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MOLECULAR MECHANISMS OF RADIATION-INDUCED BYSTANDER EFFECTS *IN VIVO*

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DEDICATION

This thesis is dedicated to my parents, Volodymyr and Lyudmyla Koturbash; my grandparents, Olga and Mykola Lytvynenko, and Theodosiy and Laryssa Koturbash; and my second half - Kristy Kutanzi.

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ABSTRACT

Ionizing radiation (IR), along with being an important diagnostic and treatment modality, is a potent tumor-causing agent, and the risk of secondary radiation treatment-related cancers is a growing clinical problem. Now some studies propose to link secondary radiation-induced cancers to an enigmatic phenomenon of bystander effects, whereby the exposed cells send signal damage and distress to their naïve neighbors and result in genome destabilization and carcinogenesis. Yet, no data existed on the bystander effects in an organ other than an exposed one. With this in mind, we focused on the analysis of existence and mechanisms of radiation-induced bystander effects *in vivo.* We have found that bystander effects occur *in vivo* in distant skin and spleen following half-body or cranial irradiation. These bystander effects resulted in elevated DNA damage, profound dysregulation of epigenetic machinery, and pronounced alterations in apoptosis, proliferation and gene expression. Bystander effects also exhibited persistency and sex specificity. The results obtained while using the animal model systems can potentially be extrapolated to different animals and humans.

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LIST OF ABBREVIATIONS

AB – Alberta
A-bomb – atomic bomb
ACB – Alberta Cancer Board
Ago – argonaute proteine
AHFMR – Alberta Heritage Foundation for Medical Research
ANOVA – analysis of variance
BALB/c – mouse strain
BE – bystander effect
C57BL/6 – mouse strain
CA - California
CAST – castrated
cGy – centigray (unit of absorbed radiation)
CHO – Chinese hamster ovary cells
COBRA – combined bisulfite restriction analysis
CpG islands – cytosine and guanine island
CT - control
CXCL12 – Chemokine (C-X-C motif) ligand 12
DAB –
dATP – Deoxyadenine triphosphate
dCTP – Deoxycytidine triphosphate
dGTP – Deoxyguanine triphosphate
dTTP – Deoxythymidine triphosphate
DNA – Deoxyribonucleic acid
DNMT – DNA (cytosine-5-)-methyltransferase
DPM – disintegrations per minute
DSB – double strand break
dsRNA – double-stranded ribonucleic acid
DTT – Dithiothreitol
E2F – transcription factor
ECL – Enhanced electrochemiluminescence
EDTA – ethylenediaminetetraacetic acid
EIF2C2 – eukaryotic translation initiation factor 2C, 2
FL – Florida
FXR – fragile X mental retardation protein
Go – phase of the cell cycle
G1 – phase of the cell cycle
G2 – phase of the cell cycle
GAPDH – Glyceraldehyde 3-phosphate dehydrogenase
Gy – Gray (unit of absorbed radiation)
H2AX – histone 2 modification
H3 – histone 3
H4 – histone 4
H & E – hematoxylin and eosin
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hpa II – restriction enzyme
HR – homologous recombination
HRP – horseradish peroxidase
HSV-tk – herpes simplex virus-thymidine kinase
IHC – immunohistochemistry
IL - Illinois
IR – ionizing radiation
KI67 – antigen identified by monoclonal antibody Ki-67
kV – kilo-Volt
LET – linear energy transfer
LINE1 – long interspersed nucleotide element 1
LOWESS – locally weighted regression
M - phase of the cell cycle
MA – Massachusetts
mA – mili-Amper
MAPK14 – mitogen-activated protein kinase 14
MBD – methyl CpG-binding domain
MD - Maryland
MECP2 – methyl CpG binding protein 2
microRNA – micro-ribonucleic acid
μl – microliter
MO – Missouri
mRNA – messenger ribonucleic acid
Msp I – restriction enzyme
NJ – New Jersey
NHEJ – non-homologous end joining
Notch1 – Notch homolog 1, translocation-associated
NSERC – Natural Sciences and Engineering Research Council of Canada
nt - nucleotide
ON – Ontario
ORF – open reading frame
OVX – ovariectomized
PACT – protein kinase, interferon-inducible double-stranded RNA-dependent activator
PBS – phosphate buffer saline
PCNA – proliferating cell nuclear antigen
PCR – polymerase chain reaction
PFA – paraformaldehyde
PMSF – phenylmethanesulphonylfluoride
pre-miRNA – pre-micro-ribonucleic acid
pri-miRNA – pri-micro-ribonucleic acid
PVDF – Polyvinylidene Fluoride
qRT-PCR – quantitative real-time polymerase chain reaction
Rad51 – homolog of the prokaryotic RecA protein
RISC – RNA -induced silencing complex
RNA – ribonucleic acid

ROPS – random oligonucleotide-primed synthesis
RT – radiation therapy
S – phase of the cell cycle
SCE – sister chromatid exchange
SD – standard deviation
SDS – sodium dodecyl sulphate
SEM – standard error of the mean
TMA – tissue microarray
Tudor-SN – tudor staphylococcal nuclease
TUNEL – terminal uridine deoxynucleotidyl transferase
TX – Texas
UK – United Kingdom
USA – United States of America
UTR – un-translated area
VA – Virginia
WI – Wisconsin
X-ray – Röntgen ray

1. GENERAL INTRODUCTION

1.1 RADIATION EXPOSURE AND CANCER

During the last 100 years, radiation has received a lot of interest from researchers. On the one hand, it is a useful tool utilized in almost all spheres of human life, and especially in medicine. In medicine, ionizing radiation (IR) is an important diagnostic and treatment modality. On the other hand, it is a severe DNA damaging agent that can lead to serious consequences including cancer (Little, 1999).

Data on radiation-induced cancers primarily come from individuals who were medically exposed, as well as from atomic-bomb and nuclear accident survivors.

Since 1902 when the first radiation-induced cancer was reported (Little, 2000), and almost a hundred years after radiation was used for the first time to treat tumors (Gramegna, 1909), it still remains the number one diagnostic and treatment tool for a majority of cancers (Erven *et* Van Limbergen, 2007; De Potter *et al*, 2006; Roof *et al*, 2003; Pollack *et al*, 2000). However, while modern cancer radiation therapy has led to increased patient survival rates, the risk of treatment-related deleterious effects, including secondary cancers, is becoming a growing problem (Hall *et* Phil, 2006; Brenner *et al*, 2005; Rund *et* Ben-Yehuda, 2004; Brenner *et al*, 2000; Leone *et al*, 1999; Boice *et al*, 1992).

Moreover, recent data suggest that even fairly low doses of IR such as those being used in diagnostic procedures (like X-ray or Computer Tomography) can lead to the development of radiation-induced cancers (Brenner *et* Hall, 2004; Liu *et a*l, 2004; Morin *et al*, 2000; Preston-Martin *et al*, 1989), especially in children or young adults who

underwent a radiation diagnostics in childhood (Kleinerman, 2006; Hall, 2002; Shu *et al*, 2002; Infante-Rivard *et al*, 2000; Hildreth *et al*, 1989).

Another group of studies that include data from the atomic bomb survivors and individuals exposed to industrial radiation accidents have shown an increased evidence of cancer development. Studies on the Hiroshina and Nagasaki A-bomb survivors cohort are the main source of information on delayed consequences of radiation exposure. Since 1945 a number of investigations have been done indicating an elevated incidence of various types of cancer among survivors: leukemia (Wakabayashi *et al*, 1983; Watanabe *et al*, 1972; Folley *et al*, 1952), breast cancer (Carmichael *et al*, 2003; Wakabayashi et al, 1983), thyroid carcinoma (Wakabayashi *et al*, 1983; Watanabe *et al*, 1972), stomach, and lung cancers (Wakabayashi *et al*, 1983).

The increased cancer incidence is also well documented in the human population exposed to radiation from nuclear power accidents and nuclear test sites (Shilnikova *et al*, 2003; Kossenko, 1996).

Due to inadequate radionuclide processing and storage, approximately 30,000 people who live near the Mayak nuclear facility in the southern Ural Mountains in Russia are constantly exposed to ionizing radiation. This has led to a significant growth in leukaemia incidence rates. Leukemia rates in the Mayak population were just slightly lower than among atomic bomb survivors (Shilnikova *et al*, 2003; Kossenko, 1996).

Another nuclear catastrophe, the most severe in uclear industry history, occurred in 1986 in Chernobyl, Ukraine, when the nuclear power reactor exploded releasing an enormous amount of radioactive isotopes into the environment. The largest human exposure was to Iodine-131, which led to a subsequent increase in a number of thyroid

carcinomas after 1990 (Williams, 2006; Bogdanova *et al*, 2006; Likhtarov *et al*, 2006). Further Chernobyl studies also described significantly elevated levels of other cancers including leukaemia and lymphoma (Balonov, 2007; Gluzman *et al*, 2005), breast cancer (Prysyazhnyuk *et al*, 2007; Pukkala *et al*, 2006), bladder cancer (Morimura *et al*, 2004), and renal-cell carcinomas (Romanenko *et al*, 2000). A majority of radiation-induced non-hematological cancers continue to occur decades after exposure. Only 20 years have passed since the Chernobyl catastrophe, therefore it is too early to make a final evaluation of that accident (Baverstock *et* Williams, 2006; Williams *et* Baverstock, 2006).

Elevated mutation and cancer rates were reported in the population of the Semipalatinsk nuclear test site in Kazakhstan – the biggest nuclear testing facility in Europe (Tanaka *et al*, 2006; Salomaa *et al*, 2002). Human population of the Semipalatinsk region exhibited significantly elevated levels of unstable chromosome aberrations such as dicentric, ring chromosomes and micronuclei in lymphocytes (Tanaka *et al*, 2006; Salomaa *et al*, 2002).

1.2 BIOLOGICAL EFFECTS OF DIRECT RADIATION EXPOSURE

IR can affect a variety of processes in exposed cells. It can cause changes in gene expression, disruption of mitochondrial processes, cell cycle arrest and apoptotic cell death (Valerie *et al*, 2007; Rodemann *et* Blaese, 2007; Jeggo *et* Lobrich, 2006; Amundson *et al*, 2003; Amundson *et* Fornace, 2003; Criswell *et al*, 2003; Fei *et* El-Deiry, 2003; Iliakis *et al*, 2003; Powell *et* Kachnic, 2003). The most important is that IR is a potent agent capable of inducing DNA damage such as cross linking, nucleotide base damage and single and double strand breaks (Huang *et al*, 2003; Little, 2000; Ward,

1995). If this damage is not correctly restored, it may cause deleterious genetic changes such as mutations and chromosomal aberrations at the initial sites of damage that are further expressed in descendants of irradiated cells. The accumulation of DNA damage caused by IR in conjunction with disrupted cellular regulation processes can lead to carcinogenesis (Sowa *et al,* 2006; Barcellos-Hoff, 2005; Little, 2000).

1.3 INDIRECT RADIATION EFFECTS

1.3.1 Radiation-induced Genomic Instability

Traditionally, the central dogma of radiation biology states that effects of IR are restricted to cells hit directly. This paradigm has been challenged by numerous observations in which cells that were not directly traversed by IR exhibit responses similar to those of the directly irradiated cells (Kovalchuk *et* Baulch, 2008). These responses were demonstrated in the cells that were descendents of directly irradited cells and were termed 'radiation-induced genomic instability' (Kovalchuk *et* Baulch, 2008; Morgan *et* Sowa, 2007; Morgan *et* Sowa, 2005; Morgan, 2003a; Morgan, 2003b; Morgan, 2003c; Hendry, 2001).

Genomic instability is characterized by an increased rate of acquisition of alterations in the genome. It occurs in irradiated cells at a delayed time after irradiation as well as in the progeny of irradiated cells for several generations after exposure (Little, 2000; Wright, 1998; Morgan *et al*, 1996). IR induced genomic instability manifests itself as an induction of chromosomal aberrations, aneuploidy, gene mutations and amplifications, microsatellite instability and cell death (Huang *et al*, 2003; Morgan, 2003a; Morgan, 2003b; Suzuki *et al*, 2003). Radiation-induced genomic instability also

includes the formation of micronuclei or chromosomal fragments that are not incorporated into the nucleus during cell division (Hamasaki *et al*, 2007; Müller *et al*, 2004).

Many signaling pathways are involved in the initiation and perpetuation of genomic instability (Limoli *et al*, 1997; Kaplan *et al*, 1997). The relative contribution of different pathways depends upon the genetic background of an irradiated cell or organism (Watson *et al*, 1997; Paquette *et* Little 1994). All these molecular changes may be causative factors in cancer development, thereby making the induction of genome instability an important prerequisite to cancer formation and a feature of cancer (Sowa *et al*, 2006; Goldberg, 2003; Little, 2003; Coleman *et* Tsongalis, 1999; Nowell, 1976).

1.3.2 Radiation-induced Bystander Effects

IR effects can also be seen in naïve cells that were in contact with directly irradiated cells or in naïve cells that received certain irradiation 'distress' signals from the cells that had been directly exposed via a growth medium. This communication of exposure is termed the 'bystander effect' (BE) (Mothersill *et* Seymour, 2006; Morgan *et* Sowa, 2005; Mothersill *et* Seymour, 2004; Morgan 2003a; Morgan 2003b; Mothersill *et* Seymour, 2003; Morgan *et al*, 2002; Zhou *et al*, 2000).

The very first bystander effect investigations were performed at the beginning of the twentieth century. Murphy, whose research interests were devoted to studies of lymphoid cells, showed morphological changes in these cells after culturing them with serum from radiation-exposed animals (Murphy *et al*, 1921; Murphy *et* Morton, 1915).

In 1954, Parsons reported the presence of soluble clastogenic factors in the circulating blood of patients who underwent radiotherapy (Parsons *et al*, 1954). These factors were capable of inducing chromosome damage in cultured cells (Emerit *et al*, 1995; Emerit *et al*, 1994; Pant *et* Kamada, 1977; Hollowell *et al*, 1968; Goh *et al*, 1968). Clastogenic activity was found in the plasma of patients receiving a high dose of radiotherapy, as well as individuals who were accidentally exposed to substantial levels of radiation (Marozik *et al*, 2007; Emerit *et al*, 1994; Pant *et* Kamada, 1977; Hollowell *et al*, 1968; Goh *et al*, 1968).

By the late 1990s, non-direct effects of radiation, including the bystander effect, started to receive a lot of interest from radiation biologists and radiation oncologists. Just then, Kadhim and colleagues published studies on the effects of α-particle irradiation on haemopoietic stem cells (Kadhim *et al*, 1994; Kadhim *et al*, 1992). In parallel, Nagasawa and Little showed α-particle irradiation-induced enhancement of sister chromatid exchanges in Chinese hamster ovary (CHO) cell cultures (Nagasawa *et* Little, 1992).

At that time, the term "radiation-induced bystander effect" was adopted from cancer gene therapy bystander effect studies. During gene therapy, cancer cells were transfected with herpes simplex virus-thymidine kinase (HSV-tk), making them susceptible to cytotoxic ganciclovir therapy (Moolten *et* Wells, 1990). Intriguingly, even non-expressing HSV-tk cells in the vicinity underwent ganciclovir-mediated apoptosis. Furthermore, it was shown that levels of apoptosis increased in several types of tumor cells, even though only one type of cell was hit (Freeman *et al*, 1993).

Over the years, a great variety of extensive radiation-induced bystander effect studies have been performed using cell culture models (Gaugler *et al*, 2007; Han *et al*,

2007; Maguire *et al*, 2007; Lyng *et al*, 2006b; Hu *et al*, 2006; Maguire *et al*, 2005; Zhou *et al*, 2005; Suzuki *et* Tsuruoka, 2004; Suzuki *et al*, 2004; Yang *et al*, 2005; Mothersill *et al*, 2001; Zhou *et al*, 2002a; Zhou *et al*, 2002b; Zhou *et al*, 2001; Lorimore *et al*, 1998) , tissue explants (Belyakov *et al*, 2006; Belyakov *et al*, 2003; Belyakov *et al*, 2002; Mothersill *et al*, 2002), spheroids (Persaud *et al*, 2005), or reconstructed human tissue models (Sedelnikova *et al*, 2007; Belyakov *et al*, 2005). As a result, bystander effects are accepted as a ubiquitous consequence of radiation exposure (Mothersill *et* Seymour 2004). By the nature of their occurrence, BE can be divided into 2 main categories:

- The gap junction communication-mediated BE that is based on the ability of intercellular gap junctions to transfer ions or metabolites from irradiated to unirradiated cells located in the vicinity (Suzuki *et* Tsuruoka, 2004; Azzam *et al*, 2003a, Azzam *et al*, 2003b, Shao *et al*, 2003; Bishayee *et al*, 2001);
- The medium-mediated BE that is based on the ability of irradiated cells to release a bystander signal into the medium with its further reception by unirradiated cells (Maguire *et al*, 2007; Liu *et al*, 2006; Lyng *et al*, 2006b; Yang *et al*, 2005; Zhou *et al*, 2002b).

Bystander effects have been reported to occur 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*, 2003; Khan *et al*, 1998). It was also shown that when one lung, either right or left, was exposed, there was a marked increase of micronuclei in the unexposed shielded lung (Khan *et al*, 2003; Khan *et al*, 1998). Similar, within-the-organ

bystander effects were observed during partial liver irradiation (Brooks, 2004; Brooks *et al*, 1974).

Bystander effects also manifest themselves in the context of an organism in its entirety. Yet compared to the bystander effect data that are based on cell cultures, conclusive data on somatic bystander effects *in vivo* are relatively scarce (Kovalchuk *et* Baulch, 2008; Mothersill *et al*, 2007; Goldberg *et* Lehnert, 2002).

Overall, bystander effects encompass a wide range of genetic alterations, including gross genome rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, gene mutations and amplifications (Han *et al*, 2007; Hamada *et al*, 2007; Smilenov *et al*, 2006; Lorimore *et al*, 2005; Klokov *et al*, 2004; Suzuki *et al*, 2003; Zhou *et al*, 2002a; Zhou *et al*, 2002b; Lorimore *et al*, 2001; Huo *et al*, 2001; Zhou *et al*, 2000). They influence gene expression, cellular proliferation, senescence and cell death (Lyng *et al*, 2006b; Liu *et al*, 2006; Sawant *et al*, 2002; Sawant *et al*, 2001) and are believed to be linked to radiation-induced genome instability (Kovalchuk *et* Baulch, 2008; Huang *et al*, 2007; Morgan, 2003c).

While a great deal of data has been accumulated on the existence and manifestation of genomic instability and bystander effects in cultured cells, 3D tissues, organs and organisms, the mechanisms of these enigmatic phenomena remain largely unexplored. High frequency of induction and persistence of bystander responses suggests their possible epigenetic background (Wright *et* Coates, 2006; Kaup *et al*, 2006; Morgan, 2003a; Morgan, 2003b; Lorimore *et al*, 2003; Nagar *et al*, 2003).

1.4 EPIGENETIC CHANGES IN CELLS

There are two main forms of information in living cells and organisms – genetic and epigenetic. The genetic changes involve alterations in a DNA sequence. Epigenetic changes are stable alterations in gene expression that include DNA methylation, histone modification, and RNA-associated silencing (Jaenisch et Bird, 2003).

1.4.1 DNA Methylation

Cytosine DNA methylation was the first epigenetic alteration identified, and it is the most widely studied epigenetic mechanism. It is important for normal development, cell proliferation and proper maintenance of genome stability of a given organism (Baylin *et* Ohm, 2006; Baylin, 2005; Jaenisch *et* Bird, 2003).

DNA methylation occurs predominantly in the context of CpG dinucleotides which are methylated at 60–80% of CpG sites (Weber *et* Schuebeler, 2007). DNA is methylated at cytosine nucleotides by DNA methyltransferases to form 5-methyl-cytosines. In mammals, three DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are primarily responsible for establishing and maintaining DNA methylation patterns at CpG sites (Goll *et* Bestor, 2005; Robertson, 2001; Rountree *et al*, 2001). Amongst those, DNMT1 is the major enzyme involved in the maintenance of a pattern of DNA methylation after DNA replication (Liang *et al*, 2002). DNMT1 is localized at the replication fork, where it can directly modify the nascent DNA immediately after replication (Weber *et* Schuebeler, 2007; Jirtle *et* Skinner, 2007; Goll *et* Bestor, 2005). DNMT3a and DNMT3b are *de novo* methyltransferases targeting unmethylated and hemimethylated sites (Weber *et* Schuebeler, 2007; Goll *et* Bestor, 2005; Okano *et al*,

1999). Deregulation of these proteins may lead to altered methylation patterns (Jirtle *et* Skinner, 2007; Weber *et* Schuebeler, 2007; Goll *et* Bestor, 2005).

DNA methylation is known to be associated with an inactive chromatin state and 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 *et* Bird, 2006). The MBD proteins, including MeCP2, MBD1, MBD2, and MBD3, selectively interact with methylated DNA and play a pivotal role in methylation-mediated chromatin remodeling and gene silencing (Bowen *et al*, 2004; Jaenisch *et* Bird, 2003; Hendrich *et* Tweedie, 2003; Robertson, 2002; Robertson *et* Wolffe, 2000). Moreover, methylated cytosines themselves can physically prevent the proper binding of transcription factors to promoter regions (Weber *et* Schuebeler, 2007). Altered global DNA methylation patterns are a well-known characteristic of cancer 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 (Weidman *et al*, 2007; Baylin *et* Ohm, 2006; Baylin, 2005; Jaenisch *et* Bird, 2003). The global loss of DNA methylation at CpG dinucleotides was the first epigenetic abnormality identified in cancer cells, and it 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 (Weber *et* Schuebeler, 2007; Weidman *et al*, 2007; Robertson, 2002; Robertson *et* Wolffe, 2000).

1.4.2 Histone Modifications

Undoubtedly, changes in DNA methylation are not isolated events; they occur in the context of global chromatin deregulation and altered histone modification levels (Weidman *et al*, 2007; Jaenisch *et* Bird, 2003). Histone modifications, including acetylation, methylation, phosphorylation and ubiquitination, are important in transcriptional regulation (Weidman *et al*, 2007; Jenuwein *et* Allis, 2001). Moreover, many of these alterations are stably maintained during the process of cell division. Acetylated histone tails lose their positive charge, thereby reducing their affinity to negatively charged DNA, which leads to a more relaxed chromatin structure. In this way, histone acetylation is linked to transcriptional activation, whereas histone deacetylation is an opposite repressive event (Jenuwein *et* Allis, 2001). Histone methylation can result in different transcriptional consequences depending upon the residue affected (Saha *et al*, 2006; Cheung *et* Lau, 2005). 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 a still unexplored histone code (He *et al*, 2007a; Weidman *et al*, 2007; Saha *et al*, 2006; Cheung *et* Lau, 2005). It was recently shown that tumors undergo a massive loss of tri-methylation at lysine 20 of histone H4 (Tryndyak *et al*, 2006a; Tryndyak *et al*, 2006b; Fraga *et al*, 2005). This loss occurs along with DNA hypomethylation and is linked to chromatin relaxation and aberrant expression. The status of lysine 20 trimethylation on H4 was suggested to be a universal marker for malignant transformation (Fraga *et al*, 2005; Sanders *et al*, 2004). Phosphorylation is another important histone modification (He *et al*, 2007a). One of the best studied modifications is phosphorylation of histone H2AX. H2AX is a member of a

H2A histone family. It becomes phosphorylated at serine 139 (γH2AX) as one of the earliest cellular responses to DSBs (Sedelnikova *et al*, 2003; Pilch *et al*, 2003; Rogakou *et al*, 1998) and accumulates in the nucleus at DSBs forming γH2AX foci. A direct correlation has been found between H2AX phosphorylation and the number of DSBs resulting from radiation (Celeste *et al*, 2003a). γH2AX is important for the repair of DNA strand breaks and for the maintenance of genome stability (Celeste *et al*, 2003b).

1.4.3 MiRNAome

Another mechanism of epigenetic control is through the involvement of small regulatory RNAs (Chuang *et* Jones, 2007; Molnar *et al*, 2007; Weber *et al*, 2007; Presutti *et al*, 2006; Nelson *et al*, 2006; Rogaev, 2005). MicroRNAs are of a special interest, as they can inhibit the translation of a variety of proteins. MicroRNAs (miRNAs) are initially expressed as part of a primary transcript (pri-miRNAs) and are formed from hairpins within the transcript. Further, dsRNA-specific ribonuclease cleavage by a member of the RNase II superfamily, *Drosha,* digests the pri-miRNA in the nucleus to release the hairpin precursor miRNA (pre-miRNA) (Esteller, 2006). Pre-miRNAs are approximately 60 nucleotide (nt) RNAs with 1-4 nt 3' overhangs, 25-30 basepair stems, and relatively small loops. They are exported to the cytoplasm where *Dicer* (RNase III) cleaves the pre-miRNA, which leads to the formation of a mature miRNA. To control the translation of target mRNAs, the mature miRNA must associate with the RNA-induced silencing complexes (RISC) (Gregory *et al*, 2005; Tang, 2005). After the association with the RISC complexes, miRNAs bind to the 3'UTR of mRNAs and serve as translational suppressors, thereby regulating the production of proteins and affecting many cellular

functions including proliferation, differentiation, and cell death (Bernstein *et* Allis, 2005). Argonaute (Ago) proteins are important components of the RISC machinery (Meister *et al*, 2005; Okamura *et al*, 2004). Specifically, *Dicer* was found to stably associate with an EIF2C2 (*Ago2*) protein (Chendrimada, 2005; Lee *et al*, 2003). In addition, PACT and several other proteins were shown to interact with *Ago2* and *Dicer* proteins (Lee *et al*, 2006). Regulatory miRNAs in association with the mRNA machinery impact cellular differentiation (Fatica *et al*, 2006; Chen *et al*, 2006; Bentwich, 2005), proliferation (Hwang *et* Mendell, 2007; Chen *et al*, 2006), apoptosis (Hwang *et* Mendell, 2007; Jovanovic *et* Hengartner, 2006), and possibly result in a predisposition to cancer (Hwang *et* Mendell, 2007; Calin *et* Croce, 2006; Kent *et* Mendell, 2006). These molecules have the ability to travel from cell to cell and from tissue to tissue to change the pattern of gene expression by targeting various regions of the genome for epigenetic modifications (Weber *et al*, 2007; Lujambio *et al*, 2007; Sevignani *et al*, 2006; Seitz *et al*, 2004).

1.5 EPIGENETIC CHANGES IN THE DIRECTLY EXPOSED TISSUE

Direct radiation exposure strongly influences epigenetic effectors. DNA damaging agents including IR have been reported to affect DNA methylation patterns (Kovalchuk *et al,* 2004; Minamoto *et al,* 1999; Tawa *et al,* 1998; Kalinich *et al*, 1989). Acute exposure to low linear energy transfer (LET) x-rays or γ-rays led to global hypomethylation (Tawa *et al,* 1998; Kalinich *et al,* 1989).

IR is known to alter DNA methylation levels in the directly exposed tissue (Kovalchuk *et al*, 2004b). It was recently shown that IR exposure leads to profound dose-dependent and sex- and tissue specific global DNA hypomethylation (Loree *et al*,

2006; Pogribny *et al*, 2005; Koturbash *et al*, 2005; Raiche *et al*, 2004; Pogribny *et al*, 2004b). IR exposure also affects methylation of a p16 tumor suppressor promoter in a sex- and tissue-specific manner (Kovalchuk *et al*, 2004b). DNA hypomethylation observed after irradiation was related to DNA repair (Pogribny *et al*, 2004a). It was also correlated with radiation-induced alterations in the expression of DNA methyltransferases, especially – *de novo* methyltransferases DNMT3a and DNMT3b (Pogribny *et al*, 2005; Raiche *et al*, 2004). Most importantly, the radiation-induced global DNA hypomethylation appeared to be linked to genome instability in the exposed tissue (Loree *et al*, 2006; Pogribny *et al*, 2005; Raiche *et al*, 2004; Pogribny *et al*, 2004b).

DNA methylation is closely connected with other components of chromatin structure. Although much attention has been given to radiation-induced changes in DNA methylation, histones have been largely overlooked. Amongst the histone modifications that change upon radiation exposure, phosphorylation of histone H2AX is being studied most intensively. Histone H2AX– a variant of histone H2A - is rapidly phosphorylated at Ser139 upon the induction of DNA strand breaks by irradiation, and it can be effectively visualized within repair foci using phospho-specific antibodies (Sedelnikova *et al*, 2003). Recent studies have also indicated that radiation-induced global loss of DNA methylation may be paralleled with changes in histone methylation, specifically the loss of histone H4 lysine trimethylation (Pogribny *et al*, 2005). Data on IR effects of microRNAome are in their infancy (Marsit *et al*, 2006; Ishii *et* Saito, 2006).

1.6 EPIGENETIC DETERMINANTS OF INDIRECT RADIATION EFFECTS: BYSTANDER EFFECT

Even though a lot of evidence points towards the epigenetic nature of radiation-induced bystander effects, until recently very few studies addressed exact epigenetic changes in indirect radiation responses. The recent pioneer work by Kaup and colleagues has proven that DNA methylation is important for the maintenance of the radiation-induced bystander effect in cultured cells (Kaup *et al*, 2006). Using cultured human keratinocytes, they have demonstrated that dysregulation of DNA methylation in naïve cells exposed to medium from irradiated cells persists for 20 passages. Over a similar period of culture under comparable conditions, these cells have also exhibited increased and persistent levels of chromosome and chromatid aberrations, reproductive cell death, apoptosis and other signs of genome instability (Kaup *et al*, 2006).

A wide range of extensive cell culture-based studies address the role of phosphorylated histone H2AX in bystander effects (Yang *et al*, 2007; Burdak-Rothkamm *et al*, 2007; Smilenov *et al*, 2006; Sokolov *et al*, 2005)

Epigenetic changes were also shown to be important in whole tissue-based bystander effect models. The reconstituted 3D human tissue model offers an excellent alternative to cell cultures (Sedelnikova *et al*, 2007). A recent study by Sedelnikova and colleagues examined the bystander effects in two reconstructed human 3D tissue models: airway and full-thickness skin. After irradiating a thin plane of cells through the tissue with a microbeam, they analyzed a variety of biological endpoints in distal bystander cells (up to 2.5 mm away from an irradiated cell plane) as a function of post-exposure time (0 hours – 7 days). They detected a significant increase in levels of phosphorylated

H2AX in bystander tissues and extensive long-term increases in apoptosis and micronucleus formation as well as the loss of nuclear DNA methylation, persistent growth arrest, and an increased fraction of senescent cells. Of special interest is the observed loss of DNA methylation in bystander cells. DNA methylation is an important epigenetic phenomenon involved in the regulation of gene expression and genome stability. Since changes in DNA methylation are linked to other epigenetic effectors, the observed alteration of DNA methylation in bystander cells may be indicative of the epigenetic nature of the bystander effect in 3D human tissue models (Sedelnikova *et al*, 2007).

In summary, from the literature we have learned that although bystander effects are thought to be linked to radiation-induced secondary cancers, relatively few studies have evaluated bystander effects *in vivo*. The mechanisms of radiation-induced bystander effects and the role of epigenetic changes still have to be delineated. Furthermore, tissue and sex specificity, strain and species-dependence, as well as long-term persistence of bystander effects need to be discerned.

1.7 HYPOTHESIS

Rationale: Epigenetic changes are important regulators of gene expression and genome stability. IR induces genome instability and also gives rise to an enigmatic phenomenon of a bystander effect. This phenomenon has not been fully explored in the epigenetic domain. While bystander effects were well studied using an *in vitro* model, its existence *in vivo* needs to be confirmed, because up to now, relatively few studies have evaluated bystander effects *in vivo*. The mechanisms of bystander effects *in vivo* need to be established, and tissue and sex specificity, strain and species-dependence as well as long-term persistence of bystander effects need to be discerned.

Based on evidence from the literature, we hypothesize that bystander effects occur *in vivo* and are epigenetically mediated and linked to genome instability. We predict that epigenetic bystander responses are correlated with levels of DNA damage accumulation and repair, proliferation and apoptosis. We beleive that the epigenetically-mediated bystander effects are persistent and distinct in males and females.

Several experiments were designed to test the proposed hypotheses. The experiments are further described as chapters of this thesis.

2. IRRADIATION INDUCES DNA DAMAGE AND MODULATES EPIGENETIC EFFECTORS IN DISTANT BYSTANDER TISSUE *IN VIVO*[1]

[1]Chapter 2 has been published in its entirety:

Koturbash I, Rugo RE, Hendricks CA, Loree J, Thibault B, Kutanzi K, Pogribny I, Yanch JC, Engelward BP, Kovalchuk O. (2006) Irradiation induces DNA damage and modulates epigenetic effectors in distant bystander tissue in vivo. *Oncogene*, **25**:4267-75

2.1 ABSTRACT

Irradiated cells induce chromosomal instability in un-irradiated bystander cells *in vitro*. Although bystander effects are thought to be linked to radiation-induced secondary cancers, almost no studies have evaluated bystander effects *in vivo*. Furthermore, it has been proposed that epigenetic changes mediate bystander effects, but few studies have evaluated epigenetic factors in bystander tissues *in vivo*. Here, we describe studies in which mice were unilaterally exposed to X-irradiation and the levels of DNA damage, DNA methylation and protein expression were evaluated in irradiated and bystander cutaneous tissue. The data show that X-ray exposure to one side of the animal body induces DNA strand breaks and causes an increase in the levels of Rad51 in unexposed bystander tissue. In terms of epigenetic changes, unilateral radiation suppresses global methylation in directly irradiated tissue, but not in bystander tissue at given time-points studied. Intriguingly, however, we observed a significant reduction in the levels of the de novo DNA methyltransferases DNMT3a and 3b and a concurrent increase in the levels of the maintenance DNA methyltransferase DNMT1 in bystander tissues. Furthermore, the levels of two methyl-binding proteins known to be involved in transcriptional silencing, MeCP2 and MBD2, were also increased in bystander tissue. Together, these results show that irradiation induces DNA damage in bystander tissue more than a centimeter away from directly irradiated tissues, and suggests that epigenetic transcriptional regulation may be involved in the etiology of radiation-induced bystander effects.

2.2 INTRODUCTION

Until recently, it has been broadly accepted that biological consequences following ionizing radiation (IR) exposure are attributable to the direct effects of DNA damage. However, a wealth of evidence now challenges this classical paradigm (Mothersill *et al*, 2004; Mothersill *et* Seymour, 2004; Hall, 2003; Ward, 2002). In particular, it has been shown that irradiated cells can elicit increased levels of mutations and chromosome aberrations in neighboring cells that were not exposed to radiation. These "bystander effects" can lead to persistent genome destabilization and ultimately may contribute to carcinogenesis (Mothersill *et al*, 2004; Mothersill *et* Seymour, 2004; Hall, 2003; Ward, 2002; Zhou *et al*, 2002b; Sawant *et al*, 2001; Zhou *et al*, 2000). 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 (Morgan, 2003a; Morgan, 2003b; Little, 1999; Little, 1998).

As such, bystander effects are a complication in radiation oncology and are thought to contribute to secondary radiation carcinogenesis (Mothersill *et* Seymour, 2004; Hall, 2003; Goldberg, 2003; Sigurdson *et* Jones, 2003; Huang *et al*, 2003; Goldberg *et* Lehnert, 2002). Despite its potential biological importance, most of what we currently know about radiation-induced bystander effects stems from studies of cultured cells, rather than from animal models (Hall, 2003; Goldberg, 2003; Goldberg *et* Lehnert, 2002). Furthermore, despite the clear link between radiation-induced bystander effects and radiation-induced genome instability (Goldberg, 2003; Sigurdson *et* Jones, 2003; Huang *et al*, 2003; Morgan, 2003a; Morgan 2003b; Morgan *et al*, 2002), very few studies have explored the potential impact of radiation exposure on distant organs and tissues (Xue *et al,* 2002).

Radiation-induced genome instability has recently been suggested to be epigenetic in nature (Nagar *et al*, 2003). Epigenetic changes are mitotically stable alterations that include DNA methylation and histone modifications (Jaenisch *et* Bird, 2003; Robertson, 2002). Aberrant cytosine DNA methylation is well documented in cancer development and is linked to genomic instability and increased rates of genome rearrangements (Feinberg *et* Tycko, 2004; Gaudet *et al*, 2003; Ehrlich, 2002; Esteller *et* Herman 2002; Robertson *et* Wolffe 2000). A variety of DNA damaging agents including IR are known to affect genome DNA methylation patterns and this may contribute to their genome destabilizing effects (Koturbash *et al*, 2005; Pogribny *et al*, 2004a; Raiche *et al*, 2004; Minamoto *et al*, 1999; Tawa *et al*, 1998; Kalinich *et al*, 1989). To our knowledge, no studies have explored the possible roles of epigenetic mechanisms on somatic *in vivo* bystander effect.

To investigate the possibility that X-irradiation induces DNA damage in bystander tissue *in vivo*, we monitored the induction and repair of DNA strand breaks in cutaneous tissue. In addition, we also explored the possibility that epigenetic mechanisms (*i.e.* DNA methylation and alterations in DNA methyltransferases and methyl-binding proteins) are involved in the generation and/or maintenance of a radiation-induced bystander effect. Here, we report that radiation exposure to one half of the body leads to elevated levels of DNA strand breaks, and alters the levels of key proteins known to modulate methylation patterns and silencing in the bystander half of the body at least 0.7 cm from the irradiated tissue. These are some of the first data to clearly demonstrate that bystander effects occur *in vivo* in distant tissues.

2.3 MATERIALS AND METHODS

2.3.1 Model and Irradiation of Animals

In this study we examined genetic and epigenetic alterations in mouse skin following *in vivo* radiation exposure. 45-days old male mice were randomly assigned to different treatment groups. Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use (1993). The procedures have been 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 exposed cohort (25 animals) received 1 Gy (2cGy/s) of X-rays (90kV, 5mA). In the bystander group (25 animals) each animal was exposed to 1Gy (2cGy/s) of X-rays (90kV, 5mA) having half of its body protected by a~2.5 mm thick lead shield, the same type as used for the human body protection in diagnostic radiology (Fig. 2.1) The protection of shielded 'bystander' tissue was complete, as verified by careful dosimetry using a RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, FL, USA). Control mice were sham treated. All animals were humanely sacrificed 6 or 4 days (precisely 96 hours) upon completion of the treatment protocol. Based on the previous research, these time points are sufficient to see the induction and persistence, if any, of the radiation-induced epigenetic changes (Koturbash *et al*, 2005; Kovalchuk *et al*, 2004a). Cutaneous tissue was sampled upon sacrifice and processed for further molecular studies.

A cohort of 5 animals was exposed to 1 Gy of X-rays while their bodies were completely shielded with a ~2.5 mm lead shield. Protection by the lead shielding was complete as verified by RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, FL, USA).

Another cohort of 5 animals was exposed to an approximate scatter dose of ~0.013Gy. To determine the bystander ventral/thigh skin dose resulting from photon scatter within the mouse itself, a Monte Carlo simulation was performed (X-5 Monte Carlo Team, 2003). The skin was assumed to be 0.1 cm thick and a 2 mm thick lead shield covered one half of the mouse. Absorbed dose was tallied within the skin region in several locations in both the unshielded dorsal regions and the shielded ventral/thigh regions. Absorbed dose to the ventral skin from a 90 kVp X-ray spectrum was determined be approximately 0.014 – 0.017 Gy for a 1 Gy dose delivered to the dorsal skin.

2.3.2 Cytosine Extension Assay to Detect Sequence Specific Changes in DNA Methylation

Total DNA was prepared from skin tissues using Trizol™ Reagent (Amersham, Baie d'Urfé, Québec) according to the manufacturer's protocol.

DNA (0.5 μg) was digested overnight with a 10-fold excess of Hp*a*II endonuclease according to manufacturers protocol (New England Biolabs, Beverly, MA). A second DNA aliquot (0.5 μg) was incubated without restriction enzyme addition and served as a background control. The single nucleotide extension reaction was performed in 25 μg of DNA, 1X PCR bufferII, 1.0 mM $MgCl_2$, 0.25 units of Taq DNA polymerase (Fisher Scientific, Ottawa, ON), [^{3}H]dCTP (57.4 Ci/mmol) (Perkin Elmer, Boston, MA) and incubated at 55°C for 1 h, then immediately placed on ice. Duplicate aliquots (25μl) from each reaction were placed on Whatman DE-81 ion-exchange filters and washed three times 10 minutes with gentle agitation with sodium phosphate buffer (0.5 M, pH 7.0) at room temperature. The filters were dried and processed by scintillation counting (Beckman Counter). Background label incorporation was subtracted from

enzyme-digested samples and results were expressed as relative [^{3}H]-dCTP incorporation/1µg of DNA or as percent change from control (Pogribny *et al*, 2005; Pogribny *et al*, 2004a; Raiche *et al*, 2004; Pogribny *et al*, 1999).

2.3.3 DNA Strand Break Measurement

A modification of the random oligonucleotide-primed synthesis assay (ROPS) was used to detect the presence of DNA strand-breaks in high molecular weight DNA (Pogribny *et al*, 2004a; Basnakian *et* James, 1996). The assay is based on the ability of Klenow fragment polymerase to initiate ROPS from the re-annealed 3'-OH ends of single stranded DNA. Briefly, 3'-OH DNA fragments present in the high molecular weight DNA are separated into single-strand fragments by heat denaturation and subsequently re-associated by cooling. The resulting random re-association of DNA strands consists primarily of single-stranded DNA fragments primed by their own tails or by other DNA fragments. These fragments serve as random primers and the excess of DNA serves as template for Klenow fragment polymerase. DNA was denatured by exposure at 100 °C for 5 min, and then immediately cooled on ice. The mixture contained 0.25 µg heat-denatured DNA, 0.1 µl [3H]dCTP (57.4 Ci/mmol) (Perkin Elmer, Boston, MA), 0.05 mM concentrations of each dGTP, dATP, dTTP, 0.6 µM dCTP, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 7.5 mM DTT, and 0.5 unit Klenow polymerase (New England Biolabs, Beverly, MA) in a total volume of 25 µl. After incubation for 30 min at 16 °C, the reaction was stopped by the addition of an equal volume of 12.5 mM EDTA. The samples were subsequently applied on Whatman DE-81 ion-exchange filters and washed three times with sodium phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed by scintillation counting. The results

were expressed as the percent difference in [3H] dCTP incorporation relative to control values.

2.3.4 Western Immunoblotting

Western immunoblotting for RAD51, γH2AX, DNMT1, DNMT3a, DNMT3b, MeCP2 and MBD2 was conducted using cutaneous 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 (20 µg) were separated by SDS-polyacrylamide electrophoresis (PAGE) in slab gels of 8 or 12% polyacrylamide, made in duplicates, and transferred to PVDF membranes (Amersham, Baie d'Urfé, Québec). Membranes were incubated with antibodies against RAD 51 (1:1000, BD Biosciences, Mountain View, CA), γH2AX (1:1000, Cell Signaling, Danvers, MA), DNMT1 (1: 1000, Abcam, Cambridge, MA), DNMT3a, DNMT3b (1:500, Abgent, San Diego, CA), MeCP2 (1:1000, Abcam, Cambridge, MA) and MBD2 (1:500, Abgent, San Diego, CA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham, Baie d'Urfé, Québec) and the ECL Plus immunoblotting detection system (Amersham, Baie d'Urfé, Québec). 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 both GAPDH and the Mr 50,000 protein which gave consistent results (values relative to Mr 50,000 are plotted).

2.3.5 Statistical analysis

Statistical analysis was performed using the MS Excel 2000, Sigma Plot and JMP5 software packages.

2.4 RESULTS

2.4.1 Accumulation of DNA Damage in Bystander Skin Tissue In Vivo

We studied bystander effects *in vivo* by placing lead shielding, one edge aligned along the spine, over half the body of a mouse, before exposure to 1 Gy of X-rays (Fig. 2.1). Animals were immobilized during exposure and bystander ventral skin was taken from the area adjacent to the thigh at least 0.7 cm from the exposed half of the mouse. The lead shielding used for these studies is the same as that is used for patients exposed to radiation in a clinic, and complete protection of shielded bystander tissue from radiation exposure was verified by careful dosimetry (see Materials and Methods).

DNA damage in mammalian bystander cells has been studied *in vitro* by directly measuring DSBs (Sokolov *et al*, 2005), and by measuring sequence rearrangements that are thought to be caused by DSBs, such as chromosomal aberrations, sister chromatid exchanges (SCEs), and homologous recombination between direct repeats (Rugo *et al*, 2005; Ponnaiya *et al*, 2004; Huo *et al*, 2001; Nagasawa *et* Little, 1992). Since single-strand breaks can become DSBs if encountered by the replication fork (Helleday, 2003; McGlynn *et* Lloyd, 2002), we measured single and DSBs in bystander tissues *in vivo* using a modified version of the random oligonucleotide-primed synthesis (ROPS) assay (Basnakian *et* James, 1996). This assay is based on the ability of Klenow polymerase to initiate ROPS from the re-annealed 3′-OH ends of single stranded DNA. DNA strand breaks were quantified in skin from unirradiated control mice, fully irradiated mice, and from irradiated and bystander sides of mice (Fig. 2.2a). DNA strand breaks were quantified at 6 hours and at 4 days (precisely 96 hours) after exposure in order to monitor the persistence and/or repair of irradiation-induced strand breaks over time. As expected, cells isolated from the skin of fully irradiated animals

as well as the skin from the irradiated side of hemi-shielded animals showed significantly increased levels of strand breaks 6 hours post-irradiation compared to controls (50% and 48%, respectively; $p<0.05$; Fig. 2.2A). Surprisingly, we also found significantly elevated levels of breaks in bystander skin from hemi-shielded mice 6 hours post-treatment (20%; $p<0.05$), indicating that damage is induced in shielded tissue *in vivo*. Four days after exposure, the number of strand breaks under all conditions had returned to control levels (Fig. 2.2A, grey bars), indicating that strand breaks induced directly (in the exposed tissue) and indirectly (in the shielded bystander tissue) had been repaired.

As an alternative approach for assessing the occurrence of DSBs, we assayed for the presence of γH2AX phosphorylation. H2AX is a member of the H2A histone family which becomes phosphorylated at S139 (γH2AX) as one of the earliest cellular responses to DSBs (Rogakou *et al*, 1998). γH2AX accumulates in the nucleus at DSBs forming the γH2AX foci and a direct correlation has been found between the H2AX phosphorylation and the number of DSBs resulting from radiation. Although the majority of γH2AX foci appear within minutes of radiation exposure, previous studies have shown that a certain subset of breaks and H2AX phosphorylation persists for many hours (Rothkamm *et* Lobrich, 2002). At 6 hours after exposure, γH2AX was undetectable by Western blot in skin from un-irradiated control mice, but was significantly increased in skin from fully irradiated mice and in skin from the irradiated sides of hemi-shielded mice (~2.2- and ~1.6-fold over control, respectively; Fig 2.2B). In addition, there was a slight increase in γH2AX in the skin from the bystander sides of hemi-shielded mice (although not readily apparent by eye, densitometry analysis shows that there is a statistically significant ~1.3 fold increase in γH2AX levels in bystander tissue compared to control cohorts; $p < 0.05$, Student's *t*-test),

which is consistent with DSBs in the bystander tissues 6 hours after irradiation. By 4 days post irradiation, γH2AX was still slightly elevated in cutaneous tissue from fully irradiated mice and from the irradiated side of hemi-shielded mice, but not in bystander skin. These data are consistent with the ROPS assay 6 hours after exposure, indicating that X-irradiation resulted in the induction of DSBs in shielded bystander tissue *in vivo*.

Mammalian cells employ homologous recombination (HR) and non-homologous end joining (NHEJ) as important pathways to repair DSBs (reviewed in Helleday, 2003; West, 2003; McGlynn *et* Lloyd, 2002; Hoeijmakers, 2001). HR allows cells to use the undamaged sister chromatid or the homologous chromosome as a template for repair and thus is considered error-free (Helleday, 2003; West, 2003; McGlynn *et* Lloyd, 2002; Hoeijmakers, 2001). NHEJ is a fast, yet error-prone process of linking broken DNA ends together without reference to the accurate base pairing (Hoeijmakers, 2001).

As we had found evidence of DSBs in bystander tissues, we next asked if HR or NHEJ related proteins were induced in bystander tissues. Rad51 is a key protein essential for repair of DSBs via HR in mammals (Lundin *et al*, 2003; Sonoda *et al*, 1998). Rad51 binds to single stranded DNA and forms a nucleoprotein filament that catalyses homology searching, strands pairing, and strand exchange (Baumann *et* West, 1998; Baumann *et al*, 1996). Ku70 is a key participant in the non-homologous end-joining (NHEJ) pathway to repair DSBs (Hoeijmakers, 2001).

Western blots were performed on lysates of skin from control mice, fully irradiated mice, and the irradiated and bystander sides of hemi-shielded mice 6 hours and 4 days post-treatment. No significant changes were found in the levels of Ku70 in the exposed and bystander mouse skin (data not shown).

Unexpectedly, we found that Rad51 expression is similarly elevated in tissues from fully exposed and the exposed and bystander sides of hemi-shielded mice at 6 hrs and 4 days after exposure (Fig. 2.2C). Rad51 activity is generally thought to be controlled by subnuclear localization. However, the increased levels of Rad51 described here are nevertheless consistent with an earlier report of a transcription-dependent increase in Rad51 found in cultured human cells after DNA damage and measured by immunofluorescence (Haaf *et al*, 1995).

2.4.2 Epigenetic Changes in Exposed and Bystander Tissue

Bystander effects are thought to arise via epigenetic mechanism(s). DNA methylation is an important epigenetic mechanism for regulating gene expression/silencing and there is increasing evidence that methylation also serves to help safeguard genome stability. For example, decreased DNA methylation has been linked to elevated levels of transposon activation, sister chromatid exchanges and other gross genome rearrangements (Robertson, 2002; Esteller *et* Herman, 2002; Gonzalgo *et* Jones, 1997).

To test if changes in methylation were observed in bystander tissue, global cytosine methylation was measured in skin from un-irradiated, irradiated, and the irradiated and bystander sides of hemi-shielded mice 6 hours and 4 days after irradiation. To monitor changes in DNA methylation, we employed the sensitive *Hpa*II-based cytosine extension assay, which measures the proportion of unmethylated CCGG sites in genomic DNA (Koturbash *et al*, 2005; Kovalchuk *et al*, 2004; Pogribny *et al*, 2004a; Raiche *et al*, 2004). Using this approach, we found that exposure to 1 Gy of X-rays led to a significant decrease in DNA methylation in exposed skin 6 hours post-treatment (decreased methylation appears as an increase in 3H-

dCTP incorporation; Fig. 2.3A). Methylation levels in the exposed tissue returned to normal in a separate cohort analyzed 4 days post-treatment (Grey bars; Fig. 2.3A). DNA methylation changes were not significant in bystander tissue, although a slight decrease was observed (Fig. 2.3A). Overall, unilateral irradiation suppressed global DNA methylation in directly irradiated, but not in bystander tissue at the time-points studied.

In mammals, three DNA methyltransferases (DNMT1, DNMT3a and DNMT3b) are primarily responsible for establishing and maintaining DNA methylation patterns at CpG sites (Raiche *et al*, 2004; Robertson, 2001; Rountree *et al*, 2001). In particular, DNMT3a and DNMT3b catalyze *de novo* methylation, while DNMT1 maintains existing methylation patterns. Deregulation of any or all of these proteins may result in perturbations of DNA methylation. To further investigate the basis of methylation loss in irradiated tissue, we monitored expression of DNMT1, DNMT3a and DNMT3b methyltransferase in skin from un-irradiated, fully irradiated, and the irradiated and bystander sides of hemi-shielded mice at 6 hours and 4 days post-irradiation. Interestingly, DNMT1 expression was significantly up-regulated in bystander tissue both 6 hours and 4 days post-treatment (~1.4 and 1.8 fold respectively; Fig. 2.3B), whereas expression remained unchanged in irradiated skin. Although these are somewhat subtle changes in the levels of DNMT1, it is striking that within the same animals, DNMT1 was consistently higher in bystander tissue than in directly irradiated tissue. Furthermore, each lane is a combined sample from 5 separate animals, and each Western was repeated 3 times, such that the fold induction reflects cohorts of at least 15 animals. In contrast to DNMT1 expression of the *de novo* methyltransferases DNMT3a and DNMT3b was slightly down-regulated both in irradiated skin as well as in bystander tissue 6 hrs post-irradiation (Fig. 2.3B). Suppression of DNMT3b was reduced at 4 days post-

irradiation and levels of DNMT3a had normalized by 96 h post-irradiation, which coincides with recovery of normal methylation levels by this time (Fig. 2.3B). (Densitometry analysis shows that the decrease is statistically significant; $p < 0.05$, Student's t-test.)

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. MBD proteins, including MeCP2, MBD1, MBD2, and MBD3, selectively interact with methylated DNA and play pivotal roles in methylation-mediated chromatin remodeling and gene silencing (Bowen *et al*, 2004; Jaenisch *et* Bird, 2003; Hendrich *et* Tweedie, 2003; Robertson, 2002; Wade, 2001; Robertson *et* Wolffe, 2000). To test if MBD expression is altered in bystander tissue, we monitored expression of MeCP2 and MBD2 in skin from un-irradiated, fully irradiated and in skin from the irradiated and bystander sides of mice at 6 and 96 hours post-irradiation. Both MeCP2 and MBD2 were significantly up-regulated (~2-fold) in bystander skin (Fig. 2.3B). As was observed with DNMT1, this induction persisted 4 days post-treatment. In addition, a slight but statistically significant increase in MeCP2 expression (~1.25 fold) was also evident in irradiated skin at 6 hours post-irradiation. Up-regulation of the methyl-binding proteins MeCP2 and MBD2 in bystander skin is consistent with the observed induction of DNMT1 methyltransferase in this tissue.

2.4.3. Bystander Effects are Not the Result of Insufficient Shielding or Radiation Scattering

As an independent measure of effective shielding, fully shielded mice were exposed to 1 Gy X-rays and ventral skin was analyzed by Western blot for expression of Rad51, DNMT1, MeCP2, and MBD2 expression. As can be seen in Figure 2.4A, there was no

induction. Furthermore, levels of the γH2AX protein, an indicator of DNA damage, also remained unchanged from the un-irradiated control (data not shown). As X-rays penetrate tissue, they can react with biological matter resulting in the deflection of their trajectories. This is referred to as scatter radiation. Low doses, close to the scatter-dose range levels were shown to induce bystander effects (Mothersill *et al*, 2005b; Maguire *et al*, 2005; Kashino *et al*, 2004; Mothersill *et* Seymour, 2002). Thus, to measure if scatter contributed to the effects seen in bystander tissue, mice were irradiated with a dose approximating the expected scatter dose (13 mGy, which was calculated as described in Materials and Methods) and ventral skin was analyzed as was done for the fully shielded samples above. There was no protein induction seen (Fig. 2.4B) indicating that the observed changes in expression were not likely to be caused by scatter radiation.

2.4.4 Exploration of Possible Effects of Physiological Asymmetry

It is well known that the mammalian body is not absolutely symmetrical and multiple organs do not have a pair. In our initial experiments we protected the right part of the animal body, exposing the left side. Thus, the spleen, an important hematopoietic organ was within the exposure field. On the other hand, irradiation of right side of the animal body exposes more of the liver. It is possible that differential exposure of organs has a role in the induction of bystander effects. To address this question, experiments were set up in which the right or the left half of the body was shielded while animals were exposed to X-rays. In a third experimental group, the heads were exposed to X-rays while the body was shielded by lead. Sham-treated animals served as experimental controls. Animals were sacrificed 6 hours and 4 days after exposure and tissue lysates analyzed by Western blot. Consistent with the

experiments shown in Figure 2.3, we found that DNMT1 expression was upregulated in bystander tissues but not on irradiated skin; this was the case regardless of which side of the body was irradiated (Fig. 2.5). In addition, Rad51 was upregulated in all irradiated and bystander tissues, regardless of which half of the body was exposed (Fig. 2.5). Interestingly, induced expression of MBD2 and MeCP2 was noted in bystander skin only when the left side of the body was irradiated, suggesting the possibility that internal organs affect upregulation of MBD2 and MeCP2 in bystander tissue (Fig. 2.5). Further studies are underway to elucidate the molecular underpinnings of the regulation of MBD2 and MECP2.

As an independent approach for studying potential bystander effects, we also adjusted the exposure procedure so that for one cohort of animals, their heads were irradiated and unexposed cutaneous tissue from their backs was analyzed. We found similar expression patterns for DNMT1 and Rad51 in bystander skin taken from the dorsal sides (at least 0.7 cm away from the irradiated tissue) of animals whose heads had been irradiated, which is consistent with the data from the unilateral exposure.

2.5 DISCUSSION

Most reported bystander effects have been observed *in vitro* and the biological significance of bystander effects *in vivo* are unclear (Goldberg, 2003; Goldberg *et* Lehnert, 2002). Ideally, research on bystander effects would be done in clinical trials. However, in the meantime, experiments involving animals could be useful for delineating the possible effects of irradiation on unexposed tissues and for dissecting the molecular basis for such effects (Goldberg, 2003; Goldberg *et* Lehnert, 2002; Balmain *et* Harris, 2000). We set out to explore DNA damage and repair in bystander tissues in a mouse model. The main findings of the present study are: (i) radiation exposure leads to the induction of DNA damage in distant (>0.7 cm from irradiated tissues), lead-shielded, bystander tissues *in vivo*; (ii) DNA damage in bystander tissues was present several hours after radiation; (iii) bystander tissues exhibited changes in expression of genes involved in methylation, indicating gene silencing by methylation may be involved in bystander effects; (iv) expression changes in bystander tissue persisted through 4 days after irradiation; (v) expression changes in bystander tissue may be, in part, linked to internal organ exposure, as the more pronounced response was observed when the left side of the body was irradiated.

2.5.1 DNA Damage and Repair

We measured DNA damage in skin from un-irradiated, irradiated and the irradiated and bystander sides of hemi-shielded mice. We found significant increases in strand breaks in irradiated and bystander samples 6 hrs after exposure but not after 4 days, which is consistent with the damage being repaired in irradiated and bystander tissues. In addition, we monitored γH2AX as a specific marker of DSBs. As the appearance of γH2AX occurs

rapidly after DSBs are formed, it is often examined soon after damage is induced, for example, after 30 minutes. The fact that we observed an increase in γH2AX in irradiated and bystander tissues 6 hrs after exposure is surprising as it indicates there may be unrepaired DSBs persisting after irradiation (Rothkamm *et* Lobrich, 2003). Alternatively, it may reflect an increase in the number of dividing cells in tissues following irradiation (Ichijima *et al*, 2005).

The induction of Rad51 in irradiated tissue was somewhat unexpected. It is thought that Rad51 activity is regulated by sub-nuclear localization (West, 2003), however there are earlier reports that are consistent with the induction of Rad51 expression by IR (Haaf *et al*, 1995). Interestingly, we found Rad51 was similarly induced in both irradiated and bystander tissues even 4 days after irradiation, suggesting a persistent upregulation of DSB repair capacity. The cellular repercussions of this finding will have to be further elucidated, since it was recently shown that overexpression of Rad51 can lead to chromosome rearrangements and genome instability (Richardson *et al*, 2004).

2.5.2 DNA Methylation

Although it is hypothesized that bystander effects result from epigenetic changes to cells, the precise mechanism by which bystander effects are induced and maintained is not known. In this study we found a decrease in global methylation in irradiated skin but not in bystander skin. The observed decrease in global DNA methylation in irradiated skin 6 hours after irradiation correlated (r^2>0.9) with the accumulation of strand breaks, as monitored by the ROPS assay, as well as with the increase in recombination activity (r^2>0.9). Thus, the loss of methylation in cells from directly irradiated tissue may be related to the repair status

of these cells. Indeed, it is known that DNA polymerases involved in repair and recombination incorporate cytosine but not methylcytosine during repair synthesis (Pogribny *et al*, 2005). Thus, the induction of DNA lesions and subsequent activation of DNA repair and recombination mechanisms may result in DNA hypomethylation (Pogribny *et al*, 2005; Koturbash *et al*, 2005; Pogribny *et al*, 2004a). We have previously shown that suppression of DNA methylation caused by exposure to gamma-irradiation is linked to activation of DNA repair (Pogribny *et al*, 2004a). Another possible mechanism by which irradiation could reduce the levels of DNA methylation could be that DNA damage interferes with the ability of DNA methyltransferases to methylate DNA (Panayiotidis *et al*, 2004; Turk *et al*, 1995). However, it seems unlikely that the number of DNA lesions induced under these conditions would yield detectable changes in global methylation.

De novo methyltransferases function primarily as regulators of cell fate and differentiation. Interestingly, we found a decrease in the expression of DNA methyltransferases involved in *de novo* methylation, DNMT3a and 3b, in both irradiated and bystander tissues. It is well established that clinical exposure to radiation therapy can induce cutaneous injury which involves complex physiological changes. Thus, one possibility is that the suppressed levels of DNMT3a and 3b reflect an early injury response. Interestingly, previous studies show a correlation between DNA hypomethylation and suppression of DNMT3a and 3b in mouse liver, spleen and thymus following whole-body exposure to X-rays (Pogribny *et al*, 2005; Raiche *et al*, 2004).

In contrast to the suppression of *de novo* methyltransferase levels, we observed an increase in the levels of the methyltransferase responsible for maintaining DNA methylation patterns, DNMT1, only in bystander tissues. It is interesting that global methylation was suppressed

in directly irradiated tissue, but not in bystander. One possibility is that increased levels of DNMT1 may offset the radiosuppression of DNA methylation that was observed in directly irradiated tissue. Along with the increased levels of DNMT1 in bystander tissue, we also found that the levels of MeCP2 and MBD2, proteins involved in transcriptional silencing, were increased in bystander but not irradiated skin. Although additional studies are necessary to delineate the potential biological significance and persistence of these shifts in protein levels, these results demonstrate that there are significant physiological changes in distant unexposed tissue of animals exposed to X-irradiation.

2.6 FIGURES AND TABLES.

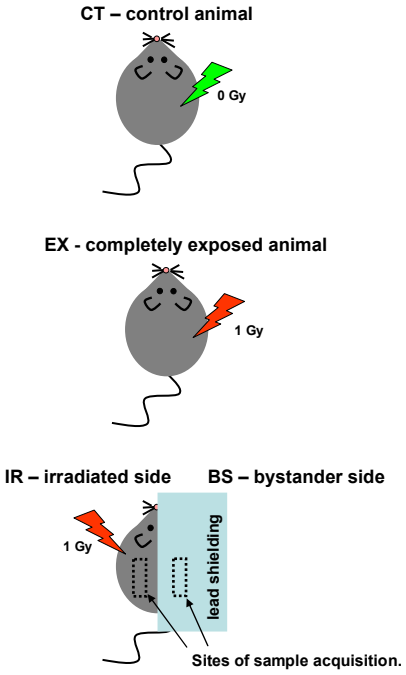

Figure 2.1. Induction of *in vivo* bystander effect.

Animals had leaded shielding covering half of their bodies during exposure to 1 Gy of X-rays. Animals in the un-irradiated control cohort were sham treated. Animals in the fully irradiated cohort received 1 Gy of whole body X-ray exposure. CT – un-irradiated skin; IR – skin from fully irradiated animals; IR½– skin from the irradiated side of hemi-shielded animals; BS ½ – skin from the shielded, bystander side of hemi-shielded animals. Note that ventral skin was taken from the area adjacent to the thigh, thus bystander samples were taken at least 0.7 cm from the edge of irradiated side.

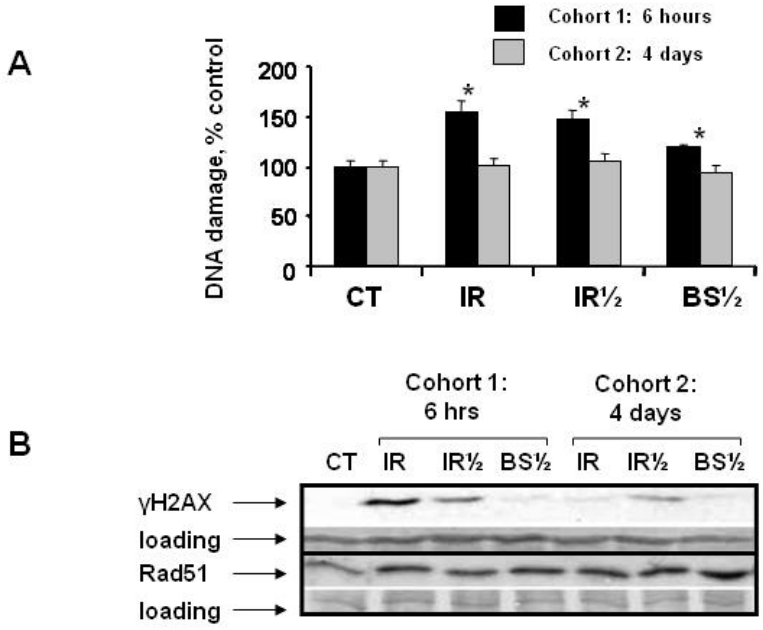

Figure 2.2. DNA damage in irradiated and bystander skin.

A. Levels of DNA strand breaks were revealed by a modified ROPS assay (see Materials and Methods). Separate cohorts were analyzed 6 hours and 4 days after exposure. Levels of DNA strand breaks are presented as mean values ± SEM, n=10; *denotes statistically significant difference compared to controls ($p<0.05$), paired Student's *t*-test.

B. Lysates from cutaneous tissue were immunoblotted using antibodies against RAD51and γH2AX. Representative blots from 3 independent experiments; each experiment included pooled lysates from 5 animals for each exposure condition, with equal representation of each animal.

CT, IR, IR½ and BS ½ are as described in Figure 2.1.

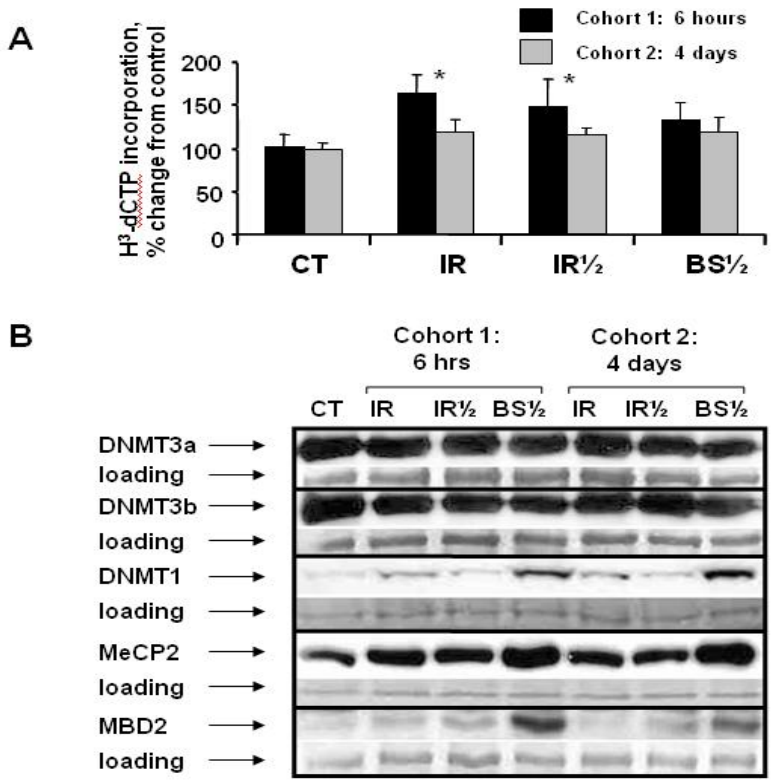

Figure 2.3. Epigenetic effects of radiation exposure.

A. Relative levels of global DNA methylation where an increase in 3H-dCTP indicates a decrease in global methylation in cutaneous tissues of un-irradiated control, fully irradiated, and the irradiated and bystander sides of animals 6 hrs and 4 days after exposure.

B. Western analysis of DNMT1, DNMT3a, DNMT3b, MeCP2, MBD2. Protein levels relative to loading controls were compared and the statistically significant fold changes relative to untreated sham controls are shown. Representative blots from 3 independent experiments; each experiment included cohorts of 5 animals for each exposure condition, with equal representation of each animal. Each lane represents pooled lysates from 5 animals; experiments were repeated 3 times independently.

CT, IR, IR½ and BS ½ are as described in Figure 2.1.

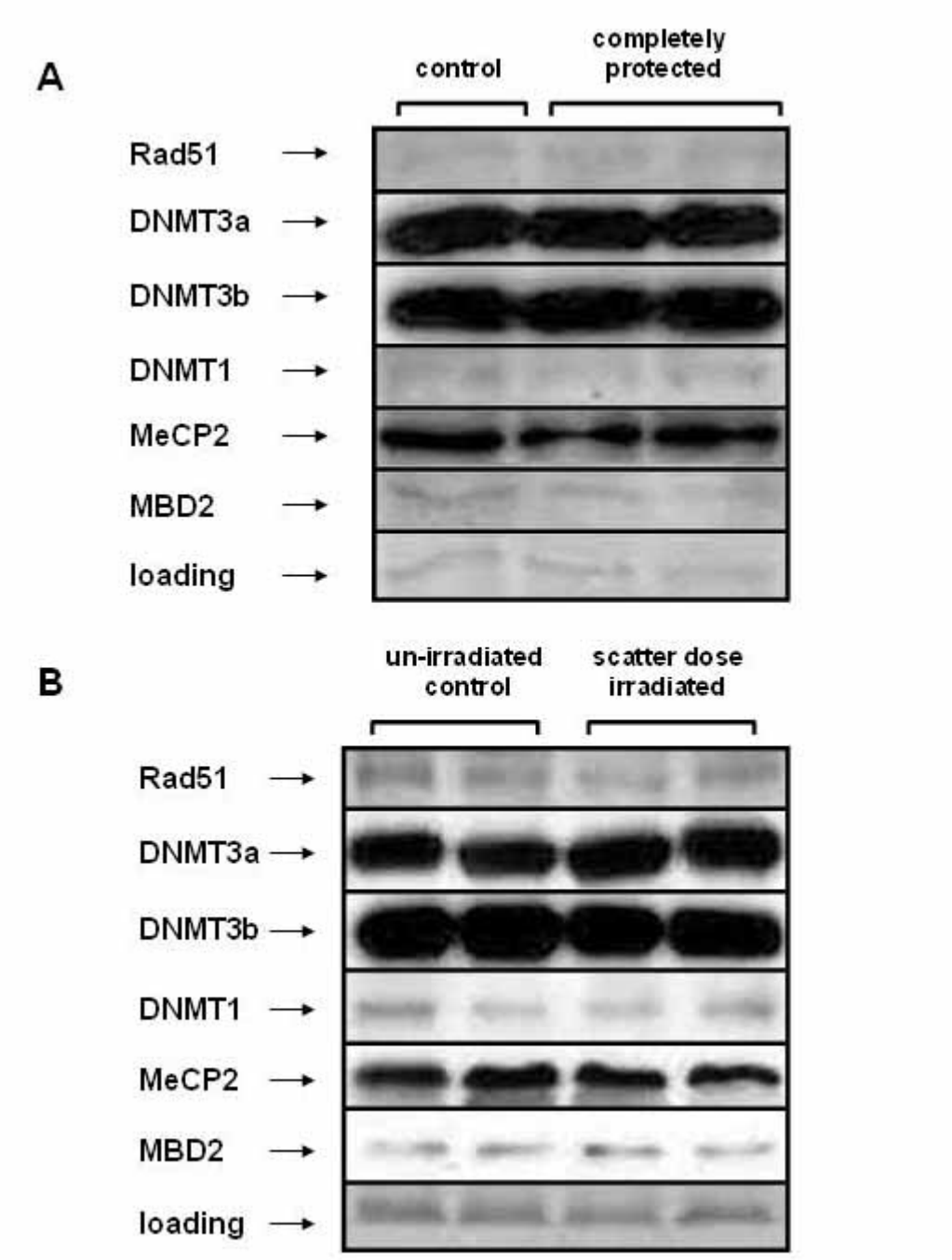

Figure 2.4. Bystander effects are not due to insufficient shielding or radiation scattering.

A. Western analysis of fully shielded animals. Animals in the control cohort were sham treated. Animals of the shielded cohort were exposed to 1 Gy of X-rays while there bodies

were completely shielded with lead. Representative blots from 3 independent technical repeats are shown; each experiment included cohorts of 5 animals for each exposure condition, with equal representation of each animal

B. Western analysis of animals exposed to the approximate dose expected from scatter. Animals in the control cohort were sham treated. Animals of the scatter-dose exposed cohort received 0.013 Gy dose (see Materials and Methods). Representative blots from among 3 independent technical repeats are shown; each experiment included pooled lysates from 5 animals for each exposure condition, with equal representation of each animal.

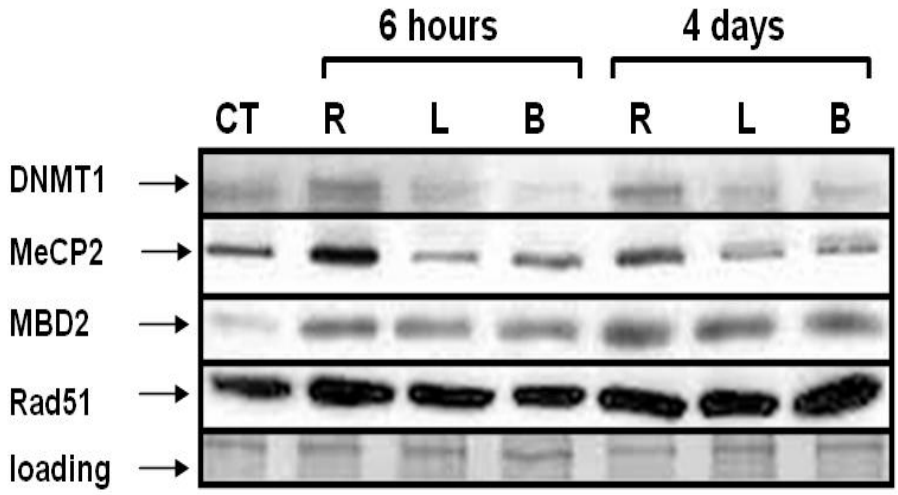

Figure 2.5. Role of internal organ exposure in the generation of bystander effects.

Animals were irradiated as described in the text, 3 animals per treatment group. Lysates from the control and shielded/bystander cutaneous tissue were immunoblotted using antibodies against DNA repair and DNA methylation-related proteins. CT – lysate from control animal skin; R – lysate from the right body side shielded skin (left side was irradiated); L - lysate from the left body side shielded skin (right side was irradiated); B - lysate from the lower animal body shielded skin (head was irradiated). Protein levels relative to loading controls were compared. Representative blots from among 3 independent technical repeats are shown; each experiment included pooled lysates from 3 animals for each exposure condition, with equal representation of each animal.

3. IN VIVO BYSTANDER EFFECT: CRANIAL X-IRRADIATION LEADS TO ELEVATED DNA DAMAGE, ALTERED CELLULAR PROLIFERATION AND APOPTOSIS, AND INCREASED P53 LEVELS IN SHIELDED SPLEEN[1]

[1]Chapter 3 has been published in its entirety:

Koturbash I, Loree J, Kutanzi K, Koganow C, Pogribny I, Kovalchuk O. (2008) In vivo bystander effect: cranial X-irradiation leads to elevated DNA damage, altered cellular proliferation and apoptosis, and increased p53 levels in shielded spleen. *Int J Radiat Oncol Biol Phys.*, **70**:554-62

3.1 ABSTRACT

It is well accepted that irradiated cells may 'forward' genome instability to non-irradiated neighboring cells, giving rise to the 'bystander effect' phenomenon. While bystander effects are well studied using cell cultures, data on somatic bystander effects *in vivo* are relatively scarce. We set out to analyze the existence and molecular nature of bystander effects in a radiation target organ spleen using a mouse model. The animal's head was exposed to X-rays while the remainder of the body was completely protected by a medical grade shield. Using immunohistochemistry, we addressed the levels of DNA damage, cellular proliferation, apoptosis, and p53 protein in the spleen of control animals, completely exposed and head exposed /body bystander animals. We have found that the localized head radiation exposure leads to the induction of bystander effects in the lead-shielded distant spleen tissue. Namely, cranial irradiation leads to the elevated levels of DNA damage and increased p53 expression, it also alters the levels of cellular proliferation and apoptosis in bystander spleen tissue. The observed bystander changes were not caused by radiation scattering, and were observed in two different mouse strains, C57BL/6 and BALB/c. Our study proves that bystander effects occur in distant somatic organs upon localized exposures. Further studies are required to characterize the nature of an enigmatic bystander signal, to analyze the long-term persistence of these effects, and the possible contribution of radiation-induced bystander effects to secondary radiation carcinogenesis.

Key words: bystander effect, mouse model, apoptosis, proliferation, DNA damage, γH2AX, p53

3.2 INTRODUCTION

Bystander effects are now accepted as ubiquitous consequences of radiation exposure (Little, 2006; Mothersill *et* Seymour, 2004; Morgan, 2003a). They are very well studied using cell cultures (Little, 2006; Sokolov *et al*, 2005; Yang *et al*, 2005; Mothersill *et* Seymour, 2004; Morgan, 2003a). Using cell culture-based assays, it has been shown that irradiated cells can elicit myriads of molecular responses in the neighboring bystander cells that have never been exposed to radiation. These responses can lead to a persistent genome destabilization, and ultimately may contribute to carcinogenesis (Mothersill *et* Seymour, 2004, Mothersill *et al*, 2004; Hall, 2003; Ward, 2002; Zhou *et al*, 2002a; Sawant *et al*, 2001; Zhou *et al*, 2000). Overall, 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 (Little, 2006; Morgan, 2003a; Morgan, 2003b; Nagasawa *et* Little, 2002). They also influence gene expression, cellular proliferation, senescence and cell death (Sedelnikova *et al*, 2007; Chaudhry, 2006; Belyakov *et al*, 2003; Belyakov *et al*, 2002).

Compared to the bystander effect data based on cell culture, the conclusive data on somatic bystander effects *in vivo* are relatively scarce (Koturbash *et al*, 2006b; Hall, 2003; Goldberg *et* Lehnert, 2002). It was shown that radiation exposure can lead to the release of soluble 'clastogenic' factors into circulating blood. These factors are capable of inducing chromosome damage in cultured cells (Marozik *et al*, 2007; Emerit *et al*, 1995; Emerit *et al*, 1994; Pant *et* Kamada, 1977; Hollowell *et* Littlefield, 1968; Goh, 1968). Such clastogenic activity was found in plasma of patients receiving high dose

radiotherapy and individuals accidentally exposed to radiation (Marozik *et al*, 2007; Emerit *et al*, 1995; Emerit *et al*, 1994; Pant *et* Kamada, 1977; Hollowell *et* Littlefield, 1968; Goh, 1968). Bystander effects were shown to be important 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*, 2003; Khan *et al*, 1998). It was also shown that when one lung, either right or left, was exposed, the marked increase of micronuclei was noted in the unexposed shielded lung (Khan *et al*, 2003; Khan *et al*, 1998). Similar within-the-organ bystander effects were observed during partial liver irradiation (Brooks *et al*, 1974).

We have 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*, 2006b). The data showed that X-ray exposure to one side of the animal body caused profound epigenetic changes in the unexposed bystander body half 4 days after exposure.

Interestingly, the data obtained in the mouse skin model showed that bystander effects were the most prominent when spleen was in the field of irradiation, suggesting a possible role of spleen in the bystander effects (Koturbash *et al*, 2006b). Spleen is an important radiation-target organ (Koturbash *et al*, 2005; Pogribny *et al*, 2004a), yet the existence, molecular and cellular mechanisms of the radiation-induced bystander effects in spleen need to be further addressed. The overall data on bystander effects in organs other than exposed ones are scarce.

Thereafter, we set out to analyze the existence and molecular nature of bystander effects in a radiation target organ spleen using a mouse model, whereby an animal head

was exposed to X-rays, while the animal body was completely protected by a medical-grade lead shield. To investigate the possibility that X-irradiation induces DNA damage in bystander tissue *in vivo*, we monitored the induction and repair of DNA strand breaks in spleen tissue. We also explored changes in the levels of cellular proliferation, apoptosis and p53 in the bystander spleen. Here, we report that cranial irradiation leads to the elevated levels of DNA strand breaks, altered levels of p53 protein, cellular proliferation and apoptosis in the bystander spleen in two different strains of laboratory mice.

3.3 MATERIALS AND METHODS

3.3.1 Model and Irradiation of Animals

In this study we examined the molecular alterations in spleen of C57BL/6 and BALB/c strains following *in vivo* cranial irradiation exposure. The 50 days old male mice (30 C57BL/6 and 30 BALB/c animals) were randomly assigned to different treatment groups. Handling and care of animals was in strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures have been 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 whole body exposed cohort (10 BALB/c and 10 C57BL/c animals) received 1 Gy of whole body exposure to X rays (5cGy/s, 90kV, 5mA). The second cohort (10 BALB/c and 10 C57BL/c animals) received 1 Gy of X rays exposure to scull only, while the rest of animal body were protected by a ~3 mm thick lead shield, the same type as used for a human body protection in diagnostic radiology. The protection of shielded 'bystander' tissue was complete, as verified by the careful dosimetry using RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, FL, USA). Control mice (10 BALB/c and 10 C57BL/c animals) were sham treated. The animals were humanely sacrificed either 6 hours or 96 hours (4 days) after exposure. These time points were chosen to analyze the initial (6 hours) as well as persistent (4 days) responses, if any. Spleen tissue was sampled upon sacrifice and processed for the further molecular studies.

The experiment was independently reproduced according to the same scheme using 18 C57BL/6 and 18 BALB/c animals. Both experiments yielded congruent results.

Another cohort of 10 animals was exposed to an approximate scatter dose of ~0.01Gy. To determine the bystander ventral//spleen/thigh skin dose resulting from photon scatter within the mouse itself, a Monte Carlo simulation was performed (X-5 Monte Carlo Team, 2003). The skin was assumed to be 0.1 cm thick, and a 2 mm thick lead shield covered one half of the mouse. An absorbed dose was tallied within the skin region in several locations in both the unshielded dorsal regions and the shielded ventral/thigh regions. The absorbed dose to the ventral skin/spleen located right beneath the skin from a 90 kVp X-ray spectrum was determined to be approximately 0.014 – 0.017 Gy for a 1 Gy dose delivered to the dorsal skin (Koturbash *et al*, 2006b). The animals were sacrificed 6 hours after exposure.

3.3.2 DNA Damage Analysis – Histone γH2AX Foci Accumulation

The spleen tissue was touch-printed onto the positively charged slides (VWR, Mississauga, ON), air-dried and fixed in 2% PFA in PBS. Upon fixation, slides were immunostained using antibodies against phosphorylated histone H2AX, as described (Nakamura *et al*, 2006; Koturbash *et al*, 2006a; Sedelnikova *et al*, 2004). Foci were counted by eye in a blinded fashion by two independent investigators. At least 100 cells from the each studied tissue of each animal were examined as recommended. Examinations were repeated 3 times. The data are presented as a mean number of foci per cell ± a standard error of the mean.

3.3.3 Proliferation Analysis – Ki67 and PCNA Immunohistochemistry

Paraffin embedding and sectioning was conducted at Central Vet Labs, Edmonton, AB. Automated staining for Ki67 was conducted at the Calgary Central

Lab Services in accordance with the standard developed protocols and using tonsil tissue as a positive control for the stain quality (Cassie *et al*, 2006). In brief, upon the citrate buffer epitope retrieval slides were rinsed and subjected to serum blocking to prevent the non-specific binding of immunoglobulin. Sections then were incubated with mouse anti-human Ki67 (DakoCytomation, Carpinteria, CA) rinsed and subjected to peroxidase blocking. Following the peroxidase blocking, the slides were incubated with the secondary biotinylated antibody, subjected to HRP-Streptavidin detection and counterstained with hematoxylin**.** Cells positive for Ki67 presented as red/pink, while negative cells stained blue. Tonsil tissue served as a positive control. Ki67 index was quantified by enumerating Ki67-positive cells in, at least, 20 high power fields. Amount of Ki67 positive cells relative to those of control animals are shown as the mean $\pm$ SD; $*p < 0.05$.

Automated staining for PCNA was conducted by Calgary Central Lab Services using primary anti-PCNA antibody (Novocastra, Newcastle, UK). Upon secondary antibody application, HRP-Streptavidin detection and counterstaining with hematoxylin, the cells positive for PCNA stained brown/purple, while the negative cells stained blue. Tonsil tissue served as a positive control. PCNA index was quantified by enumerating PCNA-positive cells in, at least, 20 high power fields. Levels of PCNA positive cells relative to those of the control animals are shown.

3.3.4 Analysis of Apoptosis

Paraffin embedding and sectioning were conducted at Central Vet Labs, Edmonton, AB. Automated staining for apoptosis was conducted using the

Peroxidase In Situ Apoptosis Detection Kit® (Chemicon, Temecula, CA) according to the manufacturer's instructions. The kit detects apoptotic cells in situ by labelling and detecting DNA strand breaks by the TUNEL method. Apoptosis was quantified by enumerating apoptotic cells in, at least, 20 high power fields. Levels of the apoptotic cells relative to those of the control animals are shown as the mean $\pm$ SD; $*p < 0.05$.

3.3.5 p53 Immunohistochemistry

Paraffin embedding and sectioning were conducted at Central Vet Labs, Edmonton, AB. Automated staining for p53 was conducted using an anti-p53 antibody (CM1) Novocastra, Newcastle, UK). This antibody recognizes both wild type and mutant forms of human p53 protein under denaturing and non-denaturing conditions and can not discriminate between functional and non-functional forms of protein. Upon the secondary antibody application, DAB detection and counterstaining, cells positive for p53 stained dark brown, while the negative cells stained light pink/orange. Tonsil tissue served as a positive control. High expressing p53 index was quantified by enumerating p53-positive cells in, at least, 20 high power fields. The number of the strongly p53 positive cells relative to those of the control animals is shown.

3.3.6 Statistical Analysis

Statistical analysis (Student's *t*-test, Tukey-Kramer test and Dunnett's test) was performed using the MS Excel 2000 and JMP5 software packages.

3.4 RESULTS

3.4.1 Accumulation of DNA Damage in Bystander Spleen Tissue In Vivo

Prior studies showed a pronounced induction of DNA strand breaks (DSBs) in bystander cells (Sokolov *et al*, 2005). Thus, we decided to measure strand breaks in bystander spleen tissue *in vivo*. We analyzed bystander effects *in vivo* by placing lead shielding over the mouse body, that only a head was exposed before exposure to 1 Gy of X-rays (Fig. 3.1). The lead shielding used for these studies is the same as used for patients exposed to radiation in the clinic. The same type of shielding was used in the published studies on the bystander effect in mouse skin (Koturbash et al, 2006b). Complete protection of shielded bystander tissue from radiation exposure was verified by the careful dosimetry (see Materials and Methods).

DSBs were analyzed in both irradiated and shielded (bystander) spleen from mice that had been exposed to 1 Gy of X-rays, while their bodies (from the neck down) were protected by a lead shield (Fig. 3.1). To analyze the presence of the DSBs, we assayed the presence of phosphorylated form of histone H2AX (γH2AX). H2AX, a variant of histone H2A, is rapidly phosphorylated at Ser139 in the chromatin micro-environment surrounding a DNA double-strand break (Paull *et al*, 2000; Rogakou *et al*, 1998). Indeed, within minutes after exposure to radiation, phosphorylated H2AX can be visualized within the repair foci using phospho-specific antibodies (Nakamura *et al*, 2006; Koturbash *et al*, 2006a; Sedelnikova *et al*, 2004; Paull *et al*, 2000; Rogakou *et al*, 1998). Quantification of phosphorylated histone H2AX foci (γH2AX foci) constitutes a sensitive and reliable method to quantify radiation-induced DNA damage (Paull *et al*, 2000; Rogakou *et al*, 1998).

Using this method, we noted that whole body X-ray exposure led to a significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in levels of γH2AX foci in spleen of C57BL/6 and BALB/c mice. There was a less pronounced, but statistically more significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in γH2AX foci in the 'bystander' spleen of the head-exposed mice. This indicates an increase in DSBs in bystander tissue. However, 4 days following irradiation, the DSBs were repaired.

3.4.2 Radiation-induced Changes in Apoptosis and Cellular Proliferation in Bystander Spleen In Vivo

In a variety of cell culture- and explant-based assays, bystander effects were shown to affect cellular proliferation and apoptosis (Belyakov *et al*, 2003; Belyakov *et al*, 2002). In this study, we addressed the levels of apoptosis and cellular proliferation in the spleen of control, completely exposed and head-exposed animals.

The whole body exposure led to a significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in the number of apoptotic cells in spleens of both mouse strains 6 hours and 4 days after exposure (Fig. 3.3A). Cranial exposure led to a significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) upregulation of apoptosis in bystander spleen tissue of C57BL/c mice but only a slight increase in the BALB/c strain. The observed elevated levels of apoptosis could be linked to the elevated levels of DNA damage.

For proper functioning, cells have to maintain a tight balance between cell death and proliferation. We studied whether cranial exposure led to alterations in cellular

proliferation levels. Immunohistochemistry techniques (Ki67) revealed that both the whole-body and cranial irradiation led to a significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in cellular proliferation in spleens of both animal strains. Ki67 is a nuclear protein expressed during late G1-, S-, G2-, and M-phases of the cell cycle, while cells in the G0 (quiescent) phase are negative for this protein (Cassie *et al*, 2006; Scholzen *et* Gerdes, 2000). Levels of Ki67 positive cells stayed elevated up to 4 days post-exposure. We also analyzed the levels of proliferating cell nuclear antigen (PCNA), an S-phase-specific protein (Maga *et* Hubscher, 2003). We did not find any significant changes in the levels of PCNA positive cells (data not shown). Therefore, we concluded that the increase in the number of the Ki67 positive cells may be due to the G1/G2 blocks, which are often seen upon exposure to DNA damaging agents (Cassie *et al*, 2006; Ishikawa *et al*, 2006).

3.4.3 Radiation Exposure Resulted in the Elevation of p53 Levels in the Exposed and Bystander Spleen Tissue

It is well accepted that p53 is a central mediator of radiation responses. Irradiation was shown to upregulate and activate p53, which can consequently induce transcription of a variety of downstream targets triggering DNA repair, cell cycle arrest, senescence and apoptosis (Fei *et* El-Deiry, 2003; Iliakis *et al*, 2003).

Having seen the induction of DNA damage and alterations in the cellular proliferation and apoptosis levels, we decided to relate these changes to p53 levels in directly irradiated and bystander cells. To do this, we analyzed the levels of p53 using immunohistochemistry. We noted that whole body irradiation led to statistically

significant p53 up-regulation 6 and 96 hours after exposure in spleen of both animal strains. Cranial irradiation also up-regulated p53 in the bystander spleen, but to a less extent than the whole-body irradiation. The up-regulation trend seen in the bystander spleen of BALB/c animals 96 hours after cranial irradiation was significant at a 90% confidence limit (Fig. 3.4). The observed p53 upregulation can be correlated with the observed proliferation and cell cycle arrest changes, elevated apoptosis, and repair of DNA damage.

3.4.4 Bystander Effects are Not the Result of Insufficient Shielding or Radiation Scattering

While passing through the tissue, X-rays can be reflected forming a small 'scatter' dose in the protected tissue. To check if the possible small scatter dose could contribute to the generation of bystander effects in spleen, we exposed a separate cohort of animals to the scatter dose of ~0.01Gy calculated according to the Monte Carlo simulation and directly measured by RAD-CHECK^{TM} (Koturbash *et al*, 2006b; X-5 Monte Carlo Team, 2003). We noted that the scatter dose application did not result in any changes in DNA damage, cellular proliferation and apoptosis in animal spleen (Fig. 3.5).

As an extra biological measure of effective shielding, the fully shielded mice were exposed to 1 Gy X-rays, and spleen was analyzed for the levels of the γH2AX protein, cellular proliferation, apoptosis and p53. Then we compared these parameters with the unexposed controls. No changes were found (data not shown).

3.5 DISCUSSION

In this report we describe a significant *in vivo* bystander effect that occurs in a mouse spleen upon cranial irradiation. The main findings of the present study are: (i) cranial X-ray irradiation results in the induction of bystander effect in the lead-shielded distant spleen tissue; (ii) cranial X-ray irradiation induces DNA damage in bystander spleen tissue; (iii) cranial exposure also results in the altered levels of cellular proliferation, apoptosis and expression of p53 protein in the bystander spleen tissue; (iv) the observed changes in the shielded bystander tissue are not due to the radiation scattering, they are observed in two different strains of mice – C57BL/6 and BALB/c.

Currently, firm and conclusive evidence that IR-induced bystander effects *in vivo* are operational is still limited. The literature evidence suggests that bystander effects may exist and have important biological consequences within an organ, as shown by irradiation of lung field and liver sectors (Khan *et al*, 2003; Khan *et al*, 1998; Brooks *et al*, 1974). Soluble clastogenic factors in blood following radiation exposure were shown to induce bystander effects (Marozik *et al*, 2007; Emerit *et al*, 1995; Emerit *et al*, 1994; Pant *et* Kamada, 1977; Hollowell *et* Littlefield, 1968; Goh, 1968). Bystander effects were also reported in the artificial human 3D tissue models upon α particle irradiation (Sedelnikova *et al*, 2007). Yet, bystander effects in distant somatic organs following localized exposure need to be further studied, especially considering their relevance to radiation-induced secondary malignancies (Marozik *et al*, 2007; Hall, 2003; Goldberg *et* Lehnert, 2002; Emerit *et al*, 1995; Emerit *et al*, 1994; Pant *et* Kamada, 1977; Hollowell *et* Littlefield, 1968; Goh, 1968).

Our laboratory has recently developed an *in vivo* model, where bystander effects were studied in the skin of mice subjected to half-body or head IR exposure, while the rest of the body is protected by a medical grade lead shield. The data obtained in this model showed that bystander effects were the most prominent when the spleen was in the field of irradiation, suggesting a possible role of the spleen in bystander effects (Koturbash *et al*, 2006b). Furthermore, some early bystander effect studies reported changes in the sternal bone marrow of children who received spleen irradiation as a part of leukemia treatment (Parsons *et al*, 1954). Therefore, we hypothesized that spleen, an organ of utmost significance for direct radiation effects, may also be targeted by the bystander responses.

As shown in the present study, spleen indeed is an important target organ for bystander effects. The bystander effects observed in the spleen tissue were characterized by a profound induction of DNA damage. This is in agreement with the existing literature (Sedelnikova *et al*, 2007; Sokolov *et al*, 2005; Mothersill *et* Seymour, 2004) and with our previous report on the *in vivo* bystander effect in skin (Koturbash *et al*, 2006b). Bystander effects have been shown to result in the DNA damage accumulation in the cell culture, 3D tissue models, and in the bystander liver and lung tissue (Sedelnikova *et al*, 2007; Koturbash *et al*, 2006b; Sokolov *et al*, 2005; Mothersill *et* Seymour, 2004; Khan *et al*, 2003; Khan *et al*, 1998).

There can be several possible, not mutually exclusive ways of an indirect DNA damage induction in bystander spleen tissue. They can be mediated by reactive oxygen species, via inflammatory cytokines or via other factors secreted by IR-damaged blood cells (reviewed in Little, 2006; Mothersill *et* Seymour, 2004; Morgan, 2003a). Amongst

those factors, microRNAs and other epigenetic effectors may be especially important (Koturbash *et al*, 2007).

Previous studies addressed the molecular changes in bystander tissues. The associated cellular changes have not been addressed in depth. This study is the first attempt to analyze the levels of cellular proliferation and apoptosis in the bystander spleen tissue *in vivo*. Here we show that the observed induction of DNA damage was paralleled by the significant elevation of apoptosis and Ki67 index in the bystander spleen tissues. However, no changes were noted in the levels of PCNA positive cells. Ki67 is a nuclear protein expressed in all cell cycle phases but the G0 (quiescent) phase. PCNA, on the other hand, is an important DNA replication protein which is strongly elevated in S phase. Radiation exposure was proven to induce G1 and G2 arrest (Cassie *et al*, 2006; Ishikawa *et al*, 2006; Fei *et* El-Deiry, 2003; Iliakis *et al*, 2003). Thus, the elevated Ki67 level observed without PCNA upregulation can be interpreted as a sign of a radiation damage-induced cell cycle block in the exposed and bystander spleen tissue (Ishikawa *et al*, 2006). Upregulation of apoptosis and cell cycle block may be viewed as a 'positive' defensive cellular response aimed to halt the cell cycle and provide cells with enough time to repair the damage. These data are in good agreement with previously reported increases of apoptosis in bystander cells and 3D tissue models (Sedelnikova *et al*, 2007; Belyakov *et al*, 2002).

Another important outcome of this study is the observed elevation of p53 levels in irradiated and bystander mouse spleen. Tumor suppressor p53, which is considered to be a guardian of the genome, coordinates DNA repair with cell cycle progression and apoptosis (Fei *et* El-Deiry, 2003). The p53 is required for the G1 checkpoint control. It

also mediates the S phase delay and partakes in the G2/M arrest following irradiation (Niida *et* Nakanishi, 2006; Jeggo *et* Lobrich, 2006; Fei *et* El-Deiry, 2003). The cell cycle arrest gives a window of time to repair the damaged DNA. Cells with unrepairable damage are then eliminated via apoptosis. The loss of p53 is linked to genome instability and carcinogenesis (Fei *et* El-Deiry, 2003). The role for p53 in bystander effects is controversial (Grosovsky, 1999). The evidence of the p53 involvement in bystander effects was initially shown in rat lung epithelial cells following the α-particle irradiation. The analysis revealed that the fraction of p53 positive cells was larger than the number of cells directly hit by α-particles (Hickman *et al*, 1994). Later, these findings were further confirmed by using confluent human fibroblast cultures (Azzam *et al*, 1998). These studies suggested that the observed p53 increases may involve direct cell-cell communications and are disrupted by gap junction inhibitors (Azzam *et al*, 1998; Hickman *et al*, 1994). Medium-transfer based bystander effect studies showed the opposite effects (Iyer *et* Lehnert, 2000). Exposure of the naïve human fibroblasts to the medium of the exposed cells led to the decreased p53 levels (Iyer *et* Lehnert, 2000). The reduced p53 levels were possibly due to elevated proliferation of cells with low p53 levels (Iyer *et* Lehnert, 2000). Alternatively, the downregulation of p53 levels may be a 'compensatory' mechanism aimed to reduce the gap junction mediated p53 increase (Iyer *et* Lehnert, 2000).

Our study in the first to show that *in vivo* cranial irradiation results in the elevated levels of p53 in the distant bystander spleen. The upregulation of p53 was persistent and correlated with the observed cell cycle, DNA repair and apoptosis changes. The mechanisms leading to the upregulation of p53 in bystander spleen need to be further

investigated. Also, the antibody used in the current study detects both functional and non-functional forms of p53 (Mothersill *et al*, 1994). In the future, more studies will be needed to dissect the roles of p53 modifications in bystander tissue.

Importantly, congruent with previous reports (Koturbash *et al*, 2007; Koturbash *et al*, 2006b), the bystander effects observed in this study, were not due to radiation scattering or insufficient shielding. In this study we used the same animal body shielding that was previously described (Koturbash *et al*, 2007; Koturbash *et al*, 2006b). The scatter dose for mouse spleen was miniscule, and exposure of animals to the scatter dose did not result in the altered levels of DNA damage, p53, cellular proliferation or cell death. Notwithstanding, the role of small scatter doses has to be further analyzed, since some other studies have shown that doses within the range of the scatter doses induced bystander effects in the cell culture based assays (Maguire *et al*, 2005; Mothersill *et al*, 2005; Kashino *et al*, 2004; Mothersill *et* Seymour, 2002).

Another central outcome of this study is the fact that comparable radiation-induced bystander effects were observed in two strains of mice. The unexpected congruency of the responses between the 2 strains was somewhat surprising. On the one hand, these strains are known to respond differently to radiation. The C57Bl/6 strain usually has a strong apoptotic response, while BALB/c strain is very susceptible to radiation lymphomogenesis and exhibits radiation-induced proliferation (Lindsay *et al*, 2007; Kataoka *et al*, 2006; Wallace *et al*, 2001; Mori *et al*, 2001). On the other hand, the recent study by Rogers and colleagues (2001) has reported a comparable induction of micronuclei in both strains upon irradiation (Rodgers *et al*, 2001). In the future more

studies are needed to define strain- as well as tissue-, dose- and exposure regime-specificities of the direct and indirect radiation responses.

These data, together with the recently reported bystander effects in mouse skin (Koturbash *et al*, 2006b) and in rat spleen (Koturbash *et al*, 2007), strongly suggest that somatic radiation-induced bystander effects that can be seen in distant organs upon half-body or cranial exposure, and they indeed represent a cross-species phenomenon. Thus, these data can potentially be extrapolated to other species and, most importantly, to humans.

Further studies are required to analyze the nature of the enigmatic bystander signal and the possible contribution of radiation-induced bystander effects to the secondary radiation carcinogenesis and cancer treatment (Mothersill *et* Seymour, 2006; Mothersill *et* Seymour, 2004, Hall, 2003; Goldberg *et* Lehnert 2002).

3.6 FIGURES AND TABLES

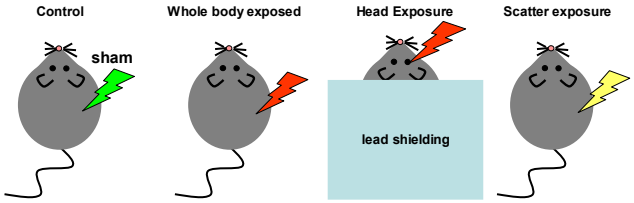

Figure 3.1. Induction of *in vivo* bystander effect in mouse spleen.

The whole-body-exposed cohort of animals received 1 Gy of whole body exposure to X-rays. The head-exposed cohort received 1 Gy of X-ray exposure to the skull only while the rest of the animal body was protected by an approximately 3-mm thick lead shield. The scatter-exposure cohort was exposed to an approximate scatter dose of 0.01 Gy. Control mice were sham treated.

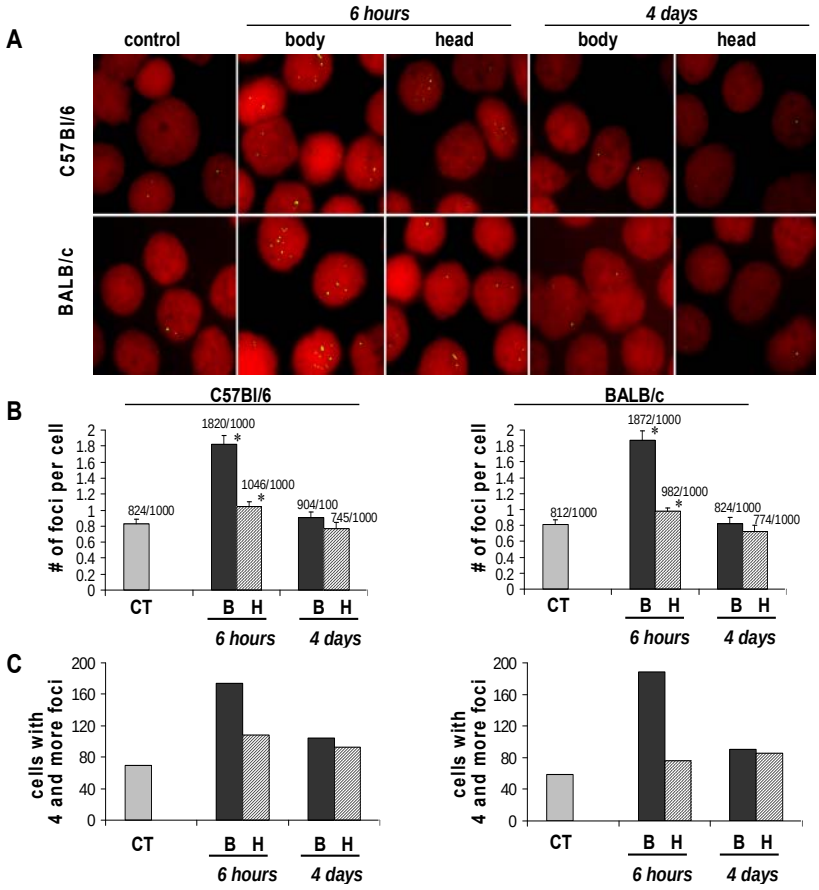

Figure 3.2. Radiation-induced accumulation of γH2AX foci in exposed and bystander murine spleen.

A. γH2AX foci in the cells.

B. Number of γH2AX foci per cell, mean values ± SEM, n= 1,000; *p<0.05, Student's *t*-test, Dunnett's test and Tukey-Kramer test. Numbers above bars show the total amount of spots/total amount of cells scored.

C. Number of cells with four and more foci of the total amount of cells scored.

CT - control animals, grey bars; B - body exposed animals, black bars; H – head exposed animals, dashed bars.

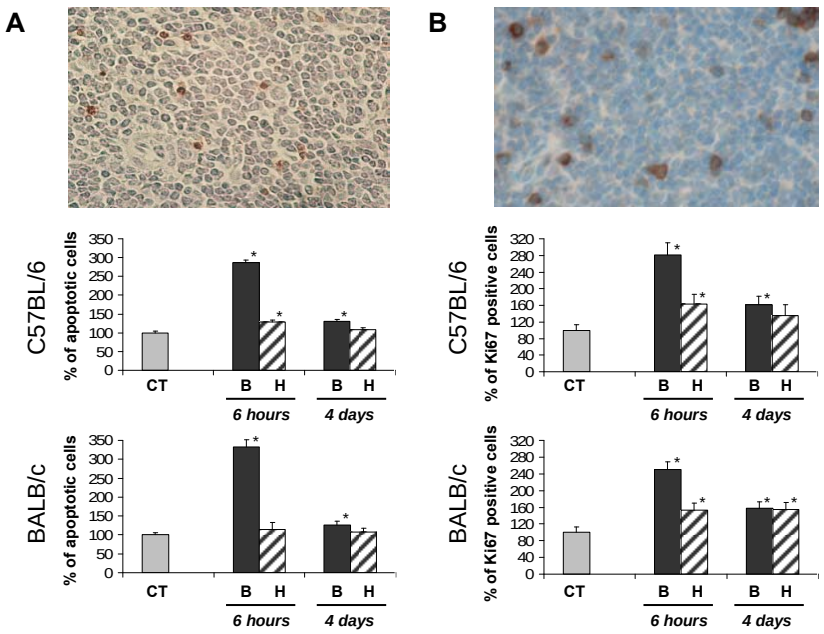

Figure 3.3. Changes in the cellular proliferation and apoptosis levels in the exposed and bystander murine spleen.

A. Levels of cellular proliferation as measured by Ki67 immunohistochemistry (IHC). Levels of Ki67 positive cells relative to control are shown (control levels taken as 100%). Mean values ± SEM, n= 1,000; *p<0.05, Student's t-test, Dunnett's test and Tukey-Kramer test. CT - control animals, grey bars; B - body exposed animals, black bars; H – head exposed animals, dashed bars.

B. Apoptosis levels as measured by ApopTag^{TM} IHC. Levels of apoptotic cells relative to control are shown (control levels taken as 100%). Mean values ± SEM, n= 1,000, *p<0.05, Student's t-test, Dunnett's test and Tukey-Kramer test.

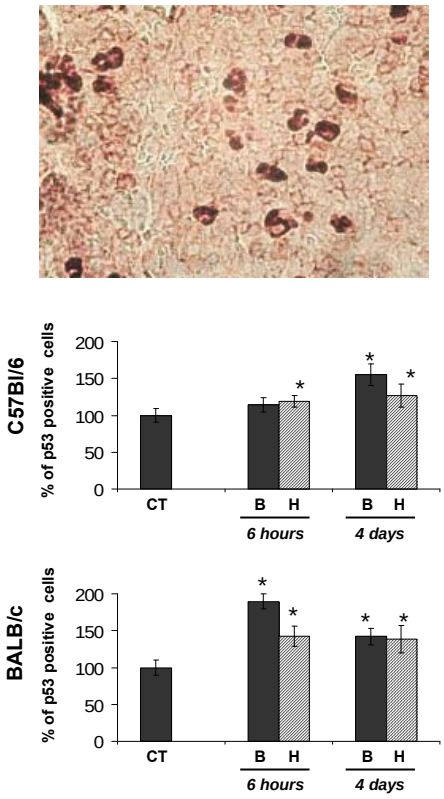

Figure 3.4. Changes in the levels of p53 positive cells in the exposed and bystander murine spleen.

Levels of p53 positive cells relative to control are shown (control levels taken as 100%). Mean values ± SEM, n=1,000; **p*<0.05, Student's *t*-test, Dunnett's test and Tukey-Kramer test. CT - control animals, grey bars; B - body exposed animals, black bars; H – head exposed animals, dashed bars.

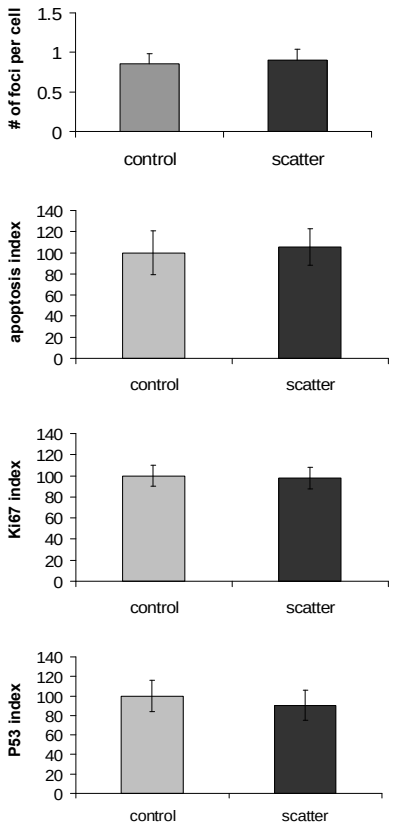

Figure 3.5. Bystander effects in murine spleen did not result from radiation scattering.

Control cohort was sham treated. The scatter-dose exposed cohort received the calculated 'scatter' dose.

4. RADIATION-INDUCED BYSTANDER EFFECTS IN VIVO ARE SEX SPECIFIC[1]

[1]Chapter 4 has been submitted in its entirety:

Koturbash I, Kutanzi K, Hendrickson K, Rodriguez-Juarez R, Kogosov D, Kovalchuk O. (2008) Radiation-induced bystander effects in vivo are sex specific. *Mutat Res.* (in press)

4.1 ABSTRACT

Ionizing radiation (IR) effects span beyond the area of direct exposure and can be observed in neighboring and distant naïve cells and organs. This phenomenon is termed the‘bystander effect’. IR effects in directly exposed tissue in vivo are epigenetically mediated and distinct in males and females. Yet, IR-induced bystander effects have never been explored in a sex-specificity domain.

We used an *in vivo* mouse model, whereby the bystander effects are studied in the spleen of male and female animals subjected to head exposure when the rest of the body is protected by a medical grade lead shield. We analyzed the induction of DNA damage and alterations in global DNA methylation. The molecular parameters were correlated with cellular proliferation and apoptosis levels. The changes observed in the bystander organs are compared to changes in unexposed animals and animals exposed to the predicted and measured scatter doses.

We have found selective induction of DNA damage levels, global DNA methylation, cell proliferation and apoptosis in the exposed and bystander spleens of male and female mice. Sex differences were significantly diminished in animals subjected to surgical removal of the gonads.

These data constitute the first evidence of sex differences in radiation-induced bystander effects in mouse spleen in vivo. We show the role of sex hormones in spleen bystander responses and discuss the implications of the observed changes.

4.2 INTRODUCTION

Over the course of years, the central dogma of radiation biology stated that ionizing radiation (IR) effects were restricted to directly exposed cells. However it was shown that IR effects can also be seen in the naïve cells that were in contact with directly irradiated cells, or in the naïve cells that received certain irradiation 'distress' signals from directly exposed cells via the growth medium (reviewed in Morgan *et* Sova, 2007; Morgan *et* Sova, 2005; Mothersill *et* Seymour, 2004; Morgan, 2003a; Morgan, 2003b). Such an exposure communication process is termed a bystander effect (Morgan *et* Sova, 2007; Morgan *et* Sova, 2005; Mothersill *et* Seymour, 2004; Morgan, 2003a; Morgan, 2003b). A variety of bystander effect studies have been performed using cell culture models (Gaugler *et al*, 2007; Han *et al*, 2007; Maguire *et al*, 2007; Hu *et al*, 2006; Liu *et al*, 2006), tissue (Belyakov *et al*, 2006; Belyakov *et al*, 2003; Belyakov *et al*, 2002), spheroids (Persaud et al, 2005), or reconstructed human tissue models (Sedelnikova *et al*, 2007; Belyakov *et al*, 2005). As a result, bystander effects are accepted as a ubiquitous consequence of IR exposure (Mothersill *et* Seymour, 2004).

Overall, bystander effects encompass a wide range of genetic alterations, including gross genome rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, gene mutations and amplifications (Han *et al*, 2007; Hamada *et al*, 2007; Smilenov *et al,* 2006; Klokov *et al*, 2004; Suzuki *et al*, 2003; Zhou *et al*, 2002; Lorimore *et al*, 2001). They also influence gene expression, cellular proliferation, senescence, and cell death (Lyng *et al*, 2006b; Sawant *et al*, 2002; Sawant *et al*, 2001), and are linked to radiation carcinogenesis (Huang *et al*, 2007; Mothersill *et al*, 2003; Goldberg *et* Lehnert, 2002).

Bystander effects also manifest themselves in the whole-organism environment

(Mothersill *et al*, 2001). Yet, compared to the bystander effect data based on cell cultures, the conclusive data on the somatic bystander effects *in vivo* are relatively uncommon (Goldberg *et* Lehnert, 2002). IR exposure was shown to result in the release of soluble 'clastogenic' factors into the circulating blood of exposed individuals. These factors are capable of inducing chromosome damage in the cultured cells (Marozik *et al*, 2007; Emerit *et al*, 1997; Pant *et* Kamada, 1977; Goh *et* Summer, 1968). The bystander effects were shown to manifest themselves within an exposed organ upon partial organ irradiation. We have recently confirmed the existence of the somatic bystander effects in vivo using rodent skin and spleen models, whereby one part of the animal body was exposed to IR, and another part was protected by a medical grade shield (Brooks, 2004; Khan *et al*, 2003). The data showed that X-ray exposure to one side of the animal body caused profound epigenetic changes in the unexposed bystander parts. The epigenetic changes being implicated in the bystander effects include DNA methylation, histone modification, and RNA-associated silencing (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b).

Interestingly, all the previous rodent model-based bystander studies were conducted using male animals. However, it has been shown that IR-induced secondary cancers occur at different frequencies in males and females (Guizard *et al*, 2001; Yoshida *et al*, 1993). Recent data also show that strikingly different patterns of gene expression and epigenetic changes are induced by IR in various tissues of male and female mice. Additionally, a limited amount of literature suggests that the bystander effects may indeed be distinct in males and females (Raiche *et al*, 2004; Pogribny *et al*, 2004; Kovalchuk *et al*, 2004a; Kovalchuk *et al*, 2004b; Mothersill *et al*, 2001).

Notwithstanding, bystander effects were never explored in a sex-specificity domain (Basnakian *et* James, 1996).

Using a mouse model, we set out to analyze the sex specificity of bystander effects in a radiation target spleen, whereby the animal head was exposed to X-rays, while the animal body was completely protected by a medical-grade lead shield. We monitored the induction of DNA strand breaks in spleen tissue of male and female mice. We also explored the epigenetic parameters such as changes in the levels of global DNA methylation and expression of methyl-binding protein MeCP2. Molecular changes were correlated with levels of cellular proliferation and apoptosis. Here, for the first time, we report that the IR-induced bystander effects in vivo are sex specific and are more pronounced in male animals.

4.3 MATERIALS AND METHODS

4.3.1 Model and Irradiation of Animals

In this study we examined the molecular changes in spleen of male and female C57BL/6 mice following *in vivo* cranial irradiation exposure. The 45 days old male mice (30 males and 30 female) were randomly assigned to different treatment groups. The whole body exposed cohort (10 male and 10 female animals) received 1 Gy of the whole body exposure to X rays (5cGy/s, 90kV, 5mA). The second cohort (10 male and 10 female animals) received 1 Gy of X rays exposure to scull only, while the rest of animal body were protected by a ~3 mm thick lead shield, the same type as used for a human body protection in diagnostic radiology. The protection of shielded 'bystander' tissue was complete, as verified by careful dosimetry using RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, NY, USA). The control mice (10 male and 10 female animals) were sham treated. The animals were humanely sacrificed either 6 hours or 96 hours (4 days) after exposure. These time points were chosen to analyze the initial (6 hours) as well as persistent (4 days) responses, if any. Spleen tissue was sampled upon sacrifice and processed for further molecular studies. The experiment was independently reproduced according to the same scheme using 15 male and 15 female animals. Both experiments yielded congruent results. Another cohort of 10 animals was exposed to an approximate scatter dose of ~0.01Gy (Koturbash *et al*, 2008; Koturbash *et al*, 2007, Koturbash *et al*, 2006b). To determine the bystander ventral/spleen/thigh skin dose resulting from photon scatter within the mouse itself, a Monte Carlo simulation was performed (Koturbash *et al*, 2006). The skin was assumed to be 0.1 cm thick, and a 2 mm thick lead shield covered one half of the mouse. An absorbed dose was tallied within

the skin region in several locations in both the unshielded dorsal regions and the shielded ventral/thigh regions. The absorbed dose to the ventral skin/spleen located right beneath the skin from a 90 kVp X-ray spectrum was determined to be approximately 0.014 – 0.017 Gy for a 1 Gy dose delivered to the dorsal skin (Hamada *et al*, 2007). Additionally, the scatter dose upon cranial was directly measured and was also found to be in the range of ~0.01Gy. The animals were sacrificed 6 hours after exposure.

To dissect the effects of sex hormones on the manifestations of bystander effects, a cohort of 30 male and 30 female animals were subjected to gonadotomy at the age of 18 days. At the age of 45 days, the castrated and ovariectomised (CAST and OVX) animals were subjected to IR treatment using the same scheme as with the intact animals.

Handling and care of animals were in strict 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.

4.3.2 DNA Damage Analysis

Total DNA was prepared from spleen tissue using Qiagen DNAeasy™ Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A modification of the random oligonucleotide-primed synthesis assay (ROPS) was used to detect the presence of DNA strand breaks in high molecular weight DNA (Pogribny *et al*, 1999). 3´-OH DNA fragments present in the high molecular weight DNA are separated into single-strand fragments by heat denaturation (95º C) and subsequently re-associated by cooling. The resulting random re-association of DNA strands consists primarily of the single-stranded DNA fragments primed by their own tails or by other DNA fragments. These

fragments serve as random primers, and the excess of DNA serves as template for Klenow fragment polymerase. DNA was denatured by exposure at 100˚C for 5 min and then immediately cooled on ice. The mixture contained 0.25 µg heat-denatured DNA, 0.1 µl [^{3}H]dCTP (57.4 Ci/mmol) (Perkin-Elmer, Boston, MA), 0.05 mM concentrations of each dGTP, dATP, and dTTP, 0.6 µM dCTP, 10 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 7.5 mM DTT, and 0.5 U Klenow polymerase (New England Biolabs, Beverly, MA) in a total volume of 25 µl. After incubation for 30 min at 16˚C, the reaction was stopped by the addition of an equal volume of 12.5 mM EDTA. The samples were subsequently applied on Whatman DE-81 ion-exchange filters and washed three times with sodium phosphate buffer (pH 7.0) at room temperature. Access of sodium phosphate was removed by extra washing with the distilled water. The filters were dried and processed using a scintillation counter (Beckman LS 5000CE, Fullerton, CA, Canada). The results were expressed as the percent difference in [^{3}H] dCTP incorporation relative to control values.

4.3.3 DNA Methylation Analysis

Total DNA was prepared from spleen tissue using Qiagen DNAeasy™ Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. A well established radiolabeled [^{3}H]dCTP extension assay was employed to evaluate the level of global DNA methylation (Nagar *et al*, 2003). 1 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˚C. A second DNA aliquot (1 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 MgCl2, 0.25 U AmpliTaq DNA polymerase, and 0.1 μl of [^{3}H]dCTP (57.4 Ci/mmol) (Perkin-Elmer, Boston, MA), and incubated at 56˚C for 1 h. The samples were applied to Whatman DE-81 ion-exchange filters and washed 3 times with 0.5 M sodium phosphate buffer (pH 7.0) at room temperature. The filters were dried and processed for counting using a scintillation counter (Beckman LS 5000CE; Fullerton, CA). 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 the undigested samples (background).

4.3.4 Analysis of Apoptosis

Paraffin embedding and sectioning were conducted at Central Vet Labs, Edmonton, AB. Staining for apoptosis was conducted using a Peroxidase In Situ Apoptosis Detection Kit® (Chemicon, Temecula, CA) according to the manufacturer's instructions. The kit detects apoptotic cells in situ by labeling and detecting DNA strand breaks by the TUNEL method. Apoptosis was quantified by enumerating apoptotic cells in, at least, 20 high power fields. Levels of the apoptotic cells relative to those of the control animals are shown as the mean amount of apoptotic cells per high resolution field of view + SD; *$p < 0.05$.

4.3.5 Proliferation Analysis – Ki67 and PCNA Immunohistochemistry

Paraffin embedding and sectioning was conducted at the Central Vet Labs, Edmonton, AB. Staining for Ki67 was conducted in accordance with the standard developed protocols and using tonsil tissue as a positive control for the stain quality

(Brooks, 2004). In brief, upon the citrate buffer epitope retrieval, slides were rinsed and subjected to serum blocking to prevent the non-specific binding of immunoglobulin. Sections then were incubated with mouse anti-human Ki67 (DakoCytomation, Carpinteria, CA), rinsed and subjected to peroxidase blocking. Following the peroxidase blocking, the slides were incubated with the secondary biotinylated antibody, subjected to HRP-Streptavidin detection, and then counterstained with hematoxylin. Cells positive for Ki67 were red/pink, while negative cells stained blue. Tonsil tissue served as a positive control. Ki67 index was quantified by enumerating Ki67-positive cells in, at least, 20 high power fields. The amount of Ki67 positive cells relative to those of control animals are shown as the mean + SD; *p <0.05.

Staining for PCNA was conducted using primary anti-PCNA antibody (Novocastra, Newcastle, UK). Upon the secondary antibody application, HRP-Streptavidin detection and counterstaining with hematoxylin, the cells positive for PCNA stained brown/purple, while the negative cells stained blue. Tonsil tissue served as a positive control. PCNA index was quantified by enumerating PCNA-positive cells in, at least, 20 high power fields. Levels of PCNA positive cells relative to those of the control animals are shown.

4.3.6 Statistical Analysis

Statistical analyses (Student's t-test with Bonferroni correction for multiple comparisons, Tukey-Kramer test and Dunnett's test) were performed using the MS Excel 2000 and JMP5 software packages.

4.4 RESULTS

4.4.1 Sex Differences in the Accumulation of DNA Damage in Bystander Spleen Tissue In Vivo

Numerous reports suggest that bystander effects are linked to the induction of DNA damage (Maguire *et al*, 2007; Sedelnikova *et al*, 2007; Han *et al*, 2007; Suzuki *et al*, 2003). Therefore, as an initial step of our study, we measured DNA strand breaks in bystander spleen tissue of male and female animals. We examined the sex specificity of bystander effects *in vivo* by placing lead shielding over the body of male or female mice, while the head was left unprotected and was exposed to 1 Gy of X-rays (Fig. 4.1). The lead shield used for these studies is the same as that used for patients exposed to radiation in a clinic. The identical shielding was used in the published studies on the bystander effect in rodent models (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b).

DNA strand breaks were analyzed in the spleen of unexposed, whole-body irradiated and head-exposed male and female mice that had been exposed to 1 Gy of X-rays, while their bodies (from the neck down) were protected by the lead shield (Fig. 4.1).

Levels of DNA strand breaks were evaluated using a modification of the random oligonucleotide-primed synthesis assay (ROPS) (Kovalchuk *et al*, 2004a; Kovalchuk *et al*, 2004b; Mothersill *et al*, 2001; Pogribny *et al*, 1999; Basnakian *et al*, 1996). The assay is based on the ability of Klenow fragment polymerase to initiate ROPS from the re-annealed 3'-OH ends of single stranded DNA. Briefly, 3'-OH DNA fragments that exist in the high molecular weight DNA are separated into single-strand fragments by heat denaturation and subsequently re-associated by cooling. The resulting random re-association of DNA strands consists

primarily of single-stranded DNA fragments primed by their own tails or by other DNA fragments. These fragments serve as random primers, and the excess DNA serves as template for Klenow fragment polymerase incorporating radioactively labeled dNTPs.

Using this method, we noted that whole body X-ray exposure led to a significant 1.7 and 1.3 fold increase in the levels of DNA strand breaks (DSBs) in spleen of male and female mice, respectively, 6 hours after treatment as compared to control animals ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) (Fig. 4.1A). The IR-induced increase in the level of DSBs in male mice was significantly higher ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) than in females. There was a less pronounced (1.4 fold) but still statistically significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in DNA strand breaks in the bystander spleen of head-exposed male mice. However, we did not detect any significant changes in the levels of breaks in the bystander female spleen (Fig. 4.1A). All the damage in the exposed and bystander spleen was repaired in 4 days after exposure (Fig. 4.1A) in both male and female animals.

Having seen the sex differences in the induction of DNA damage in the exposed and bystander spleen, we then decided to deduce the role that sex hormones may play in the aforementioned processes. To do so, weanling animals were subjected to gonadotomy - castration (CAST) and ovariectomization (OVX). At the age of 45 days, the cohorts of CAST and OVX animals were exposed to IR using the protocol described above. Our study has revealed that castration and ovariectomization strongly affect changes in the magnitude of DNA strand breaks inducted after IR exposure in male and female spleen.

Neither the whole-body nor the cranial radiation exposure has affected the levels of DNA strand breaks in the spleen tissue of CAST animals.

In the OVX animals, whole body irradiation led to a small (1.3 and 1.2 fold) but significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in the DSBs 6 and 96 hours after irradiation. A similar small but significant 1.2 fold increase was also seen in the spleen of cranially exposed OVX mice 96 hours after exposure (Fig. 4.1B).

Overall, the sex differences in the IR-induced strand break levels were somewhat less pronounced in the CAST and OVX animals as compared to the intact ones.

4.4.2 Sex-specific Radiation-induced Hypomethylation in the Bystander Spleen Tissue

Recent studies suggest that bystander effects are mediated via epigenetic mechanisms (Weber *et* Schubeler, 2007; Kaup *et al*, 2006; Wright *et* Coates, 2006; Lorimore *et al*, 2003). DNA methylation is one of the common and so far best-studied mechanisms of epigenetic regulation. It is crucial for the regulation of gene expression and silencing and serves as a safeguard of genome stability. Altered DNA methylation has been associated with gross genome rearrangements and is known as a cancer hallmark (reviewed in Baylin *et* Ohm, 2006; Klose *et* Bird, 2006; Kalinich *et al*, 1989). The direct IR exposure has been reported to affect DNA methylation patterns (Pogribny *et al*, 2005; Pogribny *et al*, 2004; Kovalchuk *et al*, 2004a; Mothersill *et al*, 2001; Tawa *et al*, 1998). Acute exposures to low LET x-rays or γ-rays were noted to result in global hypomethylation (Pogribny *et al*, 2005; Tawa *et al*, 1998). Furthermore, our laboratory

has shown that whole-body IR exposure leads to profound dose-dependent and sex-specific global DNA hypomethylation (Koturbash *et al*, 2005; Pogribny *et al*, 2004; Kovalchuk *et al*, 2004a; Brown *et* Gatter 2002).

DNA methylation levels are also affected in the bystander tissue. Previously, using both mouse and rat models, we have detected short and long-term changes in global DNA methylation in bystander spleen tissue after cranial exposure to moderate and high doses of IR (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b). Yet, all the previous bystander effect-related DNA methylation studies were conducted using male animals only, and bystander effect-induced DNA methylation changes have never been explored in a sex-specificity domain.

To test for any sex-specificity in the bystander effect-induced DNA methylation changes, we measured the levels of global cytosine DNA methylation in spleen tissue of control, whole-body exposed and head-exposed male and female mice 6 and 96 hours after exposure to 1 Gy of IR using a well-established HpaII/MspI-based cytosine extension assay (Koturbash *et al*, 2008; Koturbash *et al*, 2006b; Pogribny *et al*, 2004; Kovalchuk *et al*, 2004a; Kovalchuk *et al*, 2004b; Mothersill *et al*, 2001). The *Hpa*II-based cytosine extension assay measures the proportion of CCGG sites that lost methyl groups on both DNA strands. This overhang can be used for the subsequent single nucleotide extension with the 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 (i.e., the higher is methylation, the less [^{3}H]dCTP is incorporated). Taking into consideration that the vast majority of the frequently occurring *Hpa*II tetranucleotide recognition sequences are constitutively

methylated *in vivo*, an increase in cleavage at these sites indicates genome-wide hypomethylation.

The assay revealed that the whole body IR exposure resulted in a significant 2.8 and 1.4 fold ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in the incorporation of [^{3}H]dCTP 6 and 96 hours after exposure, respectively. Cranial irradiation also led to a significantly 1.5 and 1.4 fold elevated [^{3}H]dCTP incorporation seen 6 and 96 hours after cranial irradiation (Fig. 4.2A). These changes were indicative of a profound and persistent global genome loss of DNA methylation in the exposed and bystander spleen of male mice. In females, the DNA methylation changes were much less pronounced. In female spleen the loss of DNA methytion was seen only 96 hours after whole body irradiation (Fig. 4.2A). Intriguingly, gonadectomy resulted in profound global DNA hypomethylation ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) in the exposed spleen tissue of both CAST and OVX mice 6 hours after irradiation. In addition, all the sex differences in the DNA methylation patterns of the intact male and female animals were lost following gonadectomy (Fig. 4.2B).

4.4.3 Radiation-induced Changes in Apoptosis and Cellular Proliferation in Bystander Spleen In Vivo

The levels of cellular proliferation and apoptosis are important endpoints frequently affected in bystander cells as shown in a variety of cell culture, human 3D tissue and tissue explants studies (Sedelnikova *et al*, 2007; Belyakov *et al*, 2005). Even though their biological parameters were also significantly altered in the bystander spleen

of cranially exposed male mice (Koturbash *et al*, 2008), the sex specificity of the bystander effect-induced proliferation and apoptosis in vivo has never been considered.

In this study, we analyzed and compared the levels of cellular proliferation and apoptosis in the spleen of control, whole body exposed and head-exposed male and female mice. Apoptosis analysis was conducted using the ApopTag^{TM} method. In this assay, apoptotic cells are identified in situ by specific end labeling and immunohistochemical detection of the DNA fragments produced during the apoptotic process (Koturbash *et al*, 2008). We found that the whole-body exposure led to a significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) increase in the number of apoptotic cells in the murine spleen tissue of both sexes 6 and 96 hours after exposure. Interestingly, the number of apoptotic cells was significantly higher ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) in the female spleen tissue 6 hours after irradiation than in the male spleen tissue (Fig. 4.3).

Cranial exposure led to significant ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) up-regulation of apoptosis in bystander spleen tissue of mice of both sexes 6 hours *post* exposure. No significant changes in the levels of cell death were found 96 hours after cranial irradiation; however the higher apoptotic index in the female spleen has to be acknowledged as compared with the male spleen tissue. The detected increased levels of apoptosis could be linked to the increased levels of DNA damage.

Sterilization of animals led to diminished sex-determined differences in the majority of apoptotic cells after whole-body and cranial irradiation in both OVX and CAST animals (Fig. 4.3).

For proper functioning, the cells have to maintain a tight link between cell death and proliferation. Having observed elevated sex-specific levels of apoptosis in the exposed and bystander spleen tissue of males and females, we studied whether irradiation leads to altered cellular proliferation. To do so, we have analyzed and compared the levels of Ki67 and proliferating cell nuclear antigen (PCNA) in spleens of control and exposed male and female mice by immunohistochemistry (IHC). Ki67 is a nuclear protein expressed during the late G1-, S-, G2-, and M-phases of the cell cycle, but not in the G0 (quiescent) phase (Moldovan *et al*, 2007). PCNA is primarily functioning as a DNA polymerase cofactor during DNA replication, and therefore its levels are strongly elevated in S phase (Hall, 2003).

The analysis showed that whole-body exposure led to a significant increase ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) in the levels of Ki67-positive cells in the murine spleen tissue of both sexes (Fig. 4.4A). In the mean time, cranial exposure resulted in a significant increase ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) of Ki67-positive cells only in the male spleen tissue. Levels of Ki67 remained significantly elevated ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) 96 hours after whole-body exposure in spleen of both sexes. Sterilized male animals have shown a less pronounced, but still significant increase ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) in Ki67-positive cells in spleen 6 and 96 hours after whole body exposure (Fig. 4.4A). Sterilized female animals have shown a small, but significant increase ($p<0.05$, Student's *t*-test, Tukey-Kramer test and Dunnett's test) in Ki67-positive cells in spleen 96 hours after whole body exposure.

Levels of Ki67 in the bystander spleen tissue remained unchanged in both OVX and CAST animals 6 and 96 hours after irradiation (Fig. 4.4A).

We also analyzed the levels of PCNA in murine control, exposed and bystander spleen tissue. We did not detect any significant changes in the levels of PCNA-positive cells. We also could not identify any significant sex-determined differences in the expression of PCNA (Fig. 4.4B).

4.4.4 Bystander Effects are Not Due to Radiation Scattering

Importantly, all of the bystander effects observed in this study were not due to the insufficient shielding or radiation scattering. Radiation scattering occurs when X-rays pass through the tissue and partially get reflected. This leads to the formation of a small "scatter" dose in the shielded area. In our previous studies, exposure of the animals to the scatter doses calculated according to the Monte Carlo simulation and directly measured by using RAD-CHECK did not induce the bystander-like responses (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006). Similarly in the present study, exposure of male and female animals to the scatter dose did not result in any significant changes in DNA damage and methylation, cellular proliferation and apoptosis in spleen tissue (data not shown). As an extra measure of shielding effectiveness, the fully shielded mice were subjected to 1Gy X-ray exposure. Spleen tissue was further analyzed for the levels of DNA strand breaks, patterns of methylation and cellular proliferation and apoptosis. No changes were found in the aforementioned parameters in comparison with the unexposed control animals (data not shown).

4.5 DISCUSSION

In this study, we report significant sex-determined differences in the *in vivo* bystander effect that occurs in mouse spleen tissue upon cranial irradiation. The main findings of the present study are that: (i) cranial irradiation results in the induction of sex-specific bystander effects in lead-shielded distant spleen tissue *in vivo*; (ii) cranial X-ray irradiation induces different levels of DNA damage in bystander spleen tissue of male and female mice; (iii) cranial irradiation results in the sex-specific patterns of DNA methylation, proliferation and apoptosis in the exposed and bystander spleen tissues of male and female mice; (iv) gonadectomy leads to the significantly diminished sex differences in the bystander spleen tissue.

Ionizing radiation is an invaluable diagnostic and treatment tool. On the other hand, it is a severe damaging agent (Hall, 2003). Radiation impact is not localized only to the area of exposure, it can be extended to the unexposed naïve bystander cells and tissue (reviewed in Morgan *et* Sova, 2007; Morgan *et* Sova, 2005; Mothersill *et* Seymour, 2004; Morgan, 2003a; Morgan, 2003b) and even to other bystander living organisms (Mothersill *et al*, 2007). Bystander effects manifest themselves as changes in the levels of DNA damage and mutation, gross genome rearrangements and genome instability, cellular proliferation and death (Sedelnikova *et al*, 2007; Gaugler *et al*, 2007; Han *et al*, 2007; Maguire *et al*, 2007; Hamada *et al*, 2007; Huang *et al*, 2007; Lyng *et al*, 2006b; Smilenov *et al*, 2006; Hu *et al*, 2006; Belyakov *et al*, 2006; Liu *et al*, 2006; Persaud *et al*, 2005; Belyakov *et al*, 2005; Klokov *et al*, 2004; Mothersill *et al*, 2003; Belyakov *et al*, 2003; Suzuki *et al*, 2003; Zhou *et al*, 2002; Sawant *et al*, 2002; Belyakov *et al*, 2002; Lorimore *et al,* 2001; Sawant *et al*, 2001).

Recently, the increasing research efforts have focused on bystander effects *in vivo* in the whole organisms (Koturbash et al, 2008; Marozik et al, 2007; Koturbash et al, 2007; Koturbash et al, 2006b; Brooks, 2004; Khan et al, 2003; Emerit et al, 1997; Pant et Kamada, 1977; Goh et Summer, 1968), as those types of effects may be linked to the radiation-induced carcinogenesis (Jaenisch et Bird, 2003; Goldberg et Lehnert, 2002).

Yet, the vast majority of the aforementioned animal-based *in vivo* studies were conducted using male animals, while the sex differences in the bystander effects remained unexplored. Among the plethora of research work on bystander effect, only one study noted putative sex specificity, yet the authors did not further discuss and elucidate the phenomenon they observed (Mothersill et al, 2001).

Direct radiation exposure effects have been proven to be sex specific (Koturbash *et al*, 2005; Raiche *et al*, 2004; Pogribny *et al*, 2004; Kovalchuk *et al*, 2004a; Kovalchuk *et al*, 2004b; Brown et Gatter, 2002; Mothersill *et al*, 2001). Taking into consideration that responses in bystander cells are very reminiscent of those in directly irradiated cells, we hypothesize that bystander effects may also be distinct in males and females.

The experimental outcomes support our hypothesis. We observed a significant sex difference in the levels of radiation-induced DNA damage in the exposed and bystander spleen. Male spleen tissue exhibited higher levels of damage. We have also noted a striking difference in levels of DNA methylation in bystander male and female spleens. In male spleens, significant and persistent DNA hypomethylation was observed, while no such changes were seen in female spleens. These data are in agreement with a previous report on more pronounced loss of DNA methylation in male spleens after whole body

radiation exposure (Koturbash *et al*, 2005; Pogribny *et al*, 2004; Kovalchuk *et al*, 2004a; Brown *et* Gatter, 2002).

The observed loss of global DNA methylation in male spleen tissue was persistent. The DNA methylation changes were accompanied by a subtle decrease in the levels of methyl-binding protein MeCP2, primarily in the spleen of male animals (data not shown). The loss of DNA methylation observed in the exposed and bystander male spleen tissue was well-correlated with the accumulation of DNA strand breaks and can therefore be related to activation of DNA repair processes (Koturbash *et al*, 2005; Kovalchuk *et al*, 2004a, Brown *et* Gatter, 2002). Furthermore, DNA methylation changes observed in the spleen of cranially exposed male mice were well-correlated with the changes observed previously in a male rat spleen model (Koturbash *et al*, 2007). Interestingly, a significant sex difference in the levels of methylation in spleen tissue was eliminated in sterilized mice.

The changes in DNA methylation are not isolated events, and they occur in the context of the global chromatin deregulation and altered histone modification levels (Weidman *et al*, 2007; Jenuwein *et* Allis, 2001). Histone modifications including acetylation, methylation, phosphorylation and ubiquitination are important in transcriptional regulation (Cheung *et* Lau, 2005; Jenuwein *et* Allis, 2001). Many histone modifications are maintained during process of cell division. The acetylated histone tails lose their positive charge, reducing the affinity to the negatively charged DNA and leading to the relaxed chromatin packaging (Cheung *et* Lau, 2005). Histone methylation can result in different consequences depending upon the residue affected (Saha *et al*, 2006; Fraga *et al*, 2005; Jenuwein *et* Allis, 2001). Methylation of lysine 9 of histone H3

is associated with chromatin compaction and gene silencing. Histone residues can be mono-, di- and tri-methylated, adding an enormous complexity to the yet unexplored histone code (Saha *et al*, 2006; Fraga *et al*, 2005; Jenuwein *et* Allis, 2001). It was recently shown that tumors undergo a massive loss of tri-methylation at lysine 20 of histone H4 (Tryndyak *et a*l, 2006a; Tryndyak *et al*, 2006b). This loss occurs along with DNA hypomethylation and is linked to chromatin relaxation and aberrant expression (Tryndyak *et al*, 2006a).

We have analyzed the levels of trimethylated lysine 9 of histone H3, trimethylated lysine 20 of histone H4 and acetylated lysines 12 and 16 of histone H4. Unlike DNA methylation, no significant treatment-related histone modification changes were observed (data not shown). This correlates with the previously reported lack of histone methylation changes in the bystander rat spleen (Koturbash *et al*, 2007). No treatment related changes in levels of histone modifications were seen in the CAST and OVX animals either. Yet, we observed that overall levels of histone H3 and H4 methylation in the OVX animals were about twice as low as in intact animals of the respective groups. This interesting observation may be further studied to deduce the role of sex hormones in the maintenance of histone modification patterns.

Exposure to radiation results in gross cellular perturbations, including altered cell death and proliferation. An increased cell death was observed in the spleen tissue of male and female mice 6 hours upon whole body irradiation and was still detectable 96 hours after exposure. Cranial irradiation led to the significantly increased levels of apoptosis in bystander spleen in animals of both sexes 6 after exposure. In addition, significantly higher levels of apoptosis were detected in exposed female spleen in comparison with the

male spleen tissue. Although we did not find significant sex differences in cell death in bystander spleen, there was a clear trend towards higher levels of apoptosis in females.

Induction of apoptotic events was paralleled with significant increases in a Ki67 index in bystander spleen tissue. Interestingly, opposite to cell death, the significant increase of Ki67-positive cells in the distant bystander spleen was observed in male mice. No changes were detected in levels of PCNA-positive cells. A nuclear protein Ki67 was expressed during all stages of the cell cycle but G_0 (Moldovan *et al*, 2007). In the mean time, PCNA, a DNA replication protein, was strongly expressed in the S phase (Hall, 2003). Taking into account that exposure to radiation was previously reported to cause G_1 and G_2 cell cycle arrests, the elevated levels of apoptosis and Ki67 paralleled with the unchanged expression of PCNA might suggest a defensive cell cycle block in response to DNA damage (Cassie *et al*, 2006). We assume that this will aim to halt the cell cycle and provide cells with enough time to repair radiation-induced lesions. However, the significant sex differences in cell death and Ki67 suggest different mechanisms to be involved in male and female mice. Sterilization led to the diminished sex differences, indicating sex hormones as possible regulators of a cellular response to whole body and cranial radiation exposure.

Our previous studies showed that males are more susceptible to the whole body irradiation (Cassie *et al*, 2006; Koturbash *et al*, 2005; Pogribny *et al*, 2004; Kovalchuk *et al*, 2004a; Brown *et* Gatter, 2002). The current study confirms our previous findings and importantly, further extends them to prove that males are more susceptible to the radiation-induced induction of bystander effect. This constitutes an interesting and novel finding.

The mechanisms of sex differences have to be further explored. The observed effects may be linked to a higher incidence of leukemia and lymphoma in males in mice and humans (Guizard, 2005; Noshchenko *et al*, 2001; Yoshida, 1993). Therefore, our data can be potentially extrapolated to humans. However, additional studies are needed for a deeper understanding of the role of sex hormones, epigenetic machinery and cellular response interplay. It will help us reveal the mechanisms of secondary radiation-induced malignancies and their interrelationship with bystander effects.

4.6 ACKNOWLEDGEMENTS

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4.7 FIGURE LEGENDS.

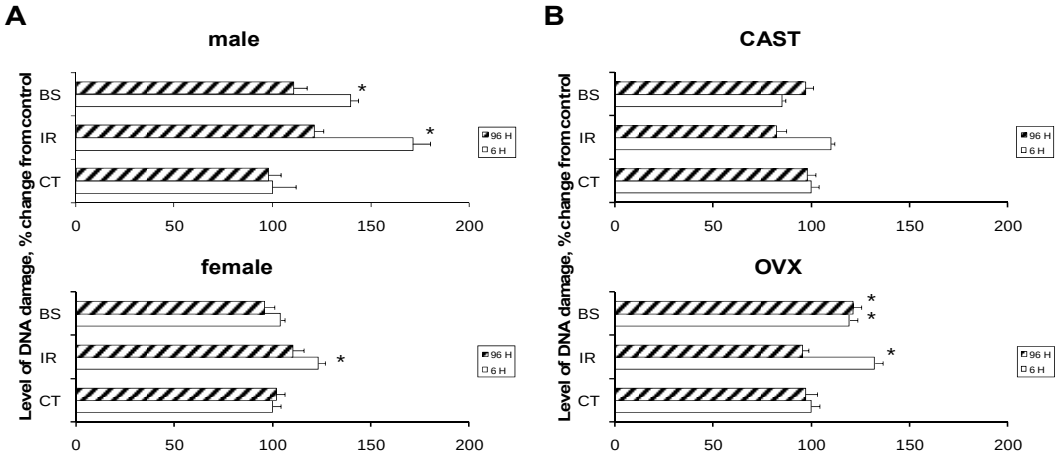

Figure 4.1. **Induction and persistence of DNA damage upon X-ray exposure in the radiation-target spleen tissue of whole body and cranially exposed male and female mice.**

The levels of DNA strand breaks were evaluated using the modification of a random oligonucleotide-primed synthesis assay (ROPS).

A. Levels of radiation-induced strand breaks in the spleen tissue of intact male and female mice.

B. Levels of radiation-induced strand breaks in the spleen tissue of castrated (CAST) or ovariectomized (OVX) animals.

The levels of DSBs relative to control are presented as mean values +/- SEM., n $\geq$ 6; ($p<0.05$), and they are significantly different from control, Student's *t*-test with Bonferroni correction for multiple comparisons, Tukey-Kramer test and Dunnett's test. White bar columns – 6 hours after exposure, dashed bar columns – 96 hours after exposure.

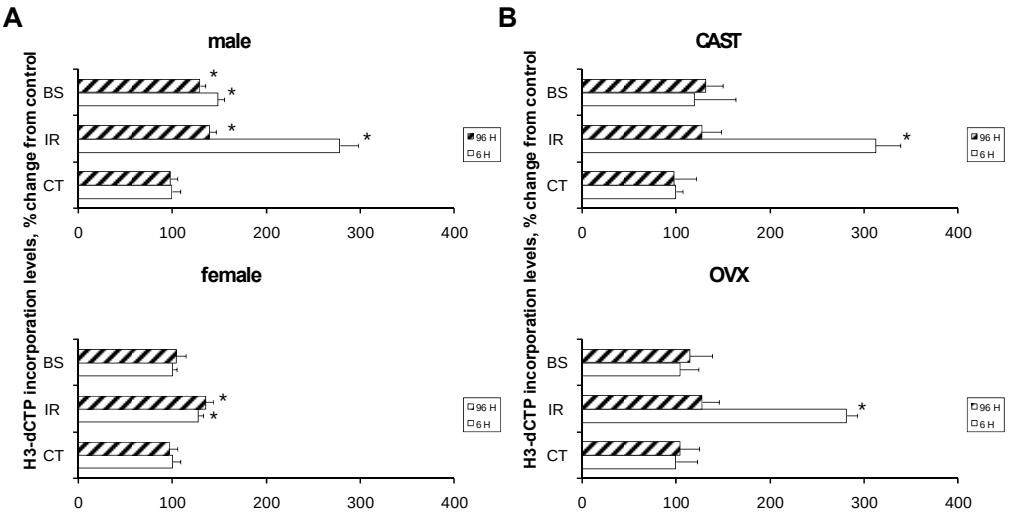

Figure 4.2. Whole body and cranial radiation exposure induces the significant and sex-specific loss of DNA methylation in the murine spleen.

The levels of global genome DNA methylation in the spleen tissue of irradiated mice were measured by the cytosine extension assay based on the treatment of DNA with a methylation-sensitive restriction enzyme *Hpa*II. This enzyme cleaves CCGG sequences in case that internal cytosine residues are unmethylated on both strands. The cleavage results in the formation of a 5'-guanine overhang that is used for the subsequent single nucleotide extension with the 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 (i.e., the higher the methylation, the lower the incorporation of [^{3}H]dCTP).

A. Levels of [^{3}H]dCTP incorporation in the intact male and female mice.

B. Levels of [^{3}H]dCTP incorporation in the CAST and OVX mice.

The levels of [^{3}H]dCTP incorporation relative to control are presented as mean values +/- SEM., n ≥ 6; (p<0.05), and they are significantly different from control, Student's *t*-test with Bonferroni correction for multiple comparisons, Tukey-Kramer test and Dunnett's test. Bars and abbreviations as defined in Figure 4.1.

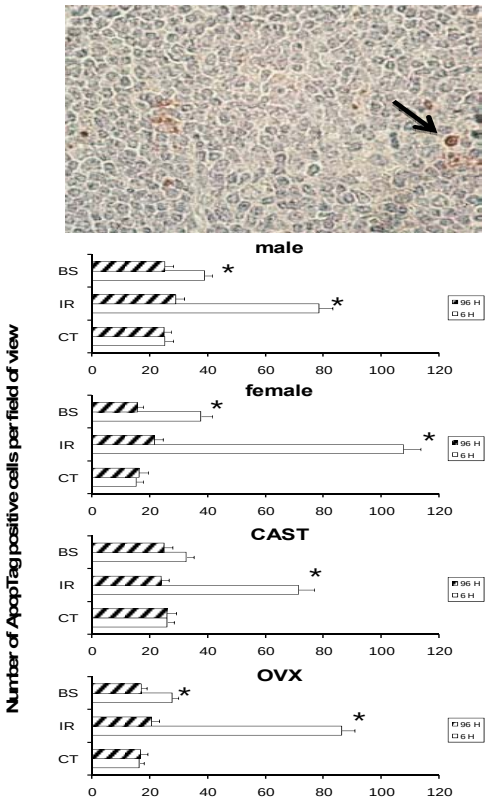

Figure 4.3. Radiation-induced induction of apoptosis in spleen tissues of the whole body head-exposed male and female mice.

The apoptosis levels were measured by ApopTag^{TM} IHC. The levels of apoptotic cells relative to control are shown, mean values ± SD, n=400; *p<0.05, Student's t-test, Dunnett's test and Tukey-Kramer test. Bars and abbreviations as defined in Figure 4.1.

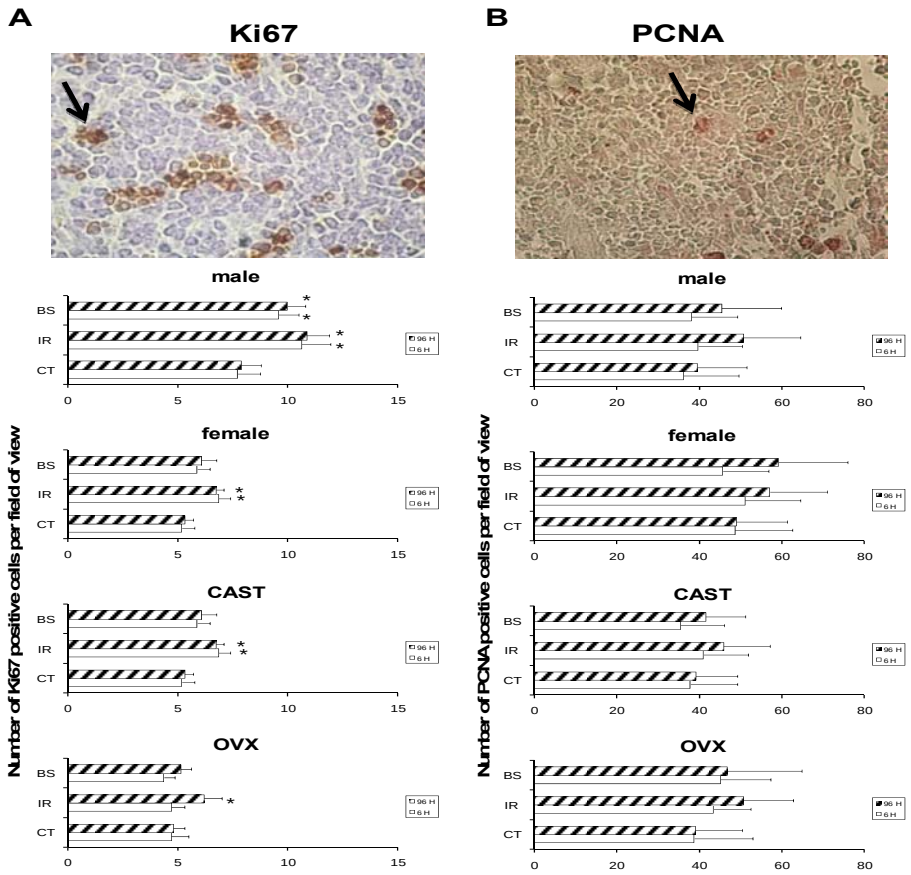

Figure 4.4. Changes in the cellular proliferation levels in the exposed and bystander spleen of male and female mice.

A. Levels of cellular proliferation as measured by Ki 67 immunohistochemistry (IHC). The levels of Ki67 positive cells relative to control are shown, mean values ± SEM, n=400; *p<0.05, Student's t-test, Dunnett's test and Tukey-Kramer test.

B. Levels of cellular proliferation as measured by PCNA immunohistochemistry (IHC). The levels of PCNA positive cells relative to control are shown, mean values ± SD, n=400; *p<0.05, Student's t-test, Dunnett's test and Tukey-Kramer test.

Bars and abbreviations as defined in Figure 4.1.

5. SEX-SPECIFIC MICRORNAOME DEREGULATION IN THE SHIELDED BYSTANDER SPLEEN OF CRANIALLY EXPOSED MICE[1]

[1]Chapter 5 has been submitted in its entirety:

Koturbash I, Zemp F, Kutanzi K, Luzhna L, Loree J, Kolb B, Kovalchuk O. (2008) Sex-specific microRNAome deregulation in the shielded bystander spleen of cranially exposed mice. *Cell Cycle* (in press)

5.1 ABSTRACT

The bystander effect whereby exposed cells signal distress to their naïve unexposed neighbors is now accepted as a ubiquitous consequence of radiation exposure. It is well documented to occur in cultured cells, 3D tissue models, and in organs and organisms. Notwithstanding, the exact mechanisms of the bystander effect remain unclear. Recent studies hinted that bystander effects may, in part, be distinct in males and females, and may be mediated via short non-coding RNAs, specifically – microRNAs. MicroRNAs are small, abundant and capable of regulating the expression of a wide variety of targets. Yet, their roles in bystander effects have not been analyzed in detail. The mechanisms of sex differences in the bystander effects also remain to be uncovered.

We hypothesized that the radiation-induced expression of microRNAs in the exposed and bystander tissue may be distinct in males and females. With this in mind, we set out to analyze the sex-specific expression of microRNAs in the shielded 'bystander' spleen of male and female mice following cranial irradiation.

Using a well-established bystander mouse model whereby the animal's head is exposed, while the body is completely protected by a medical-grade shield, we have for the first time shown that radiation exposure triggers a significant and sex-specific deregulation of microRNAome in the shielded spleen. The altered miRNA levels were paralleled by sex-specific changes in the levels of the miRNA processing enzyme Dicer and components of the RNA-induced silencing complex (RISC). Gonadectomization of animals resulted in drastic microRNAome alterations. We propose that the observed changes may be important confounding factors that may in the future help us explain sex specificity of radiation-induced tumors.

5.2 INTRODUCTION

Ionizing radiation (IR) is an invaluable diagnostic and treatment tool. On the other hand, it is a severe damaging agent and a potential carcinogen (Little, 2000). Radiation impact is localized not only in the area of exposure, but it can be extended into the tissue (Sedelnikova *et al*, 2007; Belyakov *et al*, 2005), organ (Brooks, 2004; Khan *et al*, 2003; Khan *et al*, 1998; Brooks *et al*, 1974), organism (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b) and even to other living organisms (Mothersill *et al*, 2007). Recent studies report a plethora of alterations taking place in bystander cells and tissues such as an induction of DNA strand breaks (Koturbash *et al*, 2008; Sedelnikova *et al*, 2007; Han *et al*, 2007; Smilenov *et al*, 2006; Belyakov *et al*, 2005; Suzuki *et al*, 2004; Zhou *et al*, 2002), altered gene expression (Hamada *et al*, 2007; Maguire *et al*, 2005; Zhou *et al*, 2005; Klokov *et al*, 2004), cell cycle arrest and apoptosis (Koturbash *et al*, 2008; Sedelnikova *et al*, 2007; Lyng *et al*, 2006a; Belyakov *et al*, 2005; Belyakov *et al*, 2002), and a number of epigenetic events (Koturbash *et al*, 2007; Sedelnikova *et al*, 2007, Kaup *et al*, 2006; Koturbash *et al*, 2006b), resulting in genome instability (Morgan *et* Sowa, 2007; Sedelnikova *et al*, 2007; Belyakov *et al*, 2005; Mothersill *et* Seymour, 2004; Morgan, 2003a; Morgan, 2003b; Morgan *et al*, 2002). Yet, the precise mechanisms of bystander effects and the nature of bystander signaling remain enigmatic.

A newly emerged mechanism of epigenetic control mediated through the involvement of small regulatory RNAs (Filipowicz *et al*, 2008; Niwa *et* Slack, 2007) promises to bridge the gaps in our understanding of the bystander effect phenomenon (Koturbash *et al*, 2007).

Amongst those, small regulatory RNAs, microRNAs (miRNAs) are of a particular interest. MiRNAs are evolutionally conserved, small single-stranded non-protein-coding RNA molecules which are presently recognized as major negative gene regulators (Filipowicz *et al*, 2008; Nilsen, 2007; Niwa *et* Slack, 2007). Most miRNA genes are located in noncoding regions of the genome, but about one-fourth of mammalian miRNAs are found in coding sections or introns (Ying *et* Lin, 2004). MiRNAs are initially expressed as part of primary miRNAs (pri-miRNAs). The miRNA portion of the pri-miRNA transcript forms a hairpin, which is released by a dsRNA-specific ribonuclease Drosha in the nucleus to form a precursor miRNA (pre-miRNA) (Rana, 2007; Hutvagner, 2005; Lee *et al*, 2003; Hutvagner *et* Zamore, 2002). The pre-miRNAs are then exported to the cytoplasm, where Dicer (a member of the RNAse III superfamily) cleaves the pre-miRNA leading to the formation of a mature miRNA (Rana, 2007; Hutvagner, 2005; Lee *et al*, 2003; Hutvagner *et* Zamore, 2002). To control translation of target mRNAs, mature miRNAs must associate with the RNA-induced silencing complex (RISC) proteins (Rana, 2007; Hutvagner, 2005) such as Argonaute (Ago), PACT (protein kinase, interferon-inducible double-stranded RNA-dependent activator, PRKRA), fragile X mental retardation protein (FXR), TudorSN and other proteins (Joshua-Tor, 2006; Lee *et al*, 2006; Chendrimada *et al*, 2005; Gregory *et al*, 2005; Scadden, 2005; Jin *et al*, 2004). Ago proteins are important components of the RISC machinery (Joshua-Tor, 2006). Dicer was found to stably associate with the Ago2 protein (Chendrimada *et al*, 2005). PACT has been shown to interact with Dicer without facilitating its pre-miRNA cleavage activity (Lee *et al*, 2006). Finally, FxR protein interacts with mammalian Ago2 and is associated with the Dicer activity (Jin *et al*, 2004).

Tudor-SN is another subunit of the RNA-induced silencing complex, which is important to the mechanism of RNA interference and gene regulation (Scadden, 2005).

Being associated with RISCs, miRNAs bind to a specific section of mRNAs, thus serving as translational suppressors and regulators of the proteins production (Carthew, 2006; Sevignani *et al*, 2006; Hutvagner *et* Zamore, 2002). By doing so, the regulatory miRNAs in association with the miRNA processing machinery impact cellular differentiation, proliferation, apoptosis, and even predisposition to cancer (Fabbri *et al*, 2007; Wiemer, 2007; Zhang *et al*, 2007; Esquela-Kerscher *et* Slack, 2006; Lu *et al*, 2005). Indeed, aberrant levels of miRNAs have been reported in a variety of human cancers (Wiemer, 2007; Zhang *et al*, 2007; Lu *et al*, 2005).

Furthermore, several studies pointed towards a putative role of the miRNA processing machinery in tumor cells (Chiosea *et al*, 2007; Chiosea *et al*, 2006; Karube *et al*, 2005; Kaul *et* Sikand, 2004; Cheng *et al*, 2003). However, the functions of microRNAs and miRNA processing machinery in the genotoxic stress response in general (Marsit *et al*, 2006; Cheng *et al*, 2003) and in the radiation-induced bystander in particular have yet to be studied in detail.

Our previous studies hinted the putative task of miRNAs in bystander effects (Koturbash et al, 2007). The present study was designed to dissect the role of the microRNAome and microRNA machinery in the regulation of a cellular response to the direct (whole body) or indirect (cranial) exposure to IR in the spleen tissue of male and female mice. Here, we for the first time show that radiation exposure results in a significant and sex-specific deregulation of microRNAome and miRNA processing machinery in the directly exposed and shielded bystander spleen.

5.3 MATERIALS AND METHODS

5.3.1 Animal Model

In this study, we analyzed microRNAome changes in the spleen of male and female C57BL/6 mice following *in vivo* cranial irradiation exposure. The 45 days old male mice (30 males and 30 female) were randomly assigned to different treatment groups. The whole body exposed cohort (10 male and 10 female animals) received 1 Gy of whole body exposure to X-rays (5cGy/s, 90kV, 5mA). The second cohort (10 male and 10 female animals) received 1 Gy of X-rays exposure to a scull only, while the rest of animal body were protected by a ~3 mm thick lead shield, the same type as used for a human body protection in diagnostic radiology. The protection of shielded 'bystander' tissue was complete, as verified by careful dosimetry using a RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, FL, USA). The control mice (10 male and 10 female animals) were sham treated. The animals were humanely sacrificed either 6 hours or 96 hours (4 days) after exposure. These time points were chosen to analyze the initial (6 hours) as well as persistent (4 days) responses, if any. Spleen tissue was sampled upon sacrifice and processed for further molecular studies. The experiment was independently reproduced according to the same scheme using 15 male and 15 female animals. Both experiments yielded congruent results. Another cohort of 10 animals was exposed to an approximate scatter dose of ~0.01Gy (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b). To determine the bystander ventral/spleen/thigh skin dose resulting from photon scatter within a mouse itself, a Monte Carlo simulation was performed (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b). The skin was assumed to be 0.1 cm thick, and a 2 mm thick lead shield covered one half of

the mouse. An absorbed dose was tallied within the skin region in several locations in both the unshielded dorsal regions and the shielded ventral/thigh regions. The absorbed dose to the ventral skin/spleen located right beneath the skin from a 90 kVp X-ray spectrum was determined to be approximately 0.014 – 0.017 Gy for a 1 Gy dose delivered to the dorsal skin. Additionally, the scatter dose upon cranial was directly measured, and was found to be in the range of ~0.01Gy. The animals were sacrificed 6 hours after exposure.

To dissect the effect of sex hormones on the bystander effects manifestations, a cohort of 30 male and 30 female animals were subjected to gonadotomy at the age of 18 days. At the age of 45 days, the castrated and ovariectomised (CAST and OVX) animals were subjected to IR treatment using the same scheme as the intact animals.

Handling and care of animals was in strict 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.

5.3.2 miRNA Microarray Expression Analysis

Total RNA was extracted from mouse spleen tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. Tissue from 3 animals per group was used for the analysis. The miRNA microarray analysis was performed by LC Sciences (Houston, TX). Ten micrograms of total RNA were size-fractionated (<200 nucleotides) by using a mirVana kit (Ambion, Austin, TX). Poly-A tails were added to the RNA sequences at the 3′ ends using a poly(A) polymerase, and nucleotide tags were then ligated to the poly-A tails. The tagged RNAs were hybridized

to the dual-channel microarray μParaFlo microfluidics chips (LC Sciences) containing 439 miRNA probes to rat and mouse miRNAs and then labeled with tag-specific dendrimer Cy3 and Cy5 fluorescent dyes. Dye switching was performed to eliminate the dye bias. The detection probes melting temperature was balanced by incorporating varying numbers of modified nucleotides with the increased binding affinities. Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA), and then digitized using the Array-Pro image analysis software (Media Cybernetics, Silver Spring, MD). The maximum signal level of background probes was 180. A miRNA detection signal threshold was defined as twice the maximum background signal. Normalization was performed with a cyclic LOWESS (locally weighted regression) method to remove system-related variations, as previously described (Pogribny *et al*, 2007; Bolstad *et al*, 2003). Data adjustments included data-filtering, log 2 transformation, and gene centering and normalization. The *t*-test analysis was conducted between the control, whole body exposed and bystander sample groups. MicroRNAs with p-values < 0.05 were selected for cluster analysis.

5.3.3 Quantitative real-time PCR (qRT-PCR) expression analysis

Quantitative real-time PCRs (qRT-PCRs) were performed by using SuperTaq Polymerase (Ambion) and a mirVana qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained mirVana qRT-PCR Primer Sets specific for miR-34a, miR-17-5p, miR-20a, miR-26, miR-92, miR-106, and miR-150, human 5S rRNA served as a positive control. Quantitative real-time PCR was

performed on a SmartCycler (Cepheid, Sunnyvale, CA). End-point reaction products were also analyzed on a 3.5% high-resolution agarose gel stained with ethidium bromide.

5.3.4 Immunohistochemistry

Paraffin embedding and sectioning were conducted at Central Vet Labs, Edmonton, AB. The sections were stained with hematoxylin and eosin (H&E) for the histopathological examination. Following the pathological examination, the tissues were assembled into tissue microarrays (TMAs) with 2 mm cores. TMAs offer great benefits for the fast, consistent and efficient analysis of large amounts of data. The TMAs were produced by Pantomics, Inc (www.pantomics.com). Immunohistochemical staining was conducted using the antibodies against Dicer, FXR, PACT, TUDOR-SN (Santa Cruz Biotechnology, Santa Cruz, CA), Ago 2 and p38 (Abcam, Cambridge, MA) in accordance with the manufacturer's recommendations. In brief, upon the citrate buffer the epitope retrieval slides were rinsed and subjected to serum blocking to prevent the non-specific binding of immunoglobulin. Sections then were incubated with primary antibodies, rinsed and subjected to peroxidase blocking. Following the peroxidase blocking, the slides were incubated with the secondary biotinylated antibody, subjected to HRP-Streptavidin detection and counterstained with hematoxylin**.** Positive cells stained brown/purple, while negative cells stained blue. Positive cells were enumerated in, at least, 20 high power fields.

5.3.5 Statistical analysis

Statistical analysis (Student's *t*-test, Bonferroni correction, Tukey-Kramer test and Dunnett's tests) was performed using the MS Excel 2000 and JMP5 software packages.

5.4 RESULTS

In this study, we utilized an in vivo murine model to identify and compare microRNAome changes in spleen of whole body and head-exposed animals. In the course of the study, experimental animals (males and females of comparable weight) were divided in several experimental cohorts. The 'whole body exposed' cohort received 1 Gy of whole body irradiation to X-rays. The 'bystander' cohort received 1 Gy of X-rays exposure to the scull only, while 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 identical shielding was used in the published studies on the bystander effect in rodent models (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b). The protection of the shielded 'bystander' tissue was complete as verified by careful dosimetry. Control mice were sham treated. The animals were humanely sacrificed either 6 hours or 96 hours after exposure to analyze both the initial and persistent responses.

5.4.1 MicroRNAome changes in spleen of whole-body and head exposed male and female mice

Analysis of the murine spleen microRNA profile revealed a number of intriguing patterns. First of all, the background levels of miRNA expression in male and female spleen appeared to be quite different. Specifically, 8 microRNAs were found up-regulated in the spleen tissue of intact male mice in comparison with females, whereas 4 were up-regulated in the intact female spleen in comparison with the male spleen (Fig. 5.1).

Next, we analyzed microRNAome changes upon X ray exposure in the directly irradiated and bystander spleen. Since we previously noted that both direct irradiation and bystander effects in spleen were very pronounced 96 hours after exposure and persisted even further, we decided to use this time-point for the initial analysis of radiation-induced microRNAome changes.

We found that exposure to ionizing radiation resulted in gross and sex-specific microRNAome perturbations in murine spleen tissue. In male spleen, 4 miRNAs were up-regulated and 8 miRNAs were downregulated by whole body irradiation. In female spleen, 5 microRNAs were up-regulated and 15 were down-regulated by whole body exposure. Amongst those, miR-34a was significantly upregulated and miR-150 was significantly downregulated in both sexes. Their expression was independently confirmed by qRT-PCR (data not shown).

The observed upregulation of miR-34a in the exposed spleen tissue of males and females constitutes an important novel finding. The upregulated miR-34a is known to exert a negative impact on transcription factors E2F1 via p53 pathway (He *et al*, 2007a; He *et al*, 2007b) and on E2F3 (Welch *et al*, 2007). Consequently, miR-34a has been assumed to be a tumor suppressor miRNA (He *et al*, 2007a; He *et al*, 2007b; Welch *et al*, 2007). Therefore, its upregulation in the exposed tissue may be viewed as a positive protective effect. Its role in radiation responses has to be further analyzed.

Cranial irradiation also resulted in a profound and sex-specific pattern of microRNAome changes in the shielded bystander spleen. In male mice, it led to downregulation of 6 microRNAs in the bystander spleen. In the female bystander spleen,

cranial exposure resulted in upregulation of 3 and downregulaton 6 microRNAs (Fig. 5.2).

Comparison of miRNA profiles of the exposed and bystander spleen revealed that only 1 microRNA (miR-26b) was downregulated under both conditions in males. In females, microRNA profiles of the exposed and bystander spleen tissue shared more similarities. There 4 microRNAs (miR-30e, miR-24, miR-181a and miR-422b) were down-regulated in both groups (Fig. 5.2). Their expression was also independently confirmed by qRT-PCR.

Furthermore, evaluation of miRNAome patterns of the male and female bystander spleen also revealed several interesting findings. We observed a significant ($p < 0.05$) down-regulation of microRNAs miR-23a and miR-23b (Fig. 5.2) and a slight ($p< 0.1$) down-regulation of microRNA miR-24 in the male bystander spleen tissue. Similarly, we observed a significant ($p<0.05$) down-regulation of miR-24 in the female bystander spleen. These miRNAs share a lot of similarities in their genome locations and in their targets. Specifically, miR-24 that was cloned from a mouse (Lagos-Quintana *et al*, 2002) and is supposed to stem from a hairpin originating from chromosome 13. It also has a second predicted hairpin sequence on chromosome 8. MiR-23b originates from chromosome 13 and is located in the vicinity of miR-24-1. In addition, both of them, miR-23b and miR-24, target Notch1 that is a known apoptosis and cell differentiation regulator (Fukuda *et al*, 2005). At the same time, the miR-23a hairpin derived from chromosome 8 is located in a close proximity with the predicted hairpin for miR-24-2.

The aforementioned miRNAs are also confirmed to target MAPK14 (also known as p38) (Kiriakidou et al, 2004) and chemokine CXCL12 (Lewis *et al*, 2003). MAPK14

(p38) has been shown to be implicated in the formation of a bystander response (Azzam *et al*, 2002). CXCL12 is an important cytokine (Juarez *et al*, 2007). CXCL12 can induce elevation of cytoplasmic Ca^{2+} levels (Bendall *et al*, 2005), and calcium flux was observed in bystander cells in the conditioned medium experiments (Lyng *et al*, 2006a; Shao *et al*, 2006). The fact that miR-23a, miR-23b and miR-24 were altered in the bystander spleen of both sexes allows us to propose them as putative "bystander miRNAs". Moreover, the observed down-regulation of miR-24 and miR-23 species was paralleled by a significant increase in the expression of their target protein p38 (Fig. 5.7). Based on the aforementioned facts, the exact roles of these miRNAs in bystander effects have to be further discerned.

We also saw significant changes in several miRNAs implicated in the regulation of proliferation and apoptosis in the exposed and bystander spleen (Esquela-Kerscher *et* Slack, 2006). These were miR-92, miR17-5p, miR-20a, miR-106 (Fig. 5.2). Such changes were in part expected, since radiation exposure is well known to disturb a fine balance between proliferation and apoptosis in the directly exposed and bystander tissue (Koturbash *et al*, 2008; Sedelnikova *et al*, 2007; Belyakov *et al*, 2005; Morgan, 2003a, Morgan, 2003b). Yet, further detailed analysis is required to delineate their involvement in the radiation and bystander responses.

5.4.2 Castration and ovariectomization of animals significantly impact microRNAome and its response to genotoxic stress

Having seen the marked sex differences in microRNAome changes in the exposed and bystander spleen, we decided to deduce what impact sex hormones may have on the

aforementioned phenomenon. To do so, weanling animals were subjected to gonadectomy - castration (CAST) and ovariectomization (OVX). At the age of 45 days, the cohorts of CAST and OVX animals were exposed to radiation using the protocol described above.

Our study revealed that castration and ovariectomization strongly affected the murine spleen microRNAome. We found that sterilization of animals led to an increase in the number of differentially expressed miRNAs (Fig. 5.3). In total, expression patterns of 28 microRNAs were distinct: 25 of them were up-regulated in CAST males and 3 in OVX females in comparison to the sterilized animals of the opposite sex.

Furthermore, comparison of microRNA expression in the intact and sterilized animals revealed a drastic impact of the sterilization procedure on microRNAome (Fig. 5.8).

Radiation exposure significantly influenced the microRNAome level of CAST and OVX animals. Whole body irradiation led to elevated levels of 2 miRNAs and decreased the level of 1 miRNA in CAST animals. In OVX animals, it resulted in up-regulation of 7 and down-regulation of 4 microRNAs.

Head irradiation caused up-regulation of 11 and down-regulation of 3 miRNAs in CAST males. It also led to up-regulation of 1 miRNA in OVX animals.

Surprisingly, a pattern of radiation-induced miRNAome changes observed in spayed animals was very distinct from that seen in intact animals. In the future, a detailed analysis of cellular and organismal repercussions of these changes may lead to furthering our understanding of the interplay between hormonal regulation and genotoxic stress responses.

5.4.3. Sex-specific changes in the levels of Dicer and RISC proteins upon whole body and cranial exposure in murine spleen

Production and function of miRNAs requires a set of proteins collectively referred to as the microRNA processing machinery, or miR machinery (Nilsen, 2007; Carthew, 2006; Sevignani *et al*, 2006; Gregory *et al*, 2005; Hutvagner, 2002). The altered balance of the miR machinery components may result in altered miRNA levels and influence the interaction between miRNAs and their cognate mRNAs (Nilsen, 2007; Carthew, 2006; Sevignani *et al*, 2006; Gregory *et al*, 2005; Hutvagner, 2002).

Having seen pronounced and sex-specific miRNA alterations in exposed and bystander murine spleens, we decided to further investigate radiation and bystander-induced changes in the miR machinery proteins. In this study, we analyzed and compared levels of the RNA processing enzyme Dicer and RISC machinery components – Ago2, FxR, TUDOR-SN and PACT in male and female spleens upon whole body and cranial irradiation.

The IHC analysis revealed that whole body and cranial radiation exposure led to a significant ($p<0.05$) up-regulation of Dicer expression in the spleen of male and female mice 6 and 96 hours after exposure (Fig. 5.4). Interestingly, 96 hours after exposure, up-regulation was more pronounced in males than in females. Dicer expression pattern in CAST and OVX animals differed from that in intact mice. In CAST animals whole body exposure let to a less pronounced Dicer induction, while cranial exposure led to a small but significant Dicer induction only 96 hours after irradiation. No Dicer changes were seen in CAST animals 6 hours after cranial exposure. Similarly, in the OVX spleen small

but statistically significant changes were seen only 6 hours after whole body or head exposure.

Dicer is involved in the processing of pre-miRNAs in the cytoplasm, leading to the formation of mature miRNAs (Cummins *et al*, 2006; Tijsterman *et* Plasterk, 2004). Any alterations in Dicer levels usually result in drastic changes in the microRNAome. Knockout of Dicer led to a significant down-regulation of a large number of miRNAs (Cummins *et al*, 2006). Recently, Dicer was explored in the cancer and carcinogenesis domain. Dicer was found to be up-regulated in prostate adenocarcinoma (Chiosea *et al*, 2006) and Burkitt lymphoma (Kaul *et* Sikand, 2004). In lung carcinoma, contrarily, Dicer levels were decreased (Chiosea *et al*, 2007). Our data for the first time show that Dicer levels may be changed in cells and tissues in response to genotoxic stress. Dicer, therefore, may play an important role in fine-tuning genotoxic stress responses mediated via microRNAs in both directly exposed and bystander tissues. Besides being an important RNA interference regulator, Dicer is known to affect the cell cycle G1 checkpoint and DNA damage checkpoints (Carmichael *et al*, 2004). Furthermore, Dicer is a main contributor to the maintenance of centromeric heterochromatin and chromosome segregation (Fugukawa *et al*, 2004; Volpe *et al*, 2003; Provost *et al*, 2002; Volpe *et al*, 2002). The aforementioned phenomena are affected by direct irradiation (Rodemann *et* Blaese, 2007) and bystander effects (reviewed in Morgan *et* Sowa, 2007; Mothersill *et* Seymour, 2004; Morgan, 2003a; Morgan, 2003b) Therefore, we may speculate that these processes may be partially perturbed due to Dicer dysregulation.

To regulate the expression of their target mRNAs, miRNAs need to be associated with proteins of the RISC complex. Amongst those, Ago2 is one of the most important

(Joshua-Tor, 2006; Chendrimada *et al*, 2005, Gregory *et al*, 2005). We noted that in the male spleen, whole body radiation exposure resulted in a significant ($p<0.05$) down-regulation of Ago2 96 hours after irradiation (Fig. 5.6). Cranial exposure led to a significant ($p<0.05$) up-regulation of Ago2 in the male spleen 6 hours following radiation treatment. In females, whole body irradiation induced a significant drop in Ago2 expression 6 and 96 hours after exposure ($p<0.05$). Alongside with this, cranial exposure caused a significant ($p<0.05$) decrease in Ago2 levels in the bystander spleen 96 hours after exposure (Fig. 5.6).

Interestingly, CAST and OVX animals exhibited different patterns of Ago2 expression upon irradiation. In CAST and OVX animals, both whole body and cranial irradiation led to a significant up-regulation of Ago2. Up-regulation was seen both 6 and 96 hours after treatment and was more pronounced in CAST animals than in OVX ones. Ago2 is very important for the final Dicer-mediated processing of mature miRNAs, and further for their proper interaction with the RISC members (Joshua-Tor, 2006). Notably, scarce studies showed that Ago levels were significantly elevated in multiple tumor cells lines (Jaronczyk *et al*, 2005). Therefore, up-regulation of Ago2 may have a negative influence on the exposed and bystander tissue, where it was noted. In the future dysregulation of Ago2 has to be further explored in the context of radiation- and bystander-induced carcinogenesis.

FXR levels were found to be statistically significantly elevated in the bystander spleen of cranially exposed males 6 hours after irradiation, in the irradiated spleen of the whole body exposed female mice 96 hours after treatment and in the irradiated spleen of the whole body exposed CAST animals 6 hours after the X-ray application (Fig. 5.6).

Notably, in OVX animals, all treatments led to a significant up-regulation of FXR. FXR interacts with mammalian Ago2 and is associated with Dicer activity (Jin *et al*, 2004a; Jin *et al*, 2004b). Therefore, any changes in its expression may influence production and activity of microRNAs.

PACT is another vital member of the RISC complex (Lee *et al*, 2006). It interacts with Dicer and may contribute to stabilization of the Dicer protein (Lee *et al*, 2006). PACT levels were down-regulated in the exposed male and female spleen 6 hours after exposure. Contrarily, in the bystander male spleen, PACT expression was statistically significantly elevated both 6 and 96 hours after cranial irradiation. In the bystander female spleen, PACT was down-regulated 96 hours after treatment. Interestingly, CAST and OVX animal groups exhibited different patterns of PACT induction upon irradiation. In CAST animals, PACT up-regulation was seen only 6 hours after the whole body X-ray exposure. In OVX animals, up-regulation of PACT was observed 96 hours following the whole body and head irradiation. Data on the cellular effects of PACT are relatively scarce. The up-regulation of PACT was reported in bronchoalveolar carcinoma, and higher levels of PACT were considered a poor prognostic factor (Roh *et al*, 2005). The role of PACT in genotoxic stress responses and carcinogenesis has yet to be established.

TUDOR-SN levels were significantly affected in the exposed male animals. Whole body irradiation down-regulated TUDOR-SN 6 and 96 hours after exposure. Cranial irradiation led to TUDOR-SN down-regulation 6 hours after radiation treatment. In females, contrarily, TUDOR-SN was significantly up-regulated 96 hours following whole body exposure. Patterns of TUDOR-SN induction by radiation in the spayed mice differed significantly from those in the intact ones. In CAST animals, whole body

exposure led to significantly increased levels of TUDOR-SN 6 and 96 hours after irradiation, and cranial exposure resulted in up-regulation of TUDOR-SN 96 hours after treatment. In OVX animals, TUDOR-SN was up-regulated after all treatments and all time-points. TUDOR-SN is an important RISC machinery component. It harbors a recognizable nuclease domain and could therefore contribute to the RNA degradation observed in RNAi (Scadden, 2005). Its exact role in the RISC complexes has to be further delineated. Overall, whole-body and cranial exposure-induced patterns of the miRNA machinery were significantly different in males and females, and in CAST and OVX animals.

Both the deregulated microRNAome and the altered expression of Dicer and RISC may result in significant changes in the expression of various proteins that partake in many important cellular pathways such as maintenance of genome stability, DNA repair, cell cycle control, apoptosis, proliferation, differentiation. All these processes are affected by direct irradiation. Additionally, they are the known targets of bystander effects.

The altered microRNAome and miR machinery may also be explored as potential bystander effects biomarkers. Future studies are needed to dissect the role of the miRNAs and miRNA machinery in radiation and bystander responses.

It is known that while passing through the tissue, X-rays can be reflected, and this leads to the formation of a small 'scattered' dose of radiation in the protected tissue (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b). Yet, exposure of animals to a scatter dose did not exert any changes in the exposed spleen. We therefore concluded that changes observed in the shielded spleen tissue can be classified as true

bystander effects. This conclusion is also supported by a series of our previous studies that also showed that the observed in vivo bystander effects are not initiated by the scatter-dose (Koturbash *et al*, 2008; Koturbash *et al*, 2007; Koturbash *et al*, 2006b).

5.5 DISCUSSION

In this study, we for the first time report the pronounced and sex-determined microRNAome deregulation that occurs in the mouse spleen tissue upon whole body and cranial irradiation. The main findings of the present study are that: (i) male and female spleen tissues are characterized by very distinct microRNA patterns; (ii) radiation exposure alters microRNAome of male and female animals and induces sex-specific changes of microRNA levels in the exposed spleen tissue; (iii) cranial exposure also influences spleen microRNAome and causes distinct changes in the male and female shielded bystander spleen. Importantly, these are the first data on radiation- and bystander-induced microRNAome changes in animal tissues in vivo.

Furthermore, our study is the first to investigate the role of sex hormones in microRNAome regulation *in vivo.* We provide evidence that the microRNAome of castrated and ovariectomized mice differs very much from the microRNAome of intact animals. Additionally, radiation and bystander responses of sterilized animals differ drastically from intact animal responses.

Our microRNAome data sets may provide a roadmap for further analysis of the role of microRNAome in genotoxic stress responses. Specifically, our data provide an opportunity to integrate sex hormones and stress responses.

The radiation- and bystander –induced microRNAs identified in our study may further be explored as sex-specific biomarkers of direct and indirect/bystander responses.

It is known that radiation-induced cancer occurs more frequently in males (Guizard *et al*, 2001; Noshcenko *et al*, 2001; Yoshida *et al*, 1993; http://www.infobiogen.fr/services/chromcancer/Anomalies/splenvillousID2063.html), yet

the molecular grounds of this sex-specific bias are not well understood. The microRNA patterns observed in this study in the male and female spleen tissue may be used to further explore the molecular basis of sex differences in radiation-induced carcinogenesis.

Another important outcome of this study is a proof of radiation- and bystander-inducibility of Dicer and other RISC complex members. Dicer is a key RNAse involved in the final steps of microRNA maturation. It works in tight collaboration with Ago2, PACT, FXR and TUDOR-SN. All of these proteins exhibit altered expression upon direct irradiation. They are also changed in the shielded bystander spleen. The altered levels of microRNA machinery go well together with the observed microRNAome deregulation. Cellular and organismal repercussions of the observed changes need to be further defined. Moreover, the mechanisms of sex differences in the microRNAome and microRNA processing machinery levels have to be further explored.

Altered Dicer and RISC levels, together with the altered microRNAome, may serve as novel and sex-specific biomarkers of exposure and bystander effects.

5.6 ACKNOWLEDGEMENTS

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5.7. FIGURE LEGENDS

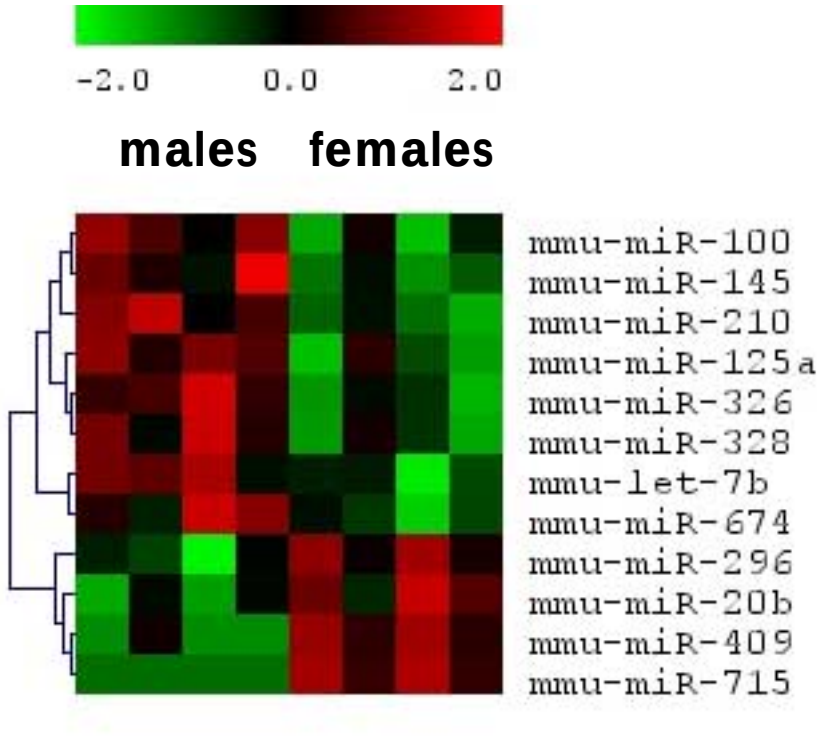

Figure 5.1. Hierarchical clustering of differentially expressed miRNA genes in male and female spleen samples.

Hierarchical clusters of significantly altered miRNAs (as determined by ANOVA) across the male and female mouse spleen. Red denotes high expression levels, whereas green depicts low expression levels. Each miRNA listed is significantly differentially expressed ($p < 0.05$) between male and female mice.

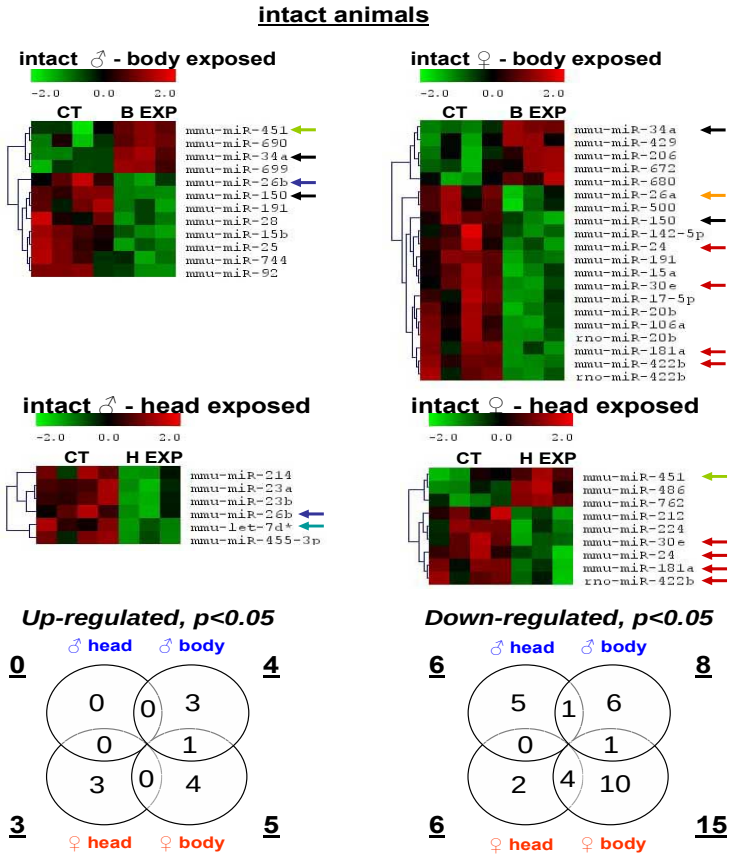

Figure 5.2. Differentially expressed miRNAs in spleen of male and female mice subjected to whole body and head exposure of 1 Gy of X rays.

Hierarchical clusters of differentially expressed miRNA genes in spleen of whole body and head exposed male and female mice (as determined by ANOVA). Each miRNA listed is significantly differentially expressed ($p < 0.05$) between control and treatment groups. Color arrows denote microRNAs expressed differentially expressed in different groups.

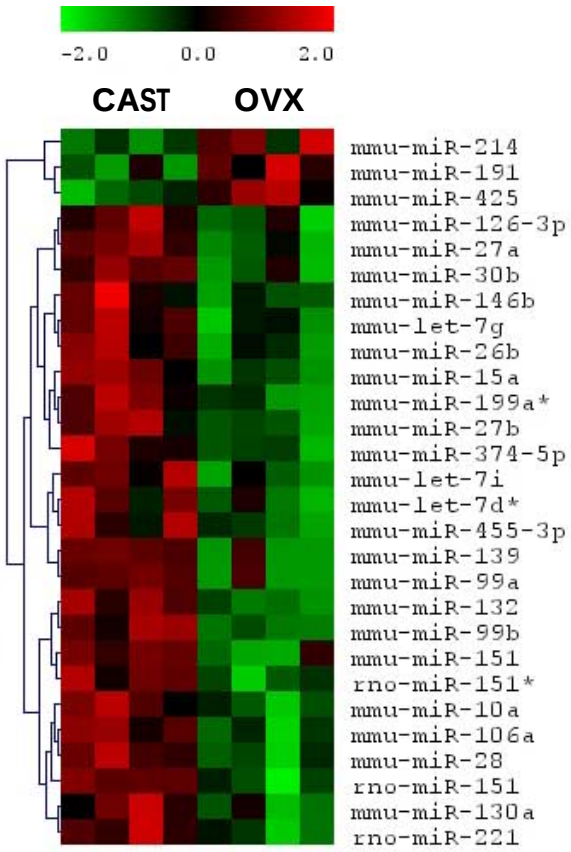

Figure 5.3. Hierarchical clustering of differentially expressed miRNA genes in castrated (CAST) and ovariectomized (OVX) spleen samples.

Hierarchical clusters of significantly altered miRNAs (as determined by ANOVA) across the CAST and OVX mouse spleen. Red denotes high expression levels, whereas green depicts low expression levels. Each miRNA listed is significantly differentially expressed ($p < 0.05$) between CAST and OVX mice.

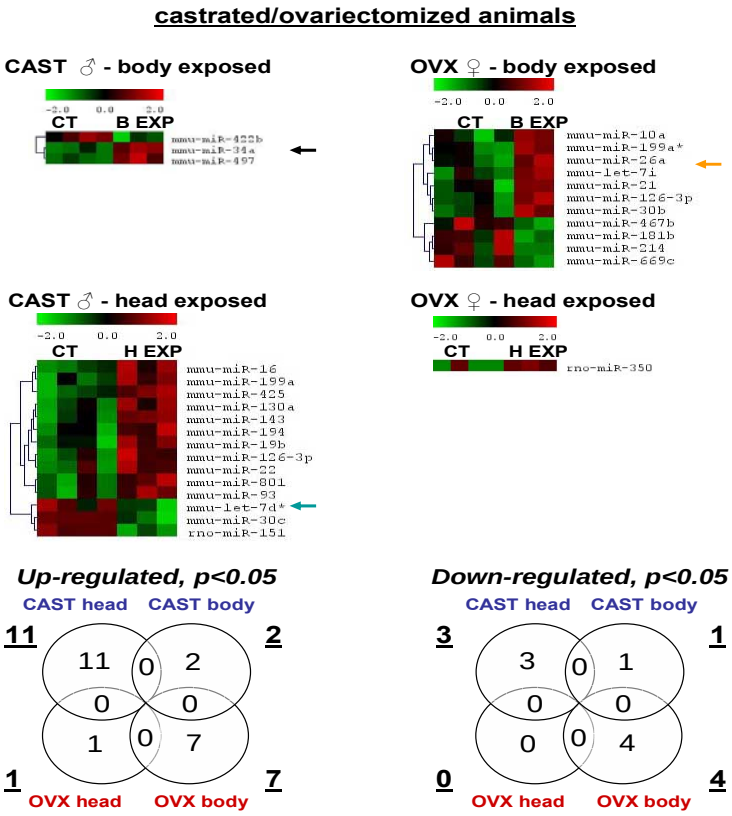

Figure 5.4. Differentially expressed miRNAs in spleen of castrated (CAST) and ovariectomized (OVX) mice subjected to whole body and head exposure of 1 Gy of X rays.

Hierarchical clusters of differentially expressed miRNA genes in spleen of whole body and head exposed CAST and OVX mice (as determined by ANOVA). Each miRNA listed is significantly differentially expressed ($p < 0.05$) between control and treatment groups. Color arrows denote microRNAs expressed differentially expressed in different groups.

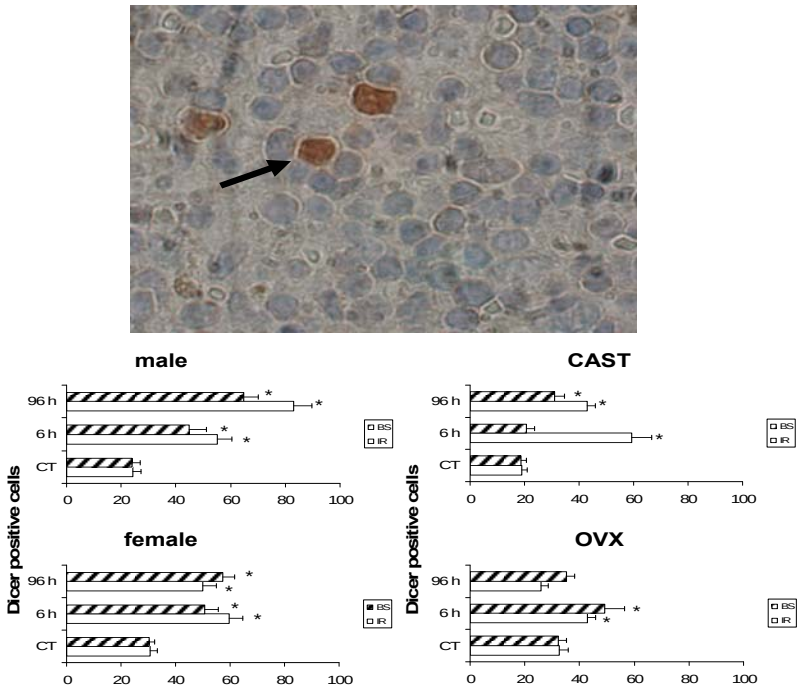

Figure 5.5. Changes in the Dicer levels in the exposed and bystander spleen of intact male, intact female, castrated (CAST) and ovariectomized (OVX) mice.

The levels of Dicer positive cells relative to control are shown, mean values ± SEM, n=400* - $p<0.05$, Student's t-test, Dunnett's test and Tukey-Kramer test.

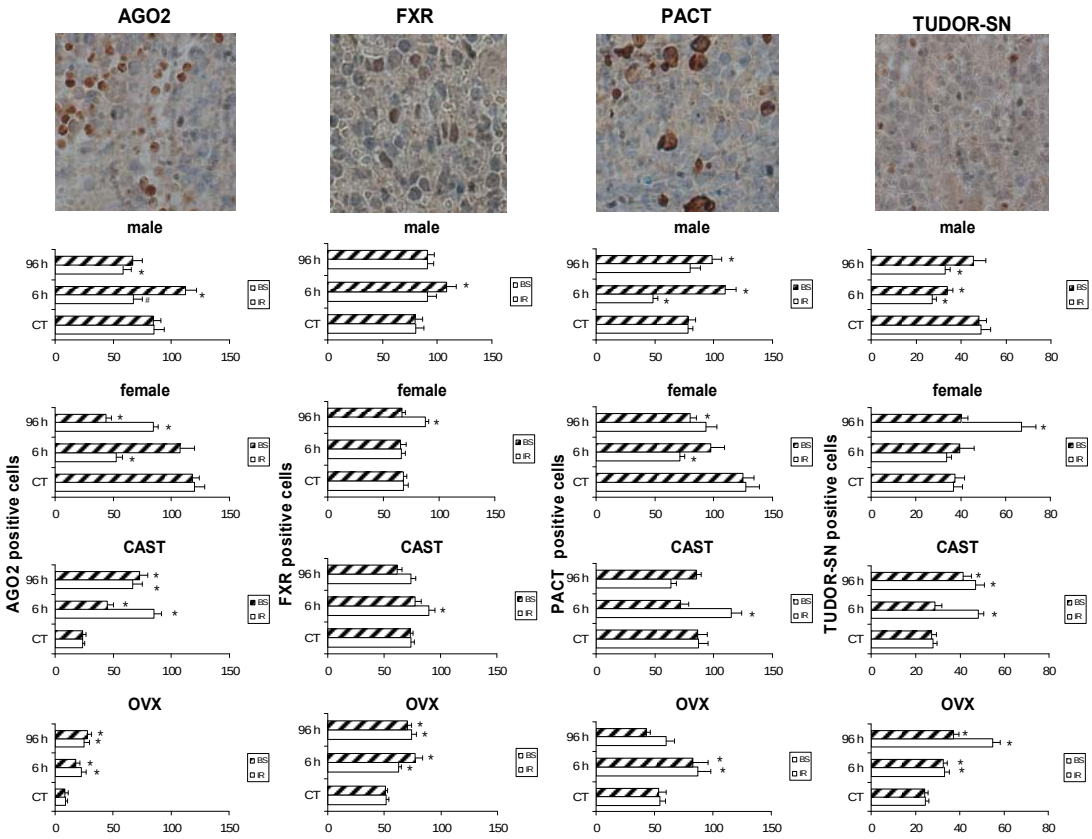

Figure 5.6. Changes in the Ago2, PACT, FXR and TUDOR-SN levels in the exposed and bystander spleen of intact male, intact female, castrated (CAST) and ovariectomized (OVX) mice.

The levels of Ago2, PACT, FXR and TUDOR-SN positive cells relative to control are shown, mean values ± SEM, n=400* - $p<0.05$, Student's t-test, Dunnett's test and Tukey-Kramer test.

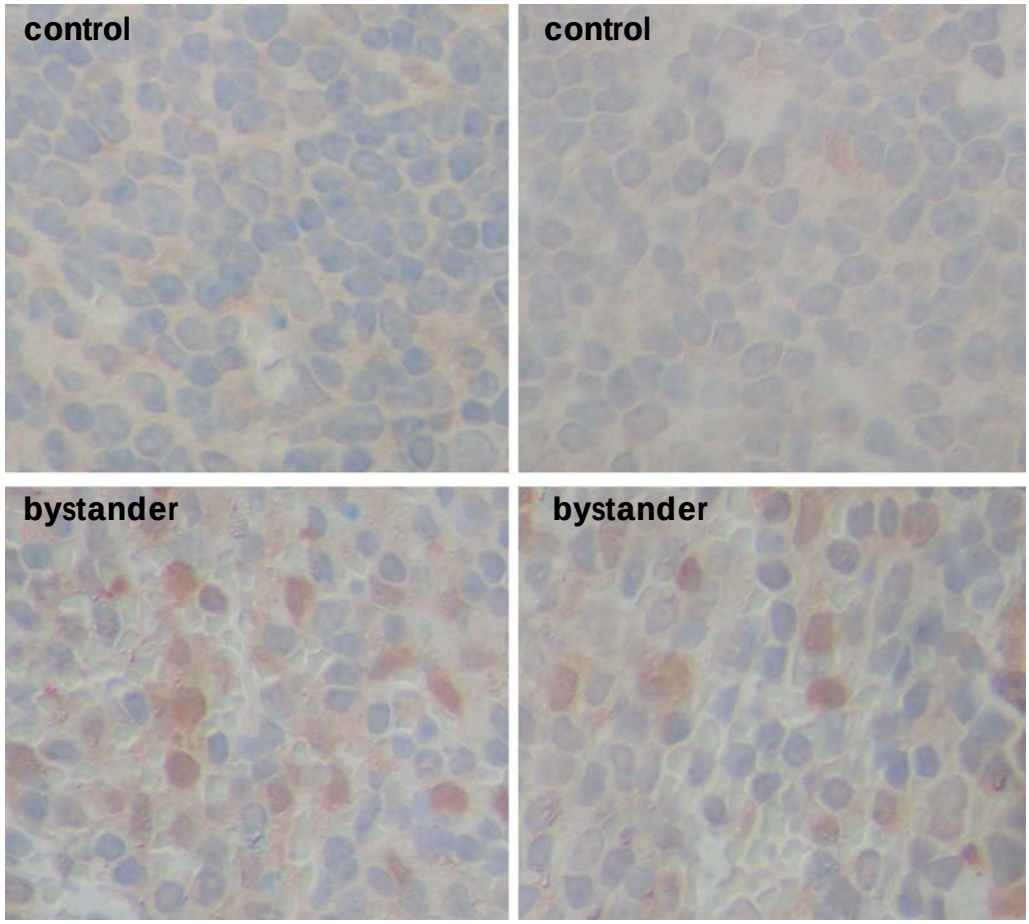

Figure 5.7. Changes in p38 levels in bystander male and female spleen.

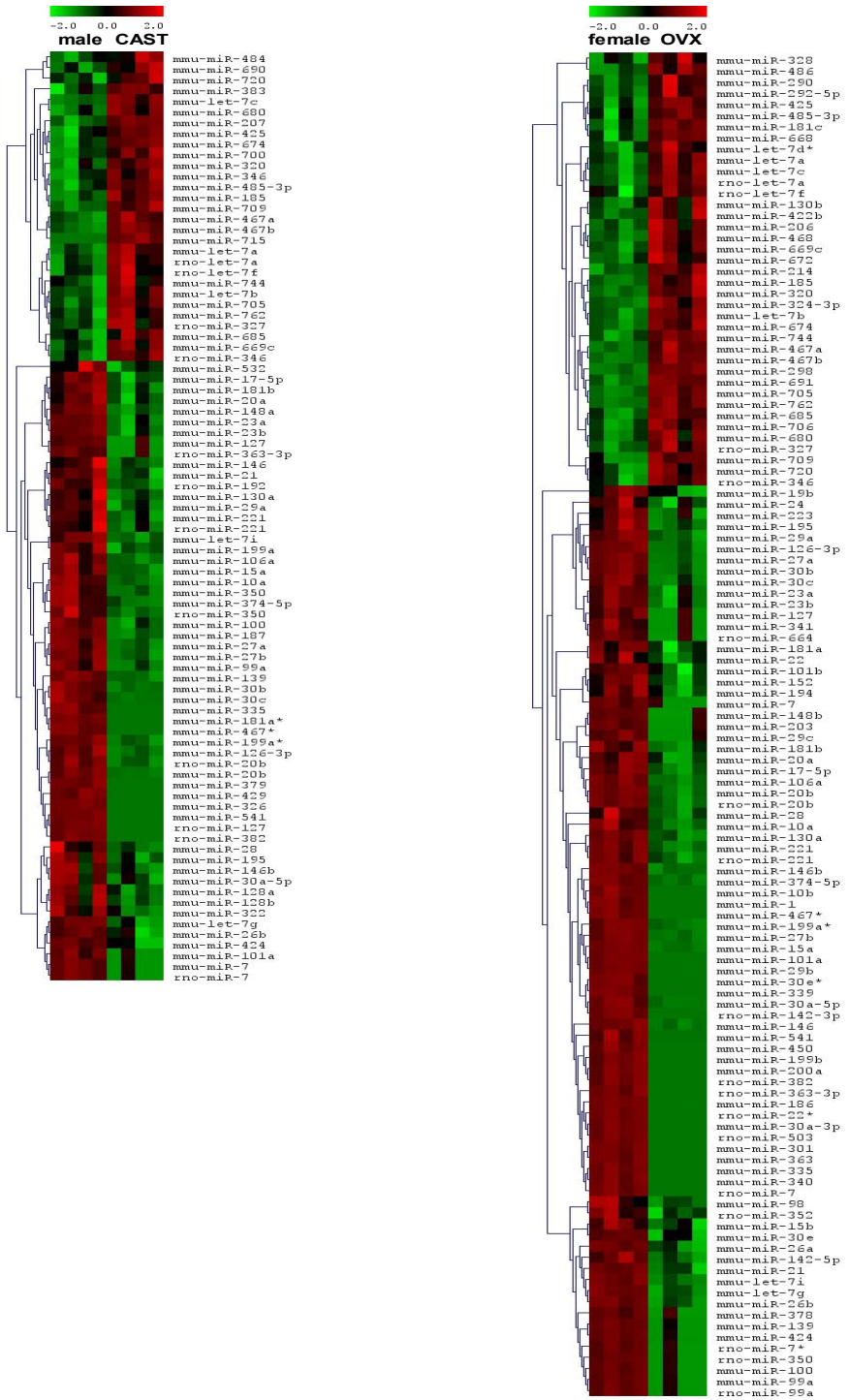

Figure 5.8. Hierarchical clustering of differentially expressed miRNA genes in intact (male/female) and sterilized (CAST/OVX) spleen samples.

6. ROLE OF EPIGENETIC EFFECTORS IN MAINTENANCE OF THE LONG-TERM PERSISTANT BYSTNADER EFFECT IN SPLEEN *IN VIVO*[1]

[1]Chapter 6 has been published in its entirety:

Koturbash I, Boyko A, Rodriguez-Juarez R, McDonald R, Tryndyak V, Kovalchuk I, Pogribny I, Kovalchuk O. (2007) Role of epigenetic effectors in maintenance of the long-term persistent bystander effect in spleen in vivo. *Carcinogenesis,* **28**:1831-8

6.1 ABSTRACT

Radiation therapy (RT) is a primary treatment modality for brain tumors, yet it has been linked to the increased incidence of secondary, post-RT cancers. These cancers are thought to be linked to indirect radiation-induced bystander effects. Bystander effects occur when irradiated cells communicate damage to nearby, non-irradiated 'bystander' cells, ultimately contributing to genome destabilization in the non-exposed cells. Recent evidence suggests that bystander effects may be epigenetic in nature; however, characterization of epigenetic mechanisms involved in bystander effect generation and its long-term persistence has yet to be defined.

To investigate the possibility that localized X-ray-irradiation induces persistent bystander effects in distant tissues, we monitored the induction of epigenetic changes (i.e., alterations in DNA methylation, histone methylation and miRNA expression) in the rat spleen tissue 24 hours and 7 months after localized cranial exposure to 20Gy of X rays.

We found that localized cranial radiation exposure led to the induction of a bystander effect in lead-shielded, distant spleen tissues. Specifically, this exposure caused a profound epigenetic dysregulation in the bystander spleen tissue which manifested as a significant loss of global DNA methylation, alterations in methylation of LINE 1 retrotransposable elements, and downregulation of DNA methyltransferases and methyl-binding protein MeCP2. Further, irradiation significantly altered expression of miR-194, a microRNA putatively targeting both DNMT3a and MeCP2. This study is the first to report conclusive evidence of the long-term persistence of bystander effects in radiation

carcinogenesis target organs (spleen) upon localized distant exposure using the doses comparable to those used for clinical brain tumor treatments.

6.2 INTRODUCTION

Radiation therapy (RT) is the number one treatment for almost all brain tumor types (Curry *et al*, 2005), malignant (Shu *et al*, 1998; Halberg *et al*, 1991; Krischer *et al*, 1991), as well as benign (Attanasio *et al*, 2003). Despite the markedly better prognosis and increased patient survival rates after RT, a serious drawback of induction of secondary tumors arose. Numerous cases of secondary cancer development following RT have been reported (Simmons *et* Laws, 1998; Brada *et al*, 1992), yet mechanisms of secondary cancers remain enigmatic. Recent studies attribute secondary carcinogenesis to indirect radiation effects, particularly – to the radiation-induced bystander effect.

Bystander effect is a phenomenon whereby irradiated cells communicate the damage to the non-irradiated nearby 'bystander' cells, thus contributing to their genome destabilization and carcinogenesis (Mothersill *et* Seymour, 2004; Hall *et* Hei, 2003; Hall, 2003). This enigmatic phenomenon is considered by some researchers to be a negative complication in radiation oncology, while others refer to it as an important protective homeostatic response (Mothersill *et* Seymour, 2005; Hall, 2003; Hall *et* Hei, 2003; Camphausen *et al*, 2003; Redpath *et al*, 2001).

The majority of the bystander effect data currently come from the studies in cell cultures, while *in vivo* data are scarce (Hall, 2003; Goldberg, 2003; Goldberg *et* Lehnert, 2002). Mechanistically, current models link radiation-induced bystander effects with the radiation-induced genome instability (Goldberg, 2003; Morgan, 2003b; Morgan, 2003c; Morgan *et al*, 2002). Radiation-induced genomic instability which encompasses a wide range of end points such as gross genome rearrangements, chromosome aberrations, gene mutation (Morgan, 2003c; Dubrova, 2003) was recently suggested to have an epigenetic

nature, yet the contribution of known epigenetic mechanisms to its generation has to be defined (Dubrova, 2003; Nagar *et al*, 2003).

Epigenetic changes are heritably stable alterations and include DNA methylation, histone modifications and RNA-mediated silencing (Jaenisch *et* Bird, 2003). Aberrant cytosine DNA methylation is associated with genomic instability, increased genome rearrangements rates and is linked to cancer development (Royo *et al*, 2006; Baylin, 2005; Jaenisch *et* Bird, 2003). A variety of DNA damaging agents, including ionizing radiation (IR), are known to affect genome DNA methylation pattern (Raiche *et al*, 2004; Pogribny *et al*, 2004a; Minamoto *et al*, 1999). DNA methylation changes are closely connected with the alterations in the other components of chromatin structure, primarily - histone modifications, which are also affected by IR exposure (Pogribny *et al*, 2005). One of the mechanisms of chromatin modification is the involvement of small regulatory RNAs (Bernstein *et* Allis, 2005). Regulatory RNAs were associated with cellular differentiation, proliferation, apoptosis, as well as with predisposition to cancer (Sevignani *et al*, 2006; Hwang *et* Mendell, 2006; Hammond, 2006). These molecules have the ability change the pattern of gene expression or target epigenetic modifications in various regions of the genome (Pogribny *et al*, 2005; Raiche *et al*, 2004; Pogribny *et al*, 2004a; Minamoto *et al*, 1999). The role of RNA-mediated silencing, specifically microRNAs (miRNAs), in bystander effects has never been addressed.

We have 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 the medical grade shield (Koturbash et al, 2006b). The data showed that X-ray exposure to one side of the animal body caused profound epigenetic

changes in the unexposed bystander body half 4 days after exposure. The potential of the localized body part exposures to induce bystander effects has never been addressed in detail.

The data obtained in the mouse skin model showed that bystander effects were the most prominent when spleen was in the field of irradiation, suggesting a possible role of spleen in the bystander effects (Koturbash *et al*, 2006b). Spleen is an important radiation-target organ (Raiche *et al*, 2004; Pogribny *et al*, 2004a), yet the existence and molecular mechanisms of the radiation-induced bystander effects in spleen have never been addressed.

Also, to be relevant for carcinogenesis the bystander effects should accumulate and/or persist over a long period of time. Yet, the long-term persistence of the distant bystander lesions in somatic organs upon irradiation has never been addressed.

To investigate the possibility that localized X-ray-irradiation induces bystander effects in the distant tissue, we monitored the induction of epigenetic changes (i.e. DNA methylation, histone methylation and miRNA expression) in spleen tissue 24 hours and 7 months after the localized cranial irradiation. Here, we report that localized cranial radiation exposure leads to decreased levels of global DNA methylation, alters the levels of key proteins known to modulate methylation patterns and silencing (e.i. de novo methyltransferase DNMT3a and methyl-binding protein MeCP2) and contributes to reactivation of LINE1 retrotransposons in the bystander spleen, located at least 16 cm from the irradiation site. We also show that radiation exposure results in the altered levels of *miR-194* in plasma and spleen of animals subjected to cranial X-irradiation. Importantly, the changes persisted for 7 months after exposure. These are some of the

first data to clearly demonstrate that bystander effects occur *in vivo* in distant tissue and, most importantly, persist over a long period of time and are epigenetically regulated.

6.3 MATERIALS AND METHODS

6.3.1 Model and Irradiation of Animals

In this study we examined the genetic and epigenetic alterations in rat spleen following *in vivo* cranial irradiation exposure. Rats (3 months old male Long Evans animals) were randomly assigned to different treatment groups. Handling and care of animals was in the strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures have been 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 whole body exposed cohort (6 animals) received 20Gy (5cGy/s) of X-rays (90kV, 5mA) applied as two doses of 10 Gy in two consecutive days. The animals were sacrificed 24 hours after the second exposure. The head-exposed cohort (18 animals) received 20 Gy (5cGy/s) of X-rays (90kV, 5mA) to the hippocampal area of the scull, applied as two doses of 10Gy in two consecutive days. The rest of the animal body 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 RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, Carle Place, NY). Six animals were humanely sacrificed 24 hours after exposure to address the early bystander effects. Twelve animals were humanely sacrificed 7 months after exposure. This time point is sufficient to see the persistence, if any, of the radiation-induced changes, as 7 months in rat life correspond to 10 human years (Quinn, 2005). Control rats (12 animals) were sham treated. For the sham treatment the animals were places into the irradiator machine and completely

shielded by lead. No radiation leakage through the shield has occurred as verified by dosimetry using RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc). Six control animals were sacrificed 24 hours after exposure, while the other six animals – 7 months after exposure. Spleen tissue was sampled upon sacrifice, snap-frozen and processed for further molecular studies. Upon sacrifice, blood from *vena cava* was collected into the polypropylene tubes with heparin and plasma was obtained by centrifugation (440g, 10 min). Plasma was carefully removed and snap frozen.

6.3.2 Cytosine Extension Assay to Detect Sequence Specific Changes in DNA Methylation

Total DNA was prepared from spleen tissue of exposed and control animals using Qiagen DNAeasy Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. The cytosine extension assay and the determination of the absolute percent of double-stranded unmethylated CCGG sites was conducted as previously described (Koturbash *et al*, 2006b; Pogribny *et al*, 2005; Pogribny *et al*, 2004b). DNA methylation changes in the exposed cohorts were related to the age-matched controls. No statistically significant age-related changes were noted.

6.3.3 Western Immunoblotting

Western immunoblotting for DNMT1, DNMT3a, DNMT3b, MeCP2 and PCNA was conducted using spleen tissue of exposed and control animals as described before (Koturbash *et al*, 2006b; Pogribny *et al*, 2005) using the following antibodies - DNMT1 (1: 1000, Abcam, Cambridge, MA), DNMT3a, DNMT3b (1:500, Abgent, San Diego,

CA), MeCP2 (1: 1000, Abcam, Cambridge, MA) and PCNA (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and the ECL Plus immunoblotting detection system (Amersham, Baie d'Urfé, Québec). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Signals were quantified using NIH ImageJ 1.63 Software and normalized to both GAPDH and the Mr 50,000 protein which gave consistent results. Protein levels in the exposed cohorts were related to the age-matched controls.

6.3.4 Analysis of LINE 1 Methylation Status by COBRA Assay

Methylation status of long interspersed nucleotide elements (LINE-1) was determined by COBRA assay, which consists of a standard bisulfite modification of genomic DNA, subsequent PCR amplification and digestion of PCR product with appropriate restriction endonuclease (Asada *et al*, 2006; Tryndyak *et al*, 2006; Xiong *et* Laird, 1997). The combination of sodium bisulfite treatment and PCR amplification results in methylation-dependent creation of new restriction endonuclease sites, such as RsaI, or methylation dependent retention of pre-existing sites, such as BstUI. Briefly, genomic DNA was treated with sodium bisulfite as previously described in detail (Tryndyak *et al*, 2006). Bisulfite-modified DNA was PCR-amplified with primers corresponding to the regulatory region of rat LINE-1 sequence (Tryndyak *et al*, 2006). The sense primer was 5'-TTTGGTGAGTTTGGGATA-3', and the antisense primer was 5'-CTCAAAAATACCCACCTAAC-3'. The PCR products were digested with RsaI or BstUI restriction endonucleases (New England Biolabs, Beverly, MA). The digested

products were separated on 3% high resolution agarose gels (Sigma, St. Louis, MO), stained with ethidium bromide, photographed, and the band intensity was analyzed by NIH ImageJ 1.63 Software.

6.3.5 mRNA Isolation and Real-time Reverse Transcription-PCR (RT-PCR)

Total mRNA was isolated from frozen spleen tissue of control and exposed animals by using TRIzol Reagent (Invitrogen, Burlington, Ontario) and first-strand cDNA template was synthesized using RevertAid™ First Strand cDNA Synthesis Kits (Fermentas, Burlington, Ontario) according to the manufacturer's instructions. The primers used in this study were synthesized by Operon Biotechnologies (Huntsville, Alabama). The LINE1 ORF1 (Long Interspersed Nuclear Element 1 open reading frame 1) primer sequences were: LINE 1 ORF1 forward 5'-AAGAAACACCTCCCGTCACA-3'; LINE1 ORF reverse 5'-CCTCCTTATGTTGGGCTTTACC- 3'. The beta actin primers were: beta actin forward 5'-CCTCTGAACCCTAAGGCCAA-3'; beta actin reverse 5'-AGCCTGGATGGCTACGTACA-3'. The data for LINE 1 ORF1 were standardized against the actin data. Control wells containing SYBR Green PCR master mix and primers without sample cDNA emitted no fluorescence after 40 cycles.

6.3.6 Histone Extraction

The acid extracts were prepared from frozen spleen tissues using lysis buffer containing 10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl , 0.5 mM DTT , 1.5 mM PMSF, followed by addition of HCl to a final concentration of 200 mM. Cell lysates

were centrifuged at 14000xg for 10 min at 4ºC, and the acid-insoluble pellets were discarded. The supernatant fractions, which contain the acid soluble proteins, were purified by sequential dialysis against 100 mM acetic acid and H_20. Protein concentrations were determined by Bradford assay (Pierce, Rockford, IL). Equal amounts of total histones (40 µg) were resolved on a 15% polyacrylamide gel. Proteins were transferred onto PVDF membranes (Pogribny *et al*, 2005).

6.3.7 Analysis of Histone Modifications

Equal amounts of total histones were resolved on 15% polyacrylamide gel and transferred onto PVDF membranes. Membranes were incubated using anti-trimethyl-histone H3 lysine 9 (1:1000), anti-trimethyl-histone H4 lysine20 (1:2000), anti-histone H3 (1:1000) and anti-histone H4 (1:1000) primary antibodies (Upstate, Charlottesville, VA). Upon secondary alkaline phosphatase-labeled antibody treatment chemifluorescence was detected with the ECL Substrate for Western Blotting (GE Healthcare, Piscataway, NJ) and measured directly by Storm Imaging System (Molecular Dynamics, Sunnyvale, CA).

6.3.8 miRNA Microarray Expression Analysis

Total RNA from frozen plasma was prepared using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to manufacturer's instructions. Total RNA from rat spleen was also extracted using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to manufacturer's instructions, size-fractionated (<200 nt) by using the *mir*Vana kit (Ambion, Austin, TX) and labeled with Cy3 and Cy5 fluorescent dyes. Dye switching

was performed to eliminate the dye bias. Pairs of labeled samples were hybridized to dual-channel microarrays. Microarray assays were performed on a µParaFlo microfluidics chip with each of the detection probes containing a nucleotide sequence of coding segment complementary to a specific miRNA sequence and a long non-nucleotide molecule spacer that extended the detection probe away from the substrate. The maximum signal level of background probes was 180. A miRNA detection signal threshold was defined as twice the maximum background signal. Normalization was performed with a cyclic LOWESS (locally-weighted regression) method to remove system-related variations. Data adjustments included data-filtering, log2 transformation, and gene centering and normalization. The *t*-test analysis was conducted between control and exposued sample groups. miRNAs with p-values < 0.05 were selected for cluster analysis. Two independent microarray hybridizations were performed, each involving 4 control and 4 exposed samples to insure the reproducibility of the results. The miRNA microarray analysis was performed by LC Sciences (Austin, TX).

6.3.9 Quantitative Real Time-PCR (RT-PCR) miRNA Expression Analysis

RT-PCRs on total spleen and plasma RNA were performed by using SuperTaq Polymerase (Ambion, Austin, TX) and the *mir*Vana qRT-PCR miRNA Detection Kit™ (Ambion, Austin, TX) following the manufacturer's instructions. Reactions contained *mir*Vana qRT-PCR Primer Sets™ specific for hsa-miR-194 and human 5S rRNA as positive controls. Real-time RT-PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA) and end-point reaction products were also analyzed on a 3.5% high-resolution agarose gel stained with ethidium bromide.

6.3.10 Statistical Analysis

Statistical analysis (Student's *t*-test, Bonferroni correction, Tukey-Kramer test and Dunnett's tests) was performed using the MS Excel 2000 and JMP5 software packages.

6.4 RESULTS

In this study we used a rat spleen model to gain further insight into the occurrence, possible persistence and molecular underpinnings of the somatic bystander effect *in vivo* after a localized cranial irradiation. The animals received either a whole-body exposure of 20Gy or the localized cranial exposure of 20Gy to the hippocampal area of the brain, while the rest of the body was shielded by lead. Shielding was complete as verified by careful dosimetry. We examined the role of epigenetic factors in the generation of bystander effects *in vivo* in the rat spleen. This important organ is frequently affected following therapeutic radiation exposures and is one of the targets of radiation oncology side effects (Koturbash *et al*, 2005).

6.4.1 Significant and Persistent Loss of Global Genome DNA Methylation in Bystander Spleen

Initially, we investigated the IR-induced changes in global cytosine DNA methylation in control, exposed and bystander spleen tissue 24 hours and 7 months after radiation treatment. To determine the absolute percent of methylated HpaII sites in DNA, we used the HpaII/MspI-based cytosine extension assay that measures the proportion of CCGG that had lost methyl groups on both strands. While HpaII cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands, its isoschizomer MspI cleaves CCGG sites in DNA regardless of CpG methylation status. The absolute percent of double-stranded unmethylated CCGG sites can be calculated by relating the data of HpaII and MspI digests (Pogribny *et al*, 2004b). Because the vast majority of the frequently occurring HpaII tetranucleotide recognition sequences are constitutively

methylated *in vivo*, an increase in cleavage at these sites is an indicator of genome-wide hypomethylation (Pogribny *et al*, 2004b). We found that whole body X-ray exposure resulted in a significant increase in the absolute percent of unmethylated CCGG sites in rat spleen as compared to controls (Fig. 6.1A). Localized head exposure also led to a significant increase in the absolute percent of unmethylated CCGG sites in the shielded bystander spleen as compared to the control (Fig. 6.1A and B). This effect, indicative of a profound global DNA hypomethylation was seen in the bystander spleen 24 hours and 7 months after cranial irradiation (Fig. 6.1A and B).

6.4.2 Hypomethylation-related Reactivation of LINE 1 Retroelements in Bystander Spleen

Having seen the significant and persistent global DNA hypomethylation in rat spleen 7 months after cranial irradiation we decided to further analyze the nature of the DNA methylation changes and the possible hypomethylation target loci. Several studies in humans and rodents have shown that global DNA demethylation resulted in activation of transposable elements contributing to genome instability (Rollins *et al*, 2006; Yoder *et al*, 1997). With this in mind, we analyzed the levels of DNA methylation of the LINE-1 transposable retroelements which constitute almost 23% of rat genome (Rat Genome Sequencing Project Consortium, 2004). Figure 6.2 shows the status of the LINE-1 regulatory region methylation in spleen of the control rats and the rats exposed to cranial irradiation, as determined by the COBRA assay (Tryndyak *et al*, 2006; Xiong *et* Laird, 1997). The assay is based on digestions of the PCR fragment amplified from the bisulfite converted DNA, where all non-methylated cytosines are converted to thymines. LINE-1-

specific primers amplified the fragment of 163 nt from the bisulfite-converted DNA (Fig. 6.2A). This fragment contains two sequences that can be recognized by BstUI and RsaI endonucleases, upon retaining and modifying the original sequence after bisulfite conversion. Digestion with BstUI is only possible if both cytosines at recognition sequence CGCG are methylated and thus, protected from conversion. Complete methylation will lead to complete cleavage resulting in two bands of 80 and 83 nucleotides long (seen as one band on the gel; Fig. 6.2B), whereas loss of methylation at any of the CpG cytosines would prevent cleavage and contribute to an "uncut" 163 nt band (Fig. 6.2B). The recognition sequence GTAC for the RsaI enzyme can be formed from GGCACG sequence in the 163nt fragment of LINE-1 promoter when non-CpG cytosine is completely unmethylated and CpG cytosine is completely methylated (Fig. 6.2C). Previous papers showed that methylation at the non-CpG sites at the LINE-1 promoter is extremely rare, with 98.2% of all the non-CpG cytosines been unmethylated (Tryndyak *et al*, 2006; Burden *et al*, 2005). Thus, it is safe to suggest that the RsaI recognition site is influenced primarily by the loss of methylation at CpG cytosine (Fig. 6.2C).

The COBRA assay revealed that LINE-1 promoters were indeed hypomethylated in the bystander rat spleen 7 months after cranial irradiation. There was a 5-10% decrease in the cleavage of PCR products using BstUI and RsaI enzymes in the group of exposed versus group of control animals (Fig. 6.2D, E). These phenomena, indicative of LINE1 reactivation, can in turn contribute to gross genome rearrangements and genome instability resulting in the neoplastic cell transformation. LINE1 activation was

previously documented in tumor cells and was paralleled by global genome hypomethylation (Garnell *et* Goodman, 2003).

To prove our hypothesis, we analyzed the steady state level of LINE-1 ORF1 RNA. RT-PCR analysis of spleen tissue revealed a significant 60% increase in expression of LINE-1 in the bystander spleens of the head-exposed rats as compared to controls (Fig. 6.2F).

6.4.3 Altered levels of DNA methyltransferases and methyl-binding proteins in bystander spleen

Having seen pronounced and persistent DNA hypomethylation in the exposed and bystander spleen we proceeded with analysis of the possible mechanisms of this phenomenon. We addressed the radiation-induced changes in the expression levels of the maintenance (DNMT1) and *de novo* (DNMT3a and DNMT3b) methyltransferases (Goll *et* Bestor, 2005) in the control, exposed and bystander spleen tissue 24 hours and 7 months after irradiation.

We noted that levels of methyltransferases DNMT1, DNMT3a and DNMT3b and the methyl-binding protein MeCP2 were statistically significantly downregulated 24 hours upon the whole-body as well as the localized head exposure as compared to the controls (Fig. 6.3). We also found that whole body exposure resulted in significant downregulation of proliferating cell nuclear antigen (PCNA) 24 hours after exposure (Fig. 6.3). Contrarily, PCNA levels were not altered in the bystander spleen 24 hours after exposure (Fig. 6.3). These data are in good agreement with whole body irradiation-induced DNA hypomethylation and reduced expression of DNMTs and MeCP2 in mouse spleen previously reported by our group (Raiche *et al*, 2004; Pogribny *et al*, 2004a).

Importantly, altered protein levels were also observed 7 months after cranial radiation exposure (Fig. 6.4). We found that DNMT1 levels were statistically significantly decreased in the spleen of the bystander group 7 months after cranial irradiation (Fig. 6.4).

At the same time, we noted that the levels of *de novo* methyltransferase DNMT3a were also statistically significantly down-regulated in bystander spleen 7 months after exposure (Fig. 6.4). We have previously shown that DNMT3a expression was reduced following IR exposure, and correlated with post-irradiation DNA hypomethylation (Raiche *et al*, 2004; Pogribny *et al*, 2004a). Also, DNMT3a expression was previously found to be down-regulated in the bystander skin (Koturbash *et al*, 2006b).

In addition to the DNA methylation loss and down-regulation of the key methyltransferases, we also noted a significantly lower level of methyl-binding protein MeCP2 in the bystander spleen 7 months after irradiation (Fig. 6.4). This protein has been shown to interact with methylated DNA and affect methylation-mediated chromatin remodeling and gene silencing (Jaenisch *et* Bird, 2003; Hendrich *et* Tweedie, 2003). The contribution of MeCP2 to IR-induced genome instability and bystander effects need to be further analyzed.

Since DNA replication is one of the most active mechanisms contributing to partial loss of methylation, it was important to show that cell proliferation was not induced in bystander spleens either 24 hours or 7 months after cranial exposure (Fig. 6.3 and Fig. 6.4). Western blot analysis did not find any changes in the level of the proliferating cells nuclear antigen, confirming that the observed changes were not linked to an increase in cellular proliferation (Fig. 6.3).

The reduced levels of DNMTs and MeCP2 seen 7 months after exposure may be linked to the LINE-1 hypomethylation and reactivation seen at the same time (Fig. 6.2). Previously, down-regulation of methyl-CpG binding protein MeCP2 was shown to pre-dispose cells to elevated levels of mutation and LINE-1 retrotransposon reactivation, and result in genome instability and aberrant gene expression rates, leading to carcinogenesis (Yu *et al*, 2001). Also, altered levels of DNMT1, DNMT3a or 3b significantly reduced DNA methylation levels in the LINE-1 repetitive elements (Xu *et al*, 1999).

Undoubtedly, DNA methylation changes and altered expression/activity of DNA methyltransferases may in part be linked to DNA damage induced by reactive oxygen species generated upon radiation exposure (Pogribny *et al*, 2005; Raiche *et al*, 2004; Pogribny *et al*, 2004a). Reactive oxygen species were implicated in the bystander effects in cell cultures, yet their exact role in bystander effects in vivo still need to be addressed.

6.4.4 Lack of Histone Methylation Changes in Bystander Spleen

Recent studies indicate that global loss of DNA methylation may be paralleled by changes in histone methylation, specifically – histones H3 and H4 hypomethylation (Fraga et al, 2005). The combined loss of DNA methylation and trimethylation of histone H3 lysine 9 (H3K9me3) and H4 lysine 20 (H4K20me3) were reported to be signs of carcinogenesis. The interplay between radiation-induced DNA methylation changes and histone modifications is still under-investigated (Pogribny *et al*, 2005). The role(s) of histone modifications in bystander effects have never been addressed. Thus, the pronounced DNA methylation changes have prompted us to investigate the changes in histones H3K9 and H4K20 trimethylation in control, exposed and bystander spleen 24

hours and 7 months after exposure. We noted that whole body exposure to 20Gy of X rays resulted in a significant decrease in the levels of H4K20 trimethylation (Fig. 6.5A). This was in agreement with the loss of H4K20 trimethylation upon irradiation in mouse thymus tissue previously reported by our group (Pogribny *et al*, 2005). Yet, we did not observe any significant changes in histone H3K9 trimethylation and histone H4K20 trimethylation levels in the bystander spleen tissue either 24 hours or 7 months after irradiation (Fig. 6.5A and B).

6.4.5 miRNA Expression in Bystander Tissue

Another mechanism of epigenetic control is through the involvement of small regulatory RNAs. Amongst those, of special interest are microRNAs (miRNAs). miRNA molecules are small non-coding RNAs that function as key regulators of RNA interference pathway (Sevignani *et al*, 2006; Hwang *et al*, 2006; Hammond, 2006). miRNAs can reduce the levels of their target transcripts as well as the amounts of protein encoded by their transcripts (Sevignani *et al*, 2006; Hwang *et al*, 2006; Hammond, 2006). By doing so miRNAs regulate RNA stability and translatability, thus contributing to gene silencing. In parallel, their roles as regulators of epigenetic status and chromatin structure have also been proposed (Bernstein *et* Allis, 2005). Thereafter, miRNAs play crucial roles in regulating cellular differentiation, proliferation and apoptosis. Further to this, miRNA expression changes are a common occurrence in cancers and were suggested to play an important role(s) in pathogenesis of human tumors (Esquela-Kerscher *et* Slack, 2006; Ruvkun, 2006).

Thus, in a search for possible regulators of long-term persistent bystander effects we analyzed the role of microRNAs. We set out to determine the expression patterns of known miRNAs in spleen from control and head-exposed animals 7 months after irradiation using miRNA microarray technology. Two independent miRNA microarray hybridizations were performed, each involving 4 control and 4 bystander spleen samples. We identified that *miR*-194 was significantly up-regulated in bystander spleen seven months after exposure. We also noted a subset of miRNAs that exhibited an up-regulation trend (90% confidence limit) (data not shown). To confirm the result of microarray analysis, we carried out RT-PCR of *miR-194*. The RT-PCR was a solid validation of the microarray result (data not shown).

We next proceeded to address the early changes in *miR*-194 expression upon whole body and cranial irradiation using the RT-PCR approach. We found that the levels of *miR-194* were significantly elevated in the spleen of whole-body and head exposed animals 24 hours after irradiation (data not shown). Furthermore, the elevated levels of miR-194 were also seen in plasma of the whole-body and head exposed animals 24 hours after irradiation (data not shown), suggesting the possible spread on miRNAs by blood.

We next proceeded with identification of putative *miR-194* targets using the known algorithms (John *et al*, 2004). To our surprise, we found that *miR-194* may be targeting DNMT3a and MeCP2, the proteins that were significantly down-regulated in the exposed and bystander spleen tissue 24 hours and 7 months after irradiation (Fig. 6.3 and Fig. 6.4).

Thus, up-regulation of *miR-194* may be seen as a putative step in maintenance of the low levels of DNMT3a and MeCP2, the key regulators of DNA methylation. The low

levels of the aforementioned proteins may consequently lead to global genome hypomethylation and locus-specific DNA methylation loss in LINE 1 retrotransposons (Fig. 6.2). Thus, *miR-194* may be one of the putative players in maintenance of an epigenetic bystander response in rat spleen. This microRNA can also regulate a variety of other targets influencing cellular stress responses.

6.5 DISCUSSION

In this report we describe a significant and persistent *in vivo* bystander effect that occurs in the rat spleen upon distant cranial irradiation. The main findings of the present study are: (i) localized cranial radiation exposure leads to the induction of a bystander effect in the lead-shielded distant spleen tissue; (ii) epigenetic dysregulation in the bystander spleen tissue was manifested as a significant loss in global DNA methylation and alterations in the methylation of LINE 1 retrotransposable elements; (iii) irradiation leads to the significant down-regulation of DNA methyltransferases and methyl-binding protein MeCP2; (iv) irradiation significantly alters the expression of *miR-194*, a microRNA that putatively targets DNMT3a and MeCP2; (v) profound epigenetic alterations can be seen 24 hours after the exposure and persisted for 7 months.

Overall, the firm and conclusive evidence that IR-induced bystander effects *in vivo* are operational is limited (Koturbash *et al*, 2006b; Hall, 2003; Goldberg, 2003; Goldberg *et* Lehnert, 2002). The clinical literature is scarce and does not provide any support for or against the somatic bystander effect *in vivo*, even though numerous studies suggest a functional bystander effect *in vitro* (reviewed by Little, 2006).

To bridge this knowledge gap, our laboratory has recently developed an *in vivo* model whereby bystander effects were studied in the skin of animals subjected to half-body or head exposure, while the rest of the body was protected by a medical grade lead shield. Using this model, we have pioneered studies of the role of epigenetic mechanisms in bystander effects in cutaneous tissue *in vivo*, and generated the first data to clearly demonstrate that bystander effects occur *in vivo* in distant tissue and are epigenetically mediated (Koturbash *et al*, 2006b). Importantly, the data obtained in the mouse skin

model showed that bystander effects were the most prominent when the spleen was in the field of irradiation, suggesting a possible role of the spleen in bystander effects (Koturbash *et al*, 2006b).

As it's shown in the present study, spleen, an important radiation-target organ, is also an important target organ for the bystander effect. The bystander effect observed in the shielded bystander spleen 24 hours and 7 months after head exposure was characterized by profound epigenetic dysregulation. This is in agreement with our previous report on the *in vivo* bystander effect in skin where epigenetic alterations also played a key role (Koturbash *et al*, 2006b). In both systems we noted a down-regulation of *de novo* DNA methyltransferase DNMT3a in bystander spleen tissue. *De novo* methyltransferases function primarily as regulators of a cell fate and differentiation. Their deregulated expression/activity may lead to deleterious effects and contribute to carcinogenesis (Goll *et* Bestor, 2005; Robertson *et* Jones, 2000).

We have also noted a significant decrease in the level of maintenance DNA methyltransferase DNMT1 and methyl-binding protein MeCP2 in bystander spleen. These results somewhat contradict our previous observations in the skin model. Yet, in our previous study we were analyzing the bystander effects that occurred after 1 Gy of X ray exposure, while the dose used in the current study was 20 times higher. Also, in the current study we addressed the persistent long-term effects 7 months after exposure. Thus the differences in DNMT1 and MeCP2 expression patterns observed in the two studies may to a certain extent reflect the dynamics of changes that occur over time. Furthermore, the discrepancy could also be due to the tissue specificity of bystander

changes. Further studies are clearly needed to address the tissue specificity and dose responsiveness of the bystander effect induction.

It should be noted that the reduction of the levels of DNA methyltransferases and methyl-binding protein MeCP2 in the exposed and bystander spleen ranges between 15 – 50%. DNA methyltransferases are responsible for setting and maintaining DNA methylation patterns in mammalian cells (Goll *et* Bestor, 2005). Importantly, it was shown that both maintenance (DNMT1) and *de novo* (DNMT3a and 3b) methyltransferases played a critical role in the development of mammalian organisms, and their loss had lethal consequences (Egger *et al*, 2006; Kaneda *et al*, 2004; Hata *et al*, 2002). Thus these seemingly 'small' methyltransferases expression changes can significantly affect cellular function. The exact impact of DNMTs and methyl-binding proteins on radiation-induced bystander effects has to be further elucidated.

Of special interest and potential importance was the loss of DNA methylation and reactivation of LINE 1 retroelements observed. Hypomethylation of LINE-1 sequences have been reported in many cancers (Roman-Gomez *et al*, 2005). It was suggested that hypomethylation of LINE-1 sequences may promote the genomic instability and facilitate tumor progression (Kazazian, 2002). However, it has not yet been established whether the epigenetic changes, including hypomethylation and reactivation of LINE1 elements often found in tumors, play a causative role in carcinogenesis, or they are merely a consequence of the transformed state. Recently, progressive hypomethylation of a LINE-1 regulatory region in liver has been found at very early stages of carcinogenesis prior to full-fledged tumor formation (Tryndyak *et al*, 2006). The result of our present study that

shows the loss of LINE-1 CpG methylation in bystander spleen may be viewed as a genome instability event predisposing cells to future carcinogenic transformations.

As X-rays penetrate tissue, they can react with biological matter resulting in the deflection of its trajectories. This is referred to as scatter radiation (Koturbash *et al*, 2006b). Low doses, close to the scatter-dose range levels, were shown to induce bystander effects (Zeng *et al*, 2006; Mothersill *et al*, 2005; Maguire *et al*, 2005; Kashino *et al*, 2004; Mothersill *et* Seymour, 2002). Yet, congruent with the previous study, the bystander effects observed in this study in the rat spleen were not due to insufficient shielding or radiation scattering. We used the same animal body shielding that was previously described (Koturbash *et al*, 2006b). The scatter dose for rat spleen was miniscule (~0.0012Gy), slightly less than the previously reported one for the mouse model (Koturbash *et al*, 2006b). Exposure of animals to the scatter dose did not affect DNA methylation and protein expression 24 hours after exposure (data not shown).

The important outcome of this study is the observation of the altered levels of *miR-194* in spleen and plasma of the whole-body and head exposed animals. miRNAs are involved in the post-translational gene regulation and gene silencing. Although the precise biological role of miRNAs has not been fully understood, they are suggested to regulate various developmental and physiological processes, such as apoptosis, cellular differentiation and proliferation. Their altered expression may contribute to diseases. Recently, the importance of miRNAs in gene silencing and chromatin dynamics has attracted scientists' increasing attention (Bernstein *et* Allis, 2005). Profiling miRNA expression under various conditions as well as correlating profiles with physiological changes may allow investigators to uncover the exact role of miRNAs in cellular life.

Here we found that up-regulated levels *miR-194* in the exposed and bystander tissue are correlated with down-regulated levels of its putative targets – *de novo* methyltransferase DNMT3a and methyl-binding protein MeCP2. The decreased levels of these proteins were linked to significant global and LINE1 locus-specific hypomethylation, thus contributing to the genome destabilization and possibly further to carcinogenesis.

It should be mentioned that *miR-194* is the only miRNA that is statistically significantly up-regulated in bystander rat spleen tissue 24 hours and 7 months after exposure. It is also strongly upregulated in the spleen and plasma of rats subjected to the whole-body irradiation. Overall, *miR-194* seems to play some role in the maintenance of the long-term response where, probably, the altered levels of just a few RNAs are enough.

Further studies are clearly needed to achieve the in-depth understanding of the complete dynamics of miRNAome patterns in the bystander effects.

A most recent report by Kemppainen *et al*, (2007) proved that miRNAs are very abundant in biofluids – plasma, serum, urine and saliva and exhibit high donor-to-donor consistency (Kemppainen *et al*, 2007). Thus, even though their means of dispersion into biofluids are illusive, microRNAs in plasma, serum, saliva and urine can be used as biomarkers for cancer and other diseases (Kemppainen *et al*, 2007).

Our study is the first that has shown the altered levels of microRNA in the plasma upon radiation exposure. More studies are needed to understand the ways of microRNA penetration/secretion into plasma and the role of blood miRNAs in mediation of distant radiation responses. Overall, this is the first report suggesting a possible role of miRNAs

in bystander effects. More studies are needed to confirm and dissect the miRNA role in this enigmatic phenomenon in detail.

Most importantly, this study is the first report that provides the conclusive evidence of the long-term persistence of the bystander effects in radiation carcinogenesis target organ (spleen) following the localized distant cranial exposure. The doses and exposure regimes used in this study are close to those used in a clinic for brain therapy treatments (Curry *et al*, 2005). Radiotherapy is one of the most common treatments for brain tumors (Curry *et al*, 2005; Shu *et al*, 1998; Halberg *et al*, 1991; Krischer *et al*, 1991). Nevertheless, the risk of the secondary radiation-induced complications, including secondary cancers which peak 10 years after exposure, has been reported (Curry *et al*, 2005; Goldberg, 2003; Goldberg *et* Lehnert, 2002). Seven months in the life cycle of a rat corresponds to ~ 10 years in human life (Quinn, 2005). Thus the data obtained in this study using a rat model can be potentially extrapolated to humans. Further studies are required to delineate the possible contribution of radiation-induced bystander effects to secondary carcinogenesis, to identify and characterize the nature of the enigmatic bystander signal, and to address the impact of reactive oxygen species and DNA damage on the mediation of the immediate and delayed bystander effects *in vivo*.

6.6 FIGURES AND TABLES.

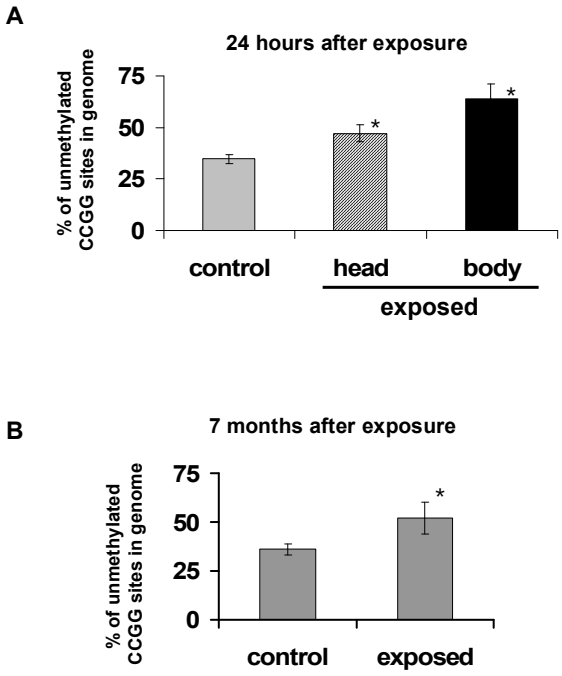

Figure 6.1. Pronounced loss of DNA methylation in rat spleen after the whole body and the localized cranial irradiation.

Levels of the global genome DNA methylation in spleen tissue were measured by the HpaII /MspI cytosine extension assay. HpaII that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. MspI is an isoschizomer of HpaII which cleaves CCGG sites in DNA regardless of CpG methylation status. The absolute percent of double-stranded unmethylated CCGG sites was calculated by relating the data of HpaII and MspI digests. Data are presented as mean values ± SD, * - $p<0.01$, Student's *t*-test, Dunnett's test and Tukey-Kramer test.

A. DNA methylation changes observed 24 hours after the whole body and head exposure;

B. DNA methylation changes seen 7 months after the head exposure.

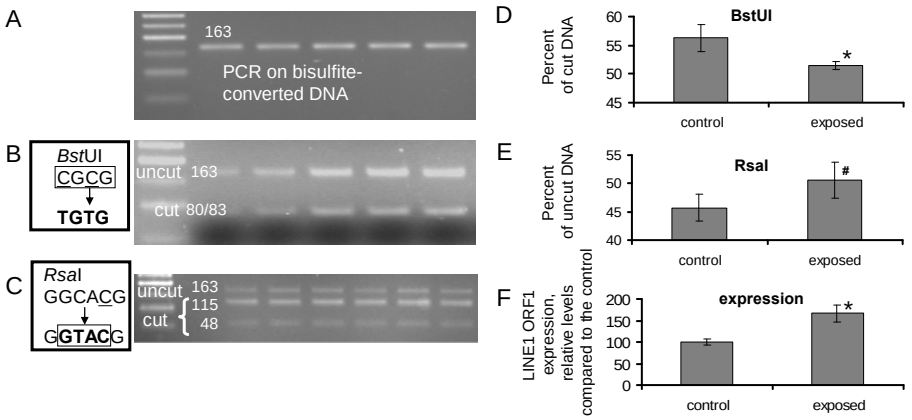

Figure 6.2. Cranial irradiation leads to altered methylation Of the LINE1 promoter, resulting in elevated expression of LINE1 ORF1 in the spleen of exposed rats.

Methylation status of LINE-1 was determined by the COBRA assay, involving bisulfite modification of genomic DNA, subsequent PCR amplification and digestion of PCR product with BstUI and RsaI enzymes. Sizes of the DNA fragments were determined using the 50bp DNA Step Ladder (Promega, Madison, WI).

A. PCR amplification of 163 nt fragment from LINE-1 promoter.

B. Methylation dependent retention of pre-existing BstUI restriction sites. Unmethylated CpG cytosines (underlined) at CGCG recognition sequence (squared) can be lost by bisulfite conversion (sequence in bold), resulting in uncut bands (163 nt). Methylation at both sites allows the cleavage, resulting in fragments of 80 and 83 nt (seen as one band).

C. CpG methylation dependent retention of cytosine (underlined) at GGCACG sequence results in generation of RsaI recognition site (squared and in

bold), leading to cleavage of the 163 nt fragment into fragments of 48 and 115 nt. Loss of methylation at CpG cytosine will prevent the cleavage.

D. Quantification of BstUI cut fragments. The "Y"axis shows the percent of cleaved 163 nt fragment presented as an average (with SD).

E. Quantification of RsaI cut fragments. The "Y"axis shows the percent of cleaved 163 nt fragment presented as an average (with SD).

F. Levels of LINE-1 ORF1 transcript detected by RT-PCR; steady state RNA levels relative to those of control animals are shown as the mean $\pm$ SD. * - significant, 95% confidence limit, $p<0.05$; # - 90% confidence limit, $p< 0.1$ (Student's *t*-test).

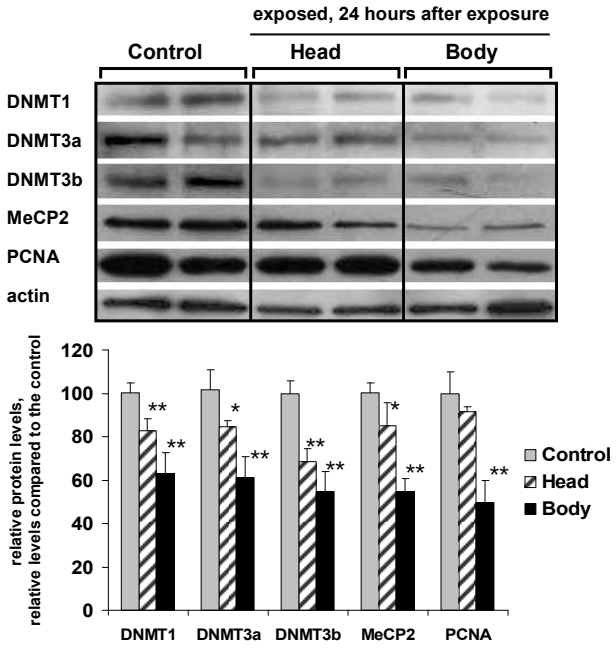

Figure 6.3. Altered expression of DNA (cytosine-5) methyltransferases, methyl-binding protein MeCP2 and proliferating cells nuclear antigen (PCNA) in spleen of rats 24 hours post whole-body or localized cranial irradiation.

Lysates of spleen tissue were subjected to immunoblotting using antibodies against DNMT1, DNMT3a, DNMT3b, MeCP2, PCNA and actin (loading control). Protein levels relative to those of control animals are shown as the mean $\pm$ SD; **p* <0.0167, Student's *t*-test with Bonferroni correction for multiple comparisons, Dunnett's test, Tukey-Kramer test). Representative western blots from among 5 independent technical repeats of the experiments are shown.

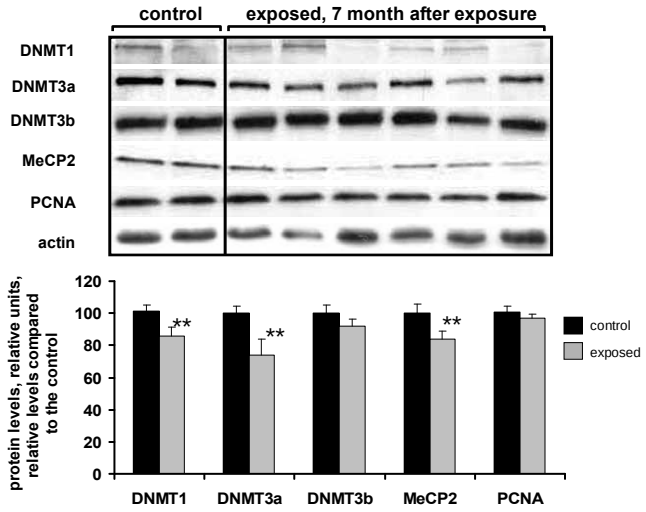

Figure 6.4. Altered expression of DNA (cytosine-5) methyltransferases, methyl-binding protein MeCP2 and proliferating cells nuclear antigen (PCNA) in spleen of rats 7 months post localized cranial irradiation.

Lysates of spleen tissue were subjected to immunoblotting using antibodies against DNMT1, DNMT3a, DNMT3b, MeCP2, PCNA and actin (loading control). Protein levels relative to those of control animals are shown as the mean $\pm$ SD; ** - significant, 99% confidence limits, p <0.01, Student's *t*-test, Dunnett's test, Tukey-Kramer test; *- significant, 95% confidence limit, p <0.05, Student's *t*-test, Dunnett's test, Tukey-Kramer test). Representative western blots from among 5 independent technical repeats of the experiments are shown. Black bars – control animals; gray bars – animals subjected to cranial irradiation.

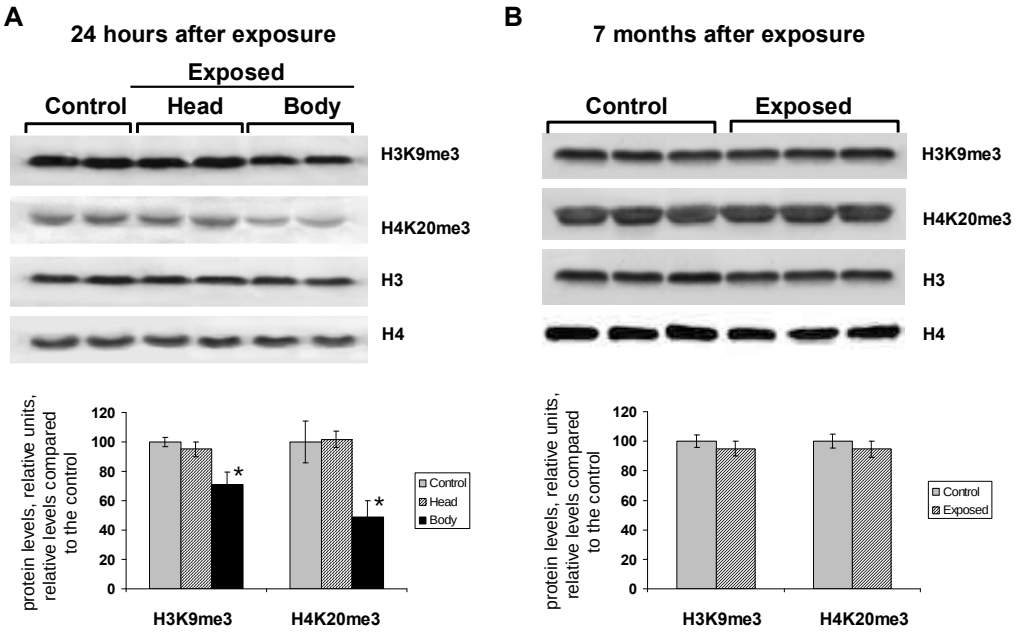

Figure 6.5. Histone methylation levels in rat spleen upon whole body and localized cranial irradiation.

Acidic spleen tissue lysates enriched in histones were subjected to immunoblotting using antibodies against histone H3K9me3, H4K20me3 and total histones H3 and H4 as loading controls. Levels relative to those of control animals are shown as the mean $\pm$ SD. Representative blots from among 3 independent experiments are shown, *p <0.0167, Student's t-test with Bonferroni correction for multiple comparisons, Dunnett's test, Tukey-Kramer test.

A. Histone methylation changes observed 24 hours after whole body and head exposure;

B. Histone methylation changes seen 7 months after head exposure.

7. FINAL DISCUSSION AND CONCLUSIONS

Currently cancer is the leading cause of death in Canadians, and the number of new cases in Alberta alone is expected to double to more than 26,000 cases per year by 2025 (Alberta Cancer Board, 2006). Most cancer patients undergo radiation diagnostics and are also treated with radiotherapy (RT). Ionizing radiation (IR), along with being an important diagnostic and treatment modality, is a potent tumor-causing agent, and the risk of secondary radiation treatment-related cancers is a growing clinical problem (Simmons *et* Laws, 1998; Brada *et al*, 1992). Notwithstanding, their molecular etiology remains elusive. Now some studies propose to link secondary RT-induced cancers to the enigmatic phenomenon of bystander effects, whereby the exposed cells signal damage and distress to their naïve neighbors and result in genome destabilization and carcinogenesis (Mothersill *et* Seymour, 2004; Hall, 2003).

7.1 RADIATION-INDUCED BYSTANDER EFFECTS

Bystander effects can be seen in naïve cells which were in contact with directly irradiated cells, or in the naïve cells which received certain irradiation 'distress' signals from directly exposed cells via a growth medium. A variety of extended bystander effect studies have been performed using cell culture models, tissue explants, spheroids (Persaud *et al*, 2005), and reconstructed human tissue models (Sedelnikova *et al*, 2007, Belyakov *et al*, 2005). As a result, bystander effects are now accepted as a ubiquitous consequence of IR exposure (Mothersill *et* Seymour, 2004). IR-induced bystander effects include a wide range of genetic alterations such as chromosome aberrations, sister chromatid exchanges, deletions, duplications, gene mutations and amplifications. These

molecular changes influence gene expression, cellular proliferation, senescence, and cell death. Importantly, they are thought to be linked to radiation-induced malignant transformation (Mothersill *et* Seymour, 2004; Mothersill *et* Seymour, 2003; Goldberg *et* Lehnert, 2002).

Bystander effects also manifest themselves in the whole-organism environment. Yet, compared to the bystander effect data based on cell cultures, conclusive data on somatic bystander effects *in vivo* are relatively uncommon (Mothersill *et al*, 2007; Koturbash *et al*, 2007; Koturbash *et al*, 2006; Hall, 2003; Goldberg *et* Lehnert, 2002).

Prior to our studies, it was shown that IR exposure led to a release of soluble 'clastogenic' factors into the circulating blood. These factors are capable of inducing chromosome damage in cultured cells. This clastogenic activity was found in the plasma of radiotherapy patients and individuals accidentally exposed to IR (Marozik *et al*, 2007; Emerit *et al*, 1995; Emerit *et al*, 1994; Pant *et* Kamada, 1977; Goh *et* Summer, 1968).

Bystander effects were shown to be important 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*, 2003; Khan *et al*, 1998). Similar within-the-organ bystander effects were observed during partial liver irradiation (Brooks, 2004; Brooks et al, 1974).

Yet, no data existed on the bystander effects in an organ other than an exposed one. With this in mind, we focused on analysis of the existence and mechanisms of radiation-induced bystander effects *in vivo*. To do so, we generated three *in vivo* models:

- an *in vivo* mouse model whereby half of an animal body was exposed and the other half was protected by a lead shield;

- an *in vivo* mouse model of cranial exposure, where the head of the experimental animal was exposed to ionizing radiation, while the rest of the body was protected with the medical-grade lead shield;
- an in vivo rat model of cranial exposure, where the head of the experimental animal was exposed to ionizing radiation, while the rest of the body was protected with the same kind of a lead shield.

Using these models, we analyzed both the induction and persistence of bystander effects and evaluated their epigenetic nature.

The main findings of this thesis are that:

1. Bystander effects occur *in vivo* in distant skin and spleen following half-body or cranial irradiation.

2. Radiation exposure leads to the induction of DNA damage in distant bystander tissues in vivo. DNA damage in bystander tissues was present several hours after radiation.

3. Epigenetic changes are involved in the generation and/or maintenance of bystander effects.

4. Specifically, bystander tissues exhibited significant and persistent changes in the levels of global and locus-specific DNA methylation. Bystander tissues also showed changes in the expression of DNA methyltarnsferases and methyl-CpG binding proteins. Contrarily, histone methylation patterns were not altered in the bystander tissue.

5. Bystander tissues exhibited altered levels of microRNAs and changed expression of the enzymes involved in microRNA maturation and microRNA-mediated translational inhibition.
6. Bystander tissues altered the levels of apoptosis, cellular proliferation and p53.
7. Bystander effects are tissue specific. Specifically, we noted that bystander changes in the spleen tissue were much more pronounced then in the skin. This can be explained by higher radiation sensitivity of the spleen tissue.
8. Bystander effects are strain and species-independent, but there are some mouse strain differences. Apoptotic and proliferative responses as well as gene expression were somewhat different between C57Bl/6 and Balb/c mouse strains.
9. Bystander effects are persistent. Molecular changes in bystander tissue persisted through 4 days after irradiation and were still detectable 7 months post exposure.
10. Bystander effects are sex-specific. Changes in the male bystander spleen tissue were distinct from the female bystander spleen. Gonadectomy led to somewhat diminished but still detectable sex differences.

Having studied the cellular and molecular grounds of radiation-induced bystander responses in vivo, we propose the following model to describe an in vivo bystander phenomenon (see Figure 7.1)

According to our model, the previously described radiation-induced changes in directly exposed cells or tissues such as DNA strand breaks (Koturbash *et al*, 2005; Yokota *et al*, 2005), epigenetic perturbations (Loree *et al*, 2006; Koturbash *et al*, 2005; Pogribny *et al*, 2005; Kovalchuk *et al*, 2004b) altered gene expression (Cassie *et al*, 2006; Loree *et al*, 2006; Besplug *et al*, 2005; Kovalchuk *et al*, 2004a), and genome instability (Morgan, 2003a; Little, 1999; Little, 1998) can be transmitted to un-irradiated bystander cells or tissues (Kovalchuk *et* Baulch, 2008; Koturbash *et al*, 2008; Koturbash *et al*, 2007; Sedelnikova *et al*, 2007; Koturbash *et al*, 2006b). Transmission occurs most likely via the blood.

We propose reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Azzam *et al*, 2003; Lyng *et al*, 2002; Lyng *et al*, 2000; Mothersill *et* Seymour, 1998), short RNAs (Koturbash *et al*, 2007), Ca2+ ions (Lyng *et al*, 2006, Shao *et al*, 2006; Lyng *et al*, 2002; Lyng *et al*, 2000) and cytokines (Facoetti *et al*, 2006; Bonner, 2003; Iyer *et* Lehnert, 2000) to be signaling molecules that communicate the radiation-induced bystander effects.

The in vivo bystander effects exhibit a strong sex-specificity (Koturbash *et al*, 2008; Koturbash *et al*, unpublished) and potentially can contribute to carcinogenesis (Mothersill *et* Seymour, 2004; Mothersill *et* Seymour, 2003; Goldberg *et* Lehnert, 2002).

7.2 SIGNIFICANCE AND RELEVANCE TO HUMAN HEALTH.

Today one third of people are likely to get cancer, and more than half are likely to receive some form of radiotherapy. While modern cancer radiation therapy has led to the increased patient survival rates, the risk of secondary radiation treatment-related deleterious complications and adverse effects is becoming a growing clinical problem (Simmons *et* Laws, 1998; Brada *et al*, 1992). A bystander effect is a negative complication in radiation oncology, and it is probably linked to secondary radiation carcinogenesis (Mothersill *et* Seymour, 2004; Hall, 2003).

Prior to our study, however there has been no firm and conclusive evidence that the IR-induced bystander effect in vivo is operational (Goldberg, 2003; Hall, 2003; Goldberg *et* Lehnert, 2002). Furthermore, information on mechanisms of the bystander effect *in vivo* was scarce.

We saw that radiation-induced changes in the bystander spleen include: γ-H2AX foci accumulation (Koturbash *et al*, 2008), global genomic DNA hypomethylation and histone modifications (Koturbash *et al*, 2007), changes in gene expression (Koturbash *et al*, 2008), altered apoptosis and proliferation processes (Koturbash et al, 2008), as well as profound dysregulation in the miRNAome (Koturbash *et al*, 2007). All these phenomena are known to be signs of genome instability and are linked to tumorigenesis (Mothersill *et* Seymour, 2004; Mothersill *et* Seymour, 2003; Goldberg *et* Lehnert, 2002) (Figure 7.2).

Prior to clinical trials, experiments with animal models could be very useful in uncovering the existence and mechanisms of the somatic bystander effect.

The results obtained while using the animal model systems can potentially be extrapolated to different animals and humans.

7.3 FUTURE DIRECTIONS:

1) IR is a well-known carcinogen and IR-induced hematological malignancies are the most common IR side effects. **Would bystander-mediated changes ultimately result in a higher frequency of leukemia and/or thymic lymphoma?** This question remains unanswered up to now. To address this important issue, cranially exposed animals need to be monitored for 6 month to analyze the frequency of occurrence of hematologic malignancies. The first sign of thymic lymphoma development in mice is breathing difficulty and enlarged thymus. The average weight of an adult mouse thymus is 0.01 g, and a thymic lymphoma is defined as a malignancy resulting in an enlarged thymus weight >0.1 g. Another sign is spleenomegaly. Should any of these signs be observed, this may be indicative of the development of the secondary bystander-induced blood malignancy, leukemia or lymphoma. Leukaemias and lymphomas can be diagnosed by microscopic examination of blood, bone marrow, spleen and thymus, as recommended (Boulton *et al*, 2002; Plumb *et al*, 1998).

2) Another important question to answer – **what is the signal that spreads bystander effects?** One could predict the involvement of reactive oxygen species (ROS), cytokines, and short RNAs in blood, exposed, and bystander tissue (Koturbash *et al*, 2007; Shareef *et al*, 2007; Facoetti *et al*, 2006). Tissue and blood samples required for measuring ROS levels (Amer *et al*, 2003; Yamato *et al*, 2003; Kobayashi *et al*, 2001), cytokines

(Neininger *et al*, 2002; Sullivan *et al*, 2000) and short RNAs (Kovalchuk *et al*, 2007; Koturbash *et al*, 2007) can be collected at different time after cranial exposure to determine the immediate (30 min – 2 hours after irradiation) and long-term (96 hours after irradiation) effects of exposure to ionizing radiation. The first time-point can be used for evaluating existence of early post-exposure changes, whereas the second one can be used for the detection of persistence of the aforementioned factors.

3) **What impact does radiation- induced dysregulation of brain function have on the development of radiation-induced bystander effect in somatic organs?** It is very important to determine if radiation-induced brain changes partake in bystander effects. This could be done by comparing effects of IR damage and other kinds of stress- or trauma-induced brain damage on distant organs. To answer this question, stroke conditions can be simulated in one group of animals (Kolb *et al*, 2007; Metz *et al*, 2005; Gonzalez *et* Kolb, 2003); another group of animals will receive stress treatment: fear conditioning (Norris *et* Strickland, 2007; Cloutier *et al*, 2005; Cordero *et al*, 2003), dietary restriction (Fitting *et al*, 2007; Smith *et* Metz, 2005) or immobilization stress (Gagliano *et al*, 2008; Grimée *et* Wülfert, 1995). Epigenetic effectors, such as global DNA methylation (Kovalchuk *et al*, 2007, Koturbash *et al*, 2007; Pogribny *et al*, 2005; Koturbash *et al*, 2005), main histone modifications (Kovalchuk *et al*, 2007; Koturbash *et al*, 2007; Pogribny *et al*, 2005), and levels of expression of DNA methyltransferases and methyl-binding proteins

(Loree *et al*, 2006; Koturbash *et al*, 2006b), as well as a miRNA profile (Kovalchuk *et al*, 2007; Koturbash *et al*, 2007) can be evaluated in somatic organs (spleen, liver) to compare bystander tissues from cranially-exposed animals and a control group. The most recent studies suggest that epigenetic mechanisms are involved in the stress response (Darnaudéry *et* Maccari, 2007), what makes this research extremely important nowadays.

4) Taking into consideration that localized cranial exposure can lead to detectable bystander effects in distant somatic organs (Koturbash *et al*, 2008; Koturbash *et al*, 2007), we could infer the influence of somatic organ exposure on a protected brain. **Would radiation exposure to somatic organs result in bystander-effects in the distant shielded brain tissue?** Direct brain exposure to ionizing radiation can lead to profound functional and morphological changes in brain tissue (Limoli *et al*, 2004; Tofilon *et* Fike, 2000; Schulthesis *et al*, 1995), declines in hippocampal proliferation and neurogenesis, cognitive dysfunction, learning and memory deficits (Monje *et* Palmer, 2003; Monje *et al*, 2002), and gene expression alterations (Silasi *et al*, 2004). Recent data suggest that ionizing radiation can lead to the blood-brain barrier disruption (Nordal *et* Wong, 2005; Kaya *et al*, 2004; Li *et al*, 2003). This potentially can lead to the deleterious changes in brain tissues described above. To evaluate the possible existence and mechanisms of the exposed somatic tissue-derived bystander effect in brain, a rat model can be developed, where experimental animals will receive a clinically relevant dose of radiation to

the liver, while the rest of the animal body will be protected by a medical-grade lead shield. Animals can be sacrificed 4 and 90 days following irradiation, to see immediate and delayed changes. The molecular and cellular changes can be studied in liver, brain and blood. Molecular and cellular changes in the brain may be correlated with behavioral patterns. Such an approach would allow us to detect the deleterious effects, if any, that radiotherapy may have on the shielded brain tissue. Such studies will help us understand, at least in part, the enigmatic phenomenon of cancer and cancer-treatment related fatigue.

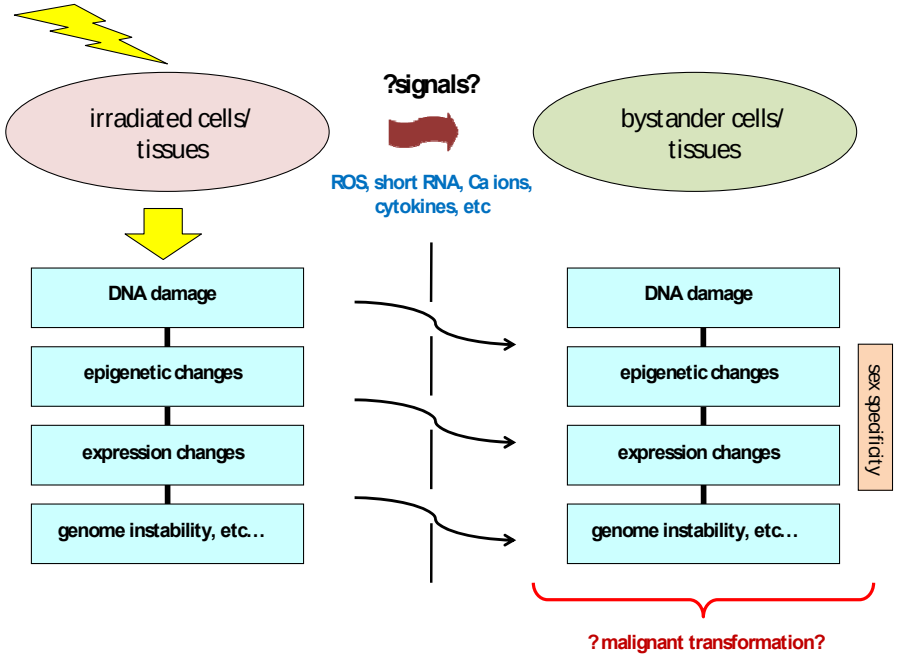

Figure 7.1 The scheme proposed to describe molecular and cellular events contributing to the in vivo bystander effects and their repercussions.

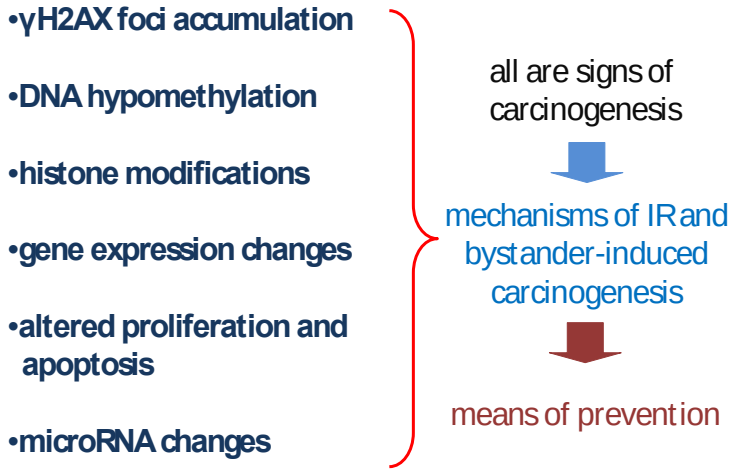

Figure 7.2. Possible linkage between radiation-induced bystander effects in vivo and carcinogenesis.

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