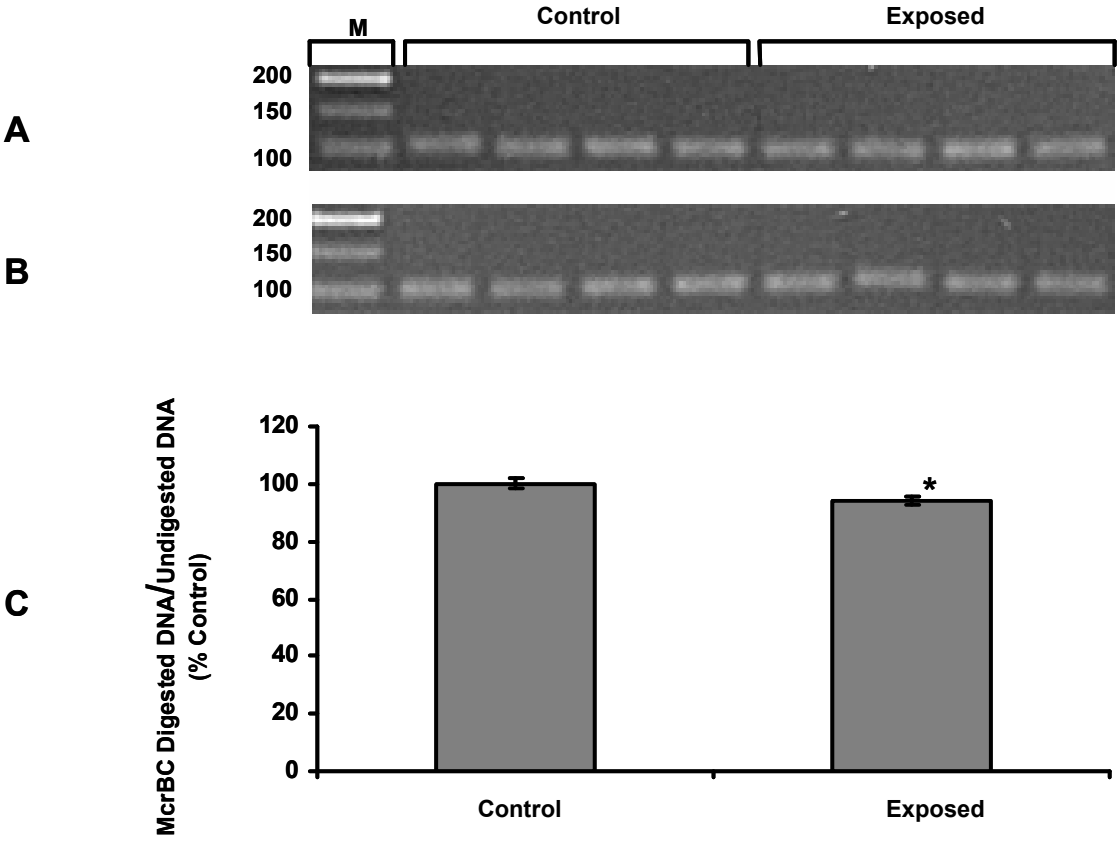

Figure 4-4. ID element methylation status in bone marrow tissue of progeny from exposed and unexposed male rats. Three bands resulted from PCR amplification (98bp, 330bp and 690bp). The 98bp band was analyzed as it shows 96-100% homology to the ID core domain (Ono et al., 2001; Pogribny et al., 2006). (A) PCR product of unmethylated DNA. (B) PCR product of methylated DNA. (C) ratio between methylated and unmethylated DNA (%control). An increase in McrBC-digested DNA/undigested DNA indicates hypomethylation. The results are presented as mean values ± SD, n=4 (however, each n value represents 3 animals pooled together); *p<0.05.

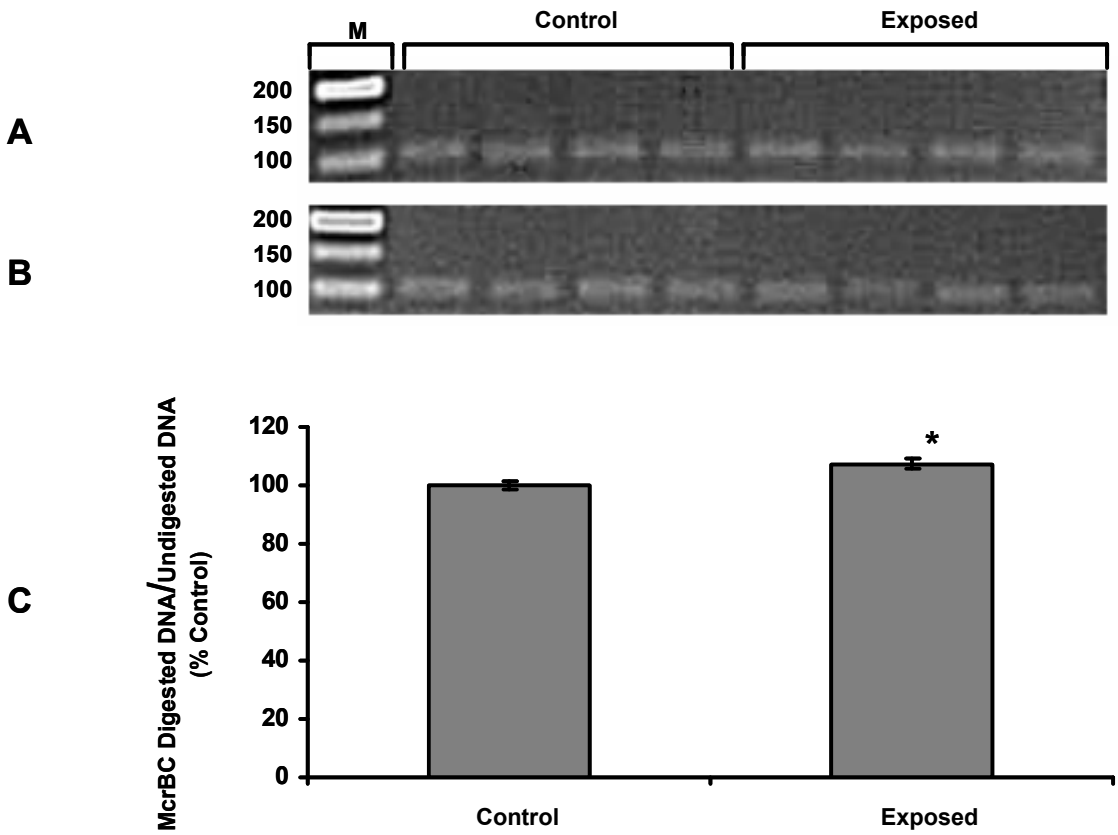

Figure 4-5. ID element methylation status in liver tissue of progeny from exposed and unexposed male rats. Three bands resulted from PCR amplification (98bp, 330bp and 690bp). The 98bp band was analyzed as it shows 96-100% homology to the ID core domain (Ono et al., 2001; Pogribny et al., 2006). (A) PCR product of unmethylated DNA. (B) PCR product of methylated DNA. (C) ratio between methylated and unmethylated DNA (%control). An increase in McrBC-digested DNA/undigested DNA indicates hypomethylation. The results are presented as mean values ± SD, n=4 (however, each n value represents 3 animals pooled together); *p<0.05.

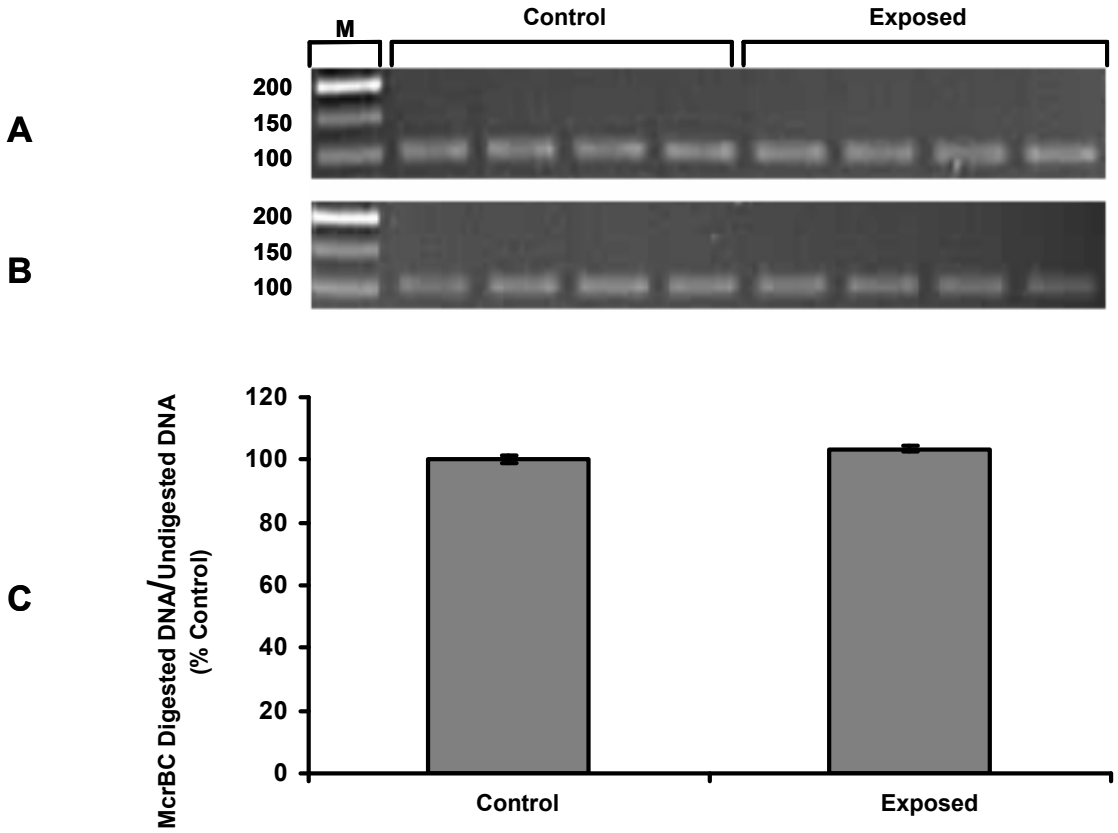

Figure 4-6. ID element methylation status in spleen tissue of progeny from exposed and unexposed male rats. Three bands resulted from PCR amplification (98bp, 330bp and 690bp). The 98bp band was analyzed as it shows 96-100% homology to the ID core domain (Ono et al., 2001; Pogribny et al., 2006). (A) PCR product of unmethylated DNA. (B) PCR product of methylated DNA. (C) ratio between methylated and unmethylated DNA (%control). An increase in McrBC-digested DNA/undigested DNA indicates hypomethylation. The results are presented as mean values ± SD, n=4 (however, each n value represents 3 animals pooled together); *p<0.05.

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CHAPTER 5

General Discussion

Major outcomes of the thesis

The current thesis investigated the role of epigenetic changes in transgenerational genome instability and dysregulation.

Humans are exposed to IR throughout every day life from natural sources, diagnostic equipment, or therapeutic devices. Parents receiving such exposure present a risk to the unborn offspring. Many transgenerational IR studies suggest that the resulting genomic dysregulation within the progeny of exposed parents may lead to an increased risk of cancer (as reviewed by Barber and Dubrova, 2006; Nomura, 2003; Nomura et al., 2004). Mounting data have suggested altered epigenetic parameters as a cause for the genome instability seen in the progeny of IR exposed parents (Jirtle and Skinner 2007; Dubrova et al., 2000; Dubrova and Plum, 2002; Koturbash et al., 2006; Morgan 2003a, 2003b; Vance et al., 2002; Wiley et al., 1997).

Specifically, several studies have shown that the IR-induced cellular reprogramming persists for several generations. A mouse preimplantation embryo chimera assay demonstrated heritable effects of paternal irradiation on embryonic cell proliferation that persisted for two generations (Wiley et al., 1997). These observations led to a hypothesis of a non-Mendelian mode of inheritance of IR-induced changes. The offspring from F_0 parental irradiation exhibited pronounced biochemical alterations (Baulch et al., 2001; Vance et al., 2002, Baulch and Raabe, 2005). Four generations of offspring from the irradiated sires exhibited changes in protein kinase C, mitogen-activated protein kinase, Tpr53 and $p21^{waf1}$ levels. These changes were not consistent in magnitude or direction of change within the offspring of a given generation, nor were they consistent between generations (Baulch and Raabe, 2005). The authors therefore

proposed that these expression parameters may be altered through an epigenetic process (Baulch and Raabe, 2005).

The significantly elevated frequency and persistence of heritable genetic effects on embryonic and spermatogenic cell proliferation rates that spanned over 2-3 generations of animals following paternal irradiation also suggested an epigenetic pattern of regulation of these transgeneration effects (Wiley et al., 1997; Baulch et al., 2002). The possible mechanism that may underlie the observed persistent transgenerational gene expression phenotype was termed 'epigenomic instability' (Baulch and Raabe, 2005). Recent studies using the neutral pH sperm comet assay have also demonstrated the effects of IR on DNA electrophoretic mobility in sperm of male mice 7 weeks post irradiation (Baulch et al., 2007). This same assay also demonstrated an unconventional, heritable DNA damage effect, or changes in chromatin conformation, in non-irradiated offspring of irradiated male mice (Baulch et al., 2007). These findings further support the proposed 'epigenomic instability' theory. Amongst the epigenetic changes, DNA methylation was suggested to be most important (Jirtle and Skinner, 2007).

In the current study, two approaches were taken to discern the transgenerational epigenetic effects of IR exposure. The first approach used a murine model whereby the animals were exposed to whole body irradiation, and the epigenetic changes were analyzed in the somatic tissues of the unexposed progeny. The second approach employed a rat model whereby male rats were subjected to the cranial irradiation. The transgenerational effects were studied in the somatic tissues of the progeny of cranially exposed parents.

Studying the epigenetic changes in the progeny of exposed animals allowed us to gain insights to the location of where epigenetic dysregulation was occurring and to compare the effects of localized and whole-body parental exposure. Importantly, both approaches yielded similar outcomes. The progeny of whole-body and cranially exposed rats exhibited significant epigenetic changes in the tissues and organs involved in hematopoiesis.

Having seen the significant changes in the levels of DNA methylation in the somatic tissues of the progeny of whole-body and cranially exposed animals, we decided to discern the exact molecular nature of those changes and identify the loci where the changes may occur. Repetitive elements make up a significant portion of the genome (Lander et al., 2001; Martens et al., 2005; Waterston et al., 2002). They are heavily methylated in order to protect the genome from their parasitic qualities (Bourc'his and Bestor, 2004; Jaenisch and Bird, 2003). Should the repetitive elements become active, severe genomic dysregulation can occur, resulting in cancer (Kazazian, 2004). For these reasons we attempted to find the location of altered epigenetics, specifically DNA hypomethylation. Overall, we noted no significant changes to the methylation status of the investigated repetitive elements (Rat ID and Mouse IAP-LTR, rDNA, β-tubulin). Epigenetic dysregulation may be occurring on other repetitive elements which were not investigated or perhaps the noted minor (not significant) changes in methylation may result in significant changes as the progeny grow and develop.

The majority of transgenerational radiation studies have used parental whole body exposures to induce changes in the offspring. However, whole body exposures are

relatively rare. In contrast, localized exposures occur far more frequently due to radiation diagnostics and therapy.

Both of our localized and whole-body exposure experiments resulted in similar epigenetic effects within the offspring. Global hypomethylation was noted along with decreased expression of DNA methyltransferases. Whole body exposure resulted in significant alteration of methylation levels and machinery in the radiation target organ, thymus, and less pronounced changes in spleen. Unfortunately, the study of the whole body irradiation effects did not include the analysis of bone marrow.

Localized exposure led to significant hypomethylation in thymus, spleen, and bone marrow tissues of the unexposed offspring. Therefore, based on our results, one can assume that a localized exposure produces transgenerational effects which may be equally damaging as the whole body exposure. The results further suggest that the bystander response to localized irradiation may be noticeably damaging to the adult gonads. Additionally, the transgenerational effects were seen in two different species – mouse and rat.

Future directions

Some of the key future investigations proposed throughout the thesis have included:

- Inspecting other repetitive elements, for example Major and Minor satellites, in an effort to determine the location of epigenetic dysregulation
- Investigating a possible mechanism into the bystander effect at distant tissues (i.e. monitor potential clastogenic factors)

Further studies are needed to establish the link between the observed transgenerational changes and transgenerational carcinogenesis. The progeny of the whole-body irradiated animals and humans was shown to be prone to hematological malignancies (Gardner, 1992; Lord, 1999; Luke et al., 1997; Mohr et al., 1999). In order to determine if the progeny of IR exposed parents do in fact develop cancer, developmental studies should be a part of future experimentation. The amount of IR exposure, both localized and whole body, the parents receive should also be taken into consideration. Furthermore, given our results showing differences between the epigenetic status of progeny of localized and whole body exposed parents, it is possible the type of IR exposure (localized or whole body) may lead to developmental differences of the progeny.

Furthermore, an exploration into the effects of parental "priming" on the transgenerational epigenetic response of the unexposed progeny may provide insight into future radiotherapy treatments. The radiaoadaptive response is defined by the induction of radioresistance to succeeding higher doses of radiation by pretreatment with lower radiation doses (priming) (Ito et al., 2007). Low dose radiation exposure (≤200mGy) has been shown to result in radioadaptive responses in both *in vivo* and *in vitro* experiments (Day et al., 2006; Day et al., 2007; Feinendegen et al., 2007; Ito et al., 2007; Yonezawa et al., 1996; Yonezawa et al., 2004). *In vivo* studies by Ito et al. (2007) and Yonezawa et al. (1996; 2004) have shown that mice exposed to low whole body doses of radiation (5-500mGy) have higher survival rates in response to a second, much higher dose, than mice not primed.

A variety of mechanisms which contribute to the radioadaptive response have been proposed. Such mechanisms include induction of antioxidative and DNA repair enzymes, amplified immune responses and an increase in HSP72 and radioprotective substances such as glutathione (reviewed by Ito et al., 2007).

Many of the experiments mentioned above have shown evidence that priming reduces the ill effects of higher doses of IR exposure in directly exposed animals. Could priming the adults also reduce the transgenerational effects seen in the progeny? Designing experiments to address this question should consider the amount of the priming dose – i.e. which dose works best? As well as the time period between priming and exposure to the effective dose.

Conclusion

The results of this thesis indicate that paternal irradiation (localized and whole body exposure) is a strong inducer of epigenetic dysregulation within the unexposed offspring (transgenerational epigenetic dysregulation). Within the hematopoietic tissue of the offspring from fathers receiving either whole body or localized irradiation, we found transgenerational epigenetic effects to consist primarily of global hypomethylation paralleled by a decrease in the expression of methylation machinery proteins. These results not only provide direct evidence for epigenetic dysregulation caused by parental irradiation, but highlight the effect of radiation bystander effects on the unexposed offspring.

Transgenerational epigenetic dysregulation mediated by a bystander response is a novel finding. This discovery may be pertinent to the clinical applications of IR, in which high doses of localized radiation are used for radiation diagnostics and therapy. Further investigation into this matter will hopefully provide insight into the molecular mechanisms responsible for mediating transgenerational epigenetic dysregulation in a bystander response manner. Understanding the mechanisms responsible for causing transgenerational epigenetic instability due to localized exposure may provide a means for preventing epigenetic dysregulation, genome destabilization and possibly carcinogenesis within the unexposed offspring.

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