

**RADIATION-INDUCED EPIGENOME DEREGULATION IN  
THE MALE GERMLINE**

**Jan Tamminga**  
**Bachelor of Science, University of Lethbridge, 2006**

A Thesis  
Submitted to the School of Graduate Studies  
of the University of Lethbridge  
in Partial Fulfilment of the  
Requirements for the Degree

**MASTER OF SCIENCE**

Department of Biological Sciences  
University of Lethbridge  
LETHBRIDGE, ALBERTA, CANADA

© Jan Tamminga, 2008

## **ABSTRACT**

Approximately 45% of men will develop cancer during their lifetime; some of which will be of reproductive age (Canadian Cancer Society, 2008). Current advances in treatment regimens such as radiotherapy have significantly lowered cancer-related mortality rates; however, one major quality-of-life issue in cancer survivors is the ability to produce healthy offspring.

Exposure to ionizing radiation (IR) leads to genomic instability in the germline, and further to transgenerational genome instability in unexposed offspring of preconceptionally exposed parents. The results presented in this thesis define, in part, the molecular consequences of direct and indirect irradiation for the male germline. Direct exposure results in a significant accumulation of DNA damage, altered levels of global DNA methylation and microRNAome dysregulation of testis tissue.

Localized cranial irradiation results in a significant accumulation of unrepaired DNA lesions and loss of global DNA methylation in the rodent (rat) germline. Biological consequences of the changes observed are discussed.

## **ACKNOWLEDGMENTS**

First and foremost, I would like to thank my parents, Jan and Rosanne Tamminga, for their endless support and encouragement.

To Dr. Olga Kovalchuk, I would like to express my great appreciation for the opportunity to learn and grow as a student in her laboratory. The experience and skill set that I have acquired will be of great use in any future endeavour.

I would also like to thank my committee members, Dr. Elizabeth Schultz and Dr. Robert McDonald for their support and guidance throughout my time at the University of Lethbridge. I also thank Dr. Francesco Marchetti, my external examiner, for the time and travel commitment to visit the University of Lethbridge for my defence.

I am very grateful for the financial support I have received from the University of Lethbridge, the Alberta Cancer Board, and the province of Alberta.

Last, but not least, I would like to thank my many colleagues from both Kovalchuk laboratories for their friendship and contributions to the following work.

## TABLE OF CONTENTS

ABSTRACT.....	iii
Acknowledgments.....	iv
Table of Contents.....	v
List of Figures.....	vii
List of Tables.....	viii
List of Abbreviations.....	ix
CHAPTER 1: General introduction.....	1
Cellular associations of the testes.....	1
Molecular mechanisms of germ cell control.....	3
The BORIS and CTCF gene family.....	6
MicroRNA and spermatogenesis.....	8
MiRNA and fertilization.....	12
Effects of radiation on the male germline.....	13
Radiation-induced apoptosis.....	17
The radiation-induced bystander effect.....	18
Mechanisms of radiation-induced bystander effects.....	21
Radiation-induced transgenerational genome instability.....	22
Mechanisms of transgenerational genome instability.....	25
Hypothesis.....	29
CHAPTER 2: DNA damage-induced upregulation of miR-709 in the germline downregulates BORIS to prevent aberrant DNA hypomethylation.....	33
Abstract.....	34
Introduction.....	35
Materials and Methods.....	35
Animal exposure.....	35
MiRNA microarray expression analysis.....	36
MicroRNA fluorescence in situ hybridization (FISH) using locked nucleic acid (LNA) probes and tyramide signal amplification.....	36
Luciferase reporter assay for targeting BORIS-3'-UTR.....	37
RNA preparation, reverse transcription and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR).....	38
Western immunoblotting.....	39
DNA extraction and DNA methylation analysis.....	40
DNA strand break measurement.....	41
Immunofluorescence.....	41
Statistical analysis.....	42
Results and discussion.....	42
CHAPTER 3: DNA damage, DNA hypomethylation and altered expression of the Brother of the Regulator of Imprinted Sites (BORIS) in the germline of radiation- exposed male mice.....	50
Abstract.....	51
Introduction.....	52
Materials and Methods.....	54
Animal model: irradiation scheme and tissue sampling.....	54

Nucleic acid extractions .....	56
Cytosine extension assay to detect sequence-specific changes in DNA methylation .....	56
DNA strand break measurement .....	57
BORIS and CTCF reverse transcriptase polymerase chain reaction (RT-PCR) .....	58
Immunofluorescence .....	59
Statistical analysis .....	60
Results .....	60
Radiation-induced DNA damage in irradiated and bystander testes tissue of the whole body and cranially exposed mice .....	60
Altered levels of global DNA methylation in the irradiated testis .....	62
Irradiation alters mRNA levels of BORIS, but not CTCF in the exposed testes tissues .....	63
Discussion .....	65
CHAPTER 4: Paternal cranial radiation exposure induces distant bystander DNA damage and alters DNA methylation in the shielded germline .....	77
Abstract .....	78
Introduction .....	79
Materials and Methods .....	81
Animal exposure .....	81
DNA extraction .....	82
DNA methylation analysis .....	82
DNA strand break measurement .....	82
DNA damage analysis – histone $\gamma$ H2AX foci accumulation .....	82
Immunocytochemistry .....	83
Immunohistochemistry .....	83
Sperm cell extraction and sperm DNA preparation .....	83
Statistical analysis .....	84
Results .....	84
Induction of bystander effect in the testes tissue of rats subjected to cranial irradiation .....	84
Accumulation of DNA damage in the testes tissue of cranially exposed rats ....	85
Bystander-mediated formation of $\gamma$ H2AX foci in the testes of cranially exposed rats .....	86
Cranial irradiation leads to unrepaired damage in the mature spermatozoa .....	87
Loss of DNA methylation in the testes of cranially exposed rats .....	88
Discussion .....	89
General Discussion and Conclusions .....	97
Future Directions .....	99
References .....	102

## LIST OF FIGURES

<b>Figure 1.0.</b> Diagrammatic representation of the 12 stages in the mouse seminiferous epithelium	<b>31</b>
<b>Figure 1.1.</b> Schematic representation of the expression pattern of BORIS, CTCF, and genome methylation during spermatogenesis	<b>32</b>
<b>Figure 2.0.</b> Radiation exposure alters microRNAome of mouse testes, leads to increased expression of miR-709, decreased expression of miR-709 target BORIS, and results in global genome hypomethylation	<b>47</b>
<b>Figure 2.1.</b> Radiation-induced DNA damage responses in the male germline	<b>49</b>
<b>Figure 3.0.</b> Levels of DNA strand breaks in murine testes following an acute exposure to 2.5Gy of X rays.	<b>72</b>
<b>Figure 3.1.</b> Effects of an acute 2.5Gy of X-rays on global DNA methylation levels in testis tissue	<b>72</b>
<b>Figure 3.2.</b> Expression levels of BORIS mRNA in murine testes tissues following an acute exposure to 2.5Gy of X-rays	<b>73</b>
<b>Figure 3.3.</b> Radiation-induced hypomethylation and altered expression of BORIS protein in testis tissue of whole-body exposed mice	<b>74</b>
<b>Figure 3.4.</b> Immunofluorescent localization of BORIS protein in the spermatocytes population of the mouse testis	<b>75</b>
<b>Figure 4.0.</b> Bystander-induced accumulation of DNA damage in the testes tissue of control, head-exposed and scattered dose exposed animals as studied by the ROPS assay and by the $\gamma$ H2AX foci formation	<b>93</b>
<b>Figure 4.1.</b> Bystander-mediated DNA damage in the mature sperm cells of cranially exposed rats	<b>95</b>
<b>Figure 4.2.</b> DNA hypomethylation in the whole rat testes and mature sperm cells after the localized cranial irradiation	<b>96</b>

## LIST OF TABLES

<b>Table 3.0:</b> RT-PCR conditions for BORIS, CTCF, and $\beta$ -actin	<b>76</b>
<b>Table 3.1:</b> Induction of DNA damage in testis tissue following 2.5Gy X-ray exposure	<b>76</b>

## LIST OF ABBREVIATIONS

- 5meC – 5-methyl-cytosine
- BORIS – brother of the regulator of imprinted sites
- bp – base pair
- CB – chromatoid body
- cDNA – complement DNA
- CT gene – cancer-testis gene
- CTCF – CCCTC binding factor
- CTCFL – CTCF-like
- DAPI – 4',6-diamidino-2-phenyl-indole
- dATP – deoxyadenine triphosphate
- DBD – DNA binding domain
- dCTP – deoxycytidine triphosphate
- DEPC - diethyl pyrocarbonate
- dGTP – deoxyguanine triphosphate
- DIG - digoxigenin
- DMR – differentially methylated region
- DNA – deoxyribonucleic acid
- DNMT – DNA methyltransferase
- dNTP - deoxynucleotide triphosphate
- DSB – double strand break
- dsRNA – double stranded RNA
- DTT - dithiothreitol

dTTP – deoxyguanine triphosphate

ECL - enhanced chemiluminescence

EDTA - ethylenediaminetetraacetic acid

ESTR – expanded simple tandem repeat

FISH – fluorescence in situ hybridization

FITC - fluorescein isothiocyanate

H&E – hematoxylin and eosin

H2AX - histone H2AX

HEK293 – human embryonic kidney cells

HPG – hypothalamic-pituitary-gonadal axis

HR – homologous recombination

ICR – imprinting control region

IF – immunofluorescent

IHC - immunohistochemical

IR – ionizing radiation

LNA – locked nucleic acid

MBD – methyl-CpG binding domain

MeCP – methyl-CpG binding proteins

miRNA – microRNA

mRNA – messenger RNA

NHEJ - nonhomologous end joining

nt – nucleotide

PBS – phosphate buffered saline

PCNA – proliferating cell nuclear antigen

PCR – polymerase chain reaction

PFA - paraformaldehyde

PGC – primordial germ cell

pre-miRNA – precursor miRNA

pri-miRNA – primary miRNA

PRMT – protein arginine methyltransferase

PVDF - polyvinylidene difluoride

RISC – RNA-induced silencing complex

RNA – ribonucleic acid

ROPS – random oligonucleotide-primed synthesis

ROS – reactive oxygen species

RT – radiation therapy

RT-PCR – reverse transcriptase polymerase chain reaction

SDS – sodium dodecyl sulfate

SEM – standard error of the mean

SSB – single strand break

SSC – sodium chloride sodium citrate

TAE - tris-acetate EDTA

TMA – tissue microarray

TNP – transition protein

UTR – untranslated region

UV – ultra violet

ZF – zinc finger

$\gamma$ H2AX – phosphorylated histone H2AX

## **CHAPTER 1: GENERAL INTRODUCTION**

### **CELLULAR ASSOCIATIONS OF THE TESTES**

The model organisms of the current study are mice and rats; the focus of this review will be the biology of the rodent germline.

The complete process of male germ cell development is called spermatogenesis and takes place within testes (reviewed in Holstein et al., 2003). A testis can be divided into several hundred conical lobules that contain the seminiferous tubules and intertubular tissue. The intertubular tissue surrounds the endocrine Leydig cells. The seminiferous tubules are coiled loops that secrete a fluid containing immature spermatozoa and are connected at both ends to the rete testis. The Rete testis is an anastomosing network of delicate tubules located in the hilum of the testicle (mediastinum testis). It carries immature sperm from the seminiferous tubules to the vasa efferentia. Immature spermatozoa are delivered to the excurrent ductal system of the epididymis where the spermatozoa mature into functional sperm (reviewed in Holstein et al., 2003).

The epithelium of the seminiferous tubule contains germ cells at various stages of development. These cells are spermatogonia, primary and secondary spermatocytes and spermatids. Spermatogonia are stem cells that line the periphery of the seminiferous tubules. As spermatogonia divide and mature into various cell types, they move progressively towards the lumen of the tubule. Normal germ cells will pass through three major phases of development which are referred to as spermatogoniogenesis, meiosis, and spermiogenesis.

There are two types of spermatogonia, namely Type A and Type B. Type A spermatogonia belong to the stem cell pool and undergo mitosis to produce one A type- and one B type-spermatogonium. Type B spermatogonia are committed to develop into spermatozoa and undergo mitosis to produce two primary spermatocytes. This marks the end of spermatogoniogenesis and the beginning of meiosis. Primary spermatocytes divide to produce secondary spermatocytes which divide again producing four round spermatids. Importantly, after germ cells divide they remain connected to one another via intercellular bridges, thus forming a syncytium (Greenbaum et al., 2007). This allows rapid communication between germ cells and ensures that they develop as a synchronous unit (Hamer et al, 2003). The process of differentiation of round spermatids into spermatozoa is called spermiogenesis.

Among these germ cells, there are somatic cells called Sertoli cells which are connected to each other by tight junctions that form a blood-testes barrier (Parreira et al., 2002). This barrier separates epithelium into basal and adluminal compartments. Once maturing germ cells pass a blood-testes barrier, they are protected from exogenous substances and the host's immune system (reviewed in Itoh et al., 2005). Sertoli cells also function as "nurse cells" that regulate the flow of nutrients and growth factors required by germ cells (reviewed in Peterson and Soder, 2006). Furthermore, Sertoli cells are also involved in the production of endocrine and paracrine substances that regulate spermatogenesis and movement of germ cells in the seminiferous epithelium (reviewed in Mruk and Cheng, 2004; Peterson and Soder, 2006).

Usually, a new cycle of spermatogenesis begins before the preceding cycle has finished. Depending on the length of spermatogenesis and frequency of new cycles, a cross section of the testes would reveal several hundred seminiferous tubules, each having a particular cellular association. For each species, these cellular associations can be artificially divided into stages (twelve in mice and fourteen in rats) that are numbered with Roman numerals (Figure 1.0).

It is important to note that the length of time that each cell remains in a particular stage is variable, and as such the frequency of time spent in each stage also varies (Hess et al., 1990). However, the seminiferous tubule is organized in such a way that these stages occur in a consecutive order. The sequential order and repetition of each stage along the tubule produce a “wave” of spermatogenesis (Timmons et al., 2002; Chung et al., 2004). The stages are in descending order from the rete testis until approximately half way along the length of the seminiferous tubule where a reversal site is found (Figure 1.1) (Hess, 1999). The presence of this wave is well documented in rodent species. Its existence in humans, however, remains debated (Schultze, 1982; Johnson, 1994; Johnson et al., 1996).

## **MOLECULAR MECHANISMS OF GERM CELL CONTROL**

Differentiation of spermatogonia through multiple steps of spermatogenesis is an intricate process requiring multiple divisions and precise levels of gene control. This level of control can be attributed to a number of factors that include testis specific transcription factors and other germline specific proteins (Hect, 1998; Grootegoed et al., 2000), as well as epigenetic mechanisms (reviewed in Rousseaux

et al., 2005). Here, I will focus on epigenetic modifications occurring during spermatogenesis, specifically DNA methylation.

Epigenetic changes are meiotically heritable and mitotically stable alterations that affect chromatin structure and function, and gene expression (Bird, 2002). DNA methylation is the most widely studied epigenetic mechanism. It takes place at the carbon 5 of cytosine residues (5meC) that are followed by guanosine residues (Jaenisch and Bird, 2003), commonly referred to as a CpG island. Methylation of the paternal germline is a dynamic process that begins at fertilization and continues through primordial germ cells (PGCs), maturing spermatogonia, spermatocytes, and possibly early spermatids (Mayer et al., 2000; La Salle and Trasler, 2006; Oakes et al., 2007). DNA methyltransferases (DNMTs) responsible for establishing methylation patterns in the testes are the *de novo* methyltransferases DNMT3a, DNMT3b, and DNMT3L (Webster et al., 2005; La Salle and Trasler, 2006; La Salle et al., 2007). The maintenance of methylation patterns is carried out by DNMT1 (La Salle et al., 2004). DNA methylation is one of the main epigenetic mechanisms that regulate germ cell-specific gene expression patterns (reviewed in Zendman et al., 2003).

The acquisition of genomic imprints is very complex. It involves erasure, establishment, and maintenance of DNA methylation at imprinted loci. The inherited methylation marks on each of the two sister chromatids are different; they must be erased and re-established in such a way that the methylation profile reflects the sex of the gametes they originate from. Upon fertilization, male and female pronuclei are epigenetically distinct and immediately undergo one of the two genome-wide

demethylation events. Interestingly, the male pronucleus is rapidly and actively demethylated prior to the first cellular division (Mayer et al., 2000; Santos et al., 2002). The maternal pronucleus, on the other hand, is demethylated in a passive manner during subsequent cell divisions (Rougier et al., 1998; Mayer et al., 2000). The imprints on both maternal and paternal genomes are maintained during the first wave of demethylation (Olek and Walter, 1997; Warnecke et al., 1998). The role that active paternal demethylation plays in embryonic development is unknown but may be an essential step in gene activation or imprinting in the early embryo (Mayer et al., 2000; Oswald et al., 2000). The second demethylation event occurs in the PGCs of the developing embryo. It is at this stage that all methylation marks are completely erased, the only exception being a subset of repeat elements (Hajkova et al., 2002). Following demethylation in PGCs, the sex-specific methylation marks are re-established during a process that is vastly different for male and female gametes. Maternal imprints are established during the growth phase of oocyte development and progress independently for each imprinted gene (Hiura et al., 2006).

The establishment of paternal imprints is less clear and appears to be initiated in prenatal prospermatogonia and completed after spermatocytes enter meiosis (Davis et al., 2000; Kerjean et al., 2000; Ueda et al., 2000). Interestingly, the paternal allele, which was previously methylated, is the first to be methylated during fetal stages, whereas methylation of the maternal allele begins at birth (Davis et al., 2000; Kerjean et al., 2000). This suggests that there is an additional epigenetic mark that survives both demethylation events identifying maternal and paternal alleles. Since the *de novo* DNMTs show no DNA binding specificity, they would require interaction with

either a bridging protein or histone modification to be effectively recruited to the imprinted control region (ICR) (Chen et al., 2004). To date, the proteins characterized to exhibit ICR recognition are CTCF and BORIS. They are further discussed below.

### **THE BORIS AND CTCF GENE FAMILY**

DNA methylation patterns are regulated in male germ cells by two paralogous proteins, a CCCTC binding factor (CTCF) and Brother of the Regulator of Imprinted Sites (BORIS) (Loukinov et al., 2002), also known as CTCF-like (CTCFL). CTCF is a nuclear protein that has been implicated in reading imprinting marks in somatic cells (reviewed in Klenova et al., 2002; Dunn and Davie, 2003). BORIS, on the other hand, can be found in the cytoplasm as well as in the nucleus (Loukinov et al., 2002), and has been implicated in the recognition and re-establishment of imprinting marks during epigenetic reprogramming of the male germline (Loukinov et al., 2002; Jelinic et al., 2006). The DNA-binding domain (DBD) found within BORIS and CTCF is composed of eleven zinc fingers (ZF), ten of which belong to the classic DNA-binding C2H2-class, and one of them represents the C2HC-class capable of binding both single-strand DNA and RNA (Klenova et al., 2002; Brown, 2005). DBDs within BORIS and CTCF are nearly 100% identical (Klenova et al., 2002). As a direct consequence, both proteins are capable of recognizing the same DNA-binding sites, and they demonstrate indistinguishable DNA-binding specificity (Loukinov et al., 2002). However, the amino- and carboxy-terminal ends of both proteins share no homology, which indicates that the functional consequence of binding would be different (Klenova et al., 2002). Importantly, these proteins do not compete for

binding sites, as their expression patterns are mutually exclusive during the development of normal male germ cells (Figure 1.3) (Loukinov et al., 2002).

*BORIS* expression is controlled by three alternative promoters regulated negatively by DNA methylation, CTCF and p53 (Vatolin et al., 2005; Hoffmann et al., 2006; Woloszynska-Read et al., 2007; Renaud S et al., 2007). Consequently, *BORIS* expression is restricted to primary spermatocytes which do not express *CTCF* and are void of DNA methylation, as it was previously visualized using antibodies against 5meC (Loukinov et al., 2002). There has been some debate regarding the extent of DNA-demethylation in spermatocytes, as immunohistochemical staining would not be able to detect minute changes in DNA methylation (Marchal et al., 2004 and references therein). Furthermore, the *de novo* methylation and demethylation events that occur during spermatogenesis are sequence-specific (Oakes et al., 2007). Regardless, it appears that *BORIS* expression followed by *CTCF* expression takes place during erasure and re-establishment of methylation marks (Loukinov et al., 2002). *BORIS* might associate with a demethylase that was not discovered yet (Loukinov et al., 2002); however, since no such enzyme has been found, its role in the demethylation process remains obscure.

Several studies have implicated *BORIS* in the derepression of numerous members of the cancer-testis (CT) gene family (Vatolin et al., 2005, Hong et al., 2005; Kouprina et al., 2007). The CT gene family is normally expressed exclusively in germ cells of the testis, occasionally in female reproductive organs, and aberrantly in different malignancies (reviewed in Zendman et al., 2003). *BORIS* itself is a member of the CT gene family, as it is known to be expressed in a large number of

malignant tumors (Ulaner et al., 2003; Vatolin et al., 2005; Hong et al., 2005; D'Arcy et al., 2006; Hoffmann et al., 2006; Risinger et al., 2007; Woloszynska-Read et al., 2007). The main mechanism by which CT genes are silenced in somatic tissues is through methylation of CpG islands in the promoter region (reviewed in Zendman et al., 2003).

Germline expression of some CT genes involves switching BORIS for CTCF at various promoter regions. This results in demethylation of respective promoters and derepression of expression (Hong et al., 2005; Vatolin et al., 2005; Kouprina et al., 2007). Indeed, a large number of CT genes are likely to be activated in the same manner.

Recently, BORIS has been shown to interact with histones H1, H2A, H3 and H4 as well as with a protein arginine methyltransferase, PRMT7 (Jelinic et al., 2006). Jelinic and colleagues have shown that the N-terminus of BORIS interacts with PRMT7 and directs methylation of H2A and H4 (Jelinic et al., 2006). Moreover, this process takes place at imprinted loci and occurs shortly after erasure of methylation marks in PGCs (Hajkova et al., 2002; Jelinic et al., 2006). Methylated histones and bridging proteins that recognize modified histones are assumed to be responsible for the recruitment of de novo DNMTs for ICR methylation (Li, 2002; Jelinic et al., 2006).

#### **MICRORNA AND SPERMATOGENESIS**

Another mechanism of epigenetic control is accomplished through the involvement of small regulatory RNAs (Chuang and Jones, 2007; Weber et al., 2007;

Presutti et al., 2006; Rogaev, 2005), microRNAs being of special interest. MicroRNAs (miRNAs) are small (21-25 nucleotide), single-stranded, non-coding RNAs that regulate gene expression at the post-transcriptional level. MicroRNA genes can be transcribed as independent genes, or they can be clustered together and transcribed as a polycistron (Chen and Meister, 2005). There are also a large number of intragenic miRNAs that are transcribed from regions within introns or exons of protein coding and non-protein coding genes (Rodriguez et al., 2004). 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) (Han et al., 2004). 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 in which *Dicer* (RNase III enzyme) cleaves the pre-miRNA which leads to the formation of a mature miRNA. To control the translation of target mRNAs, mature miRNAs must associate with the RNA-induced silencing complexes (RISC) (Gregory et al, 2005; Tang, 2005). Being associated with 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 and Allis, 2005). The mechanisms by which miRNAs reduce protein yield still need to be studied. Currently, miRNAs with a high degree of complementarity to their target mRNAs are thought to be able to repress expression through mRNA cleavage, whereas miRNAs with imperfect

complementary sequences to their target mRNAs can repress translation without cleavage (Yekta et al., 2004; Doench and Sharp, 2004). Furthermore, individual miRNAs can target multiple transcripts, and multiple miRNAs can target a single mRNA, the outcome of which is the fine-tuned gene expression program (reviewed in Shivdasani, 2006).

A number of miRNAs are known to be preferentially or exclusively expressed in testes, thus suggesting that germ cell-specific miRNAs play a significant role in spermatogenesis (Ro et al., 2007; Hayashi et al., 2008). In fact, miRNAs have been found in all cell types of the seminiferous epithelium including somatic Sertoli cells (Ro et al., 2007; Hayashi et al., 2008; Marcon et al., 2008). Taking into consideration that testes-specific mRNAs can be found at various stages of spermatogenesis, it is not surprising that numerous miRNAs are also expressed in the same testes-specific manner. In fact, seminiferous tubules of mice that lack Dicer were void of spermatocytes and arrested at the spermatogonial stage, suggesting that miRNAs are crucial for proliferation and/or differentiation of spermatogonia (Hayashi et al., 2008). Furthermore, the expression profile of miRNAs overlaps with a peak in active transcription and suppressed translation in late meiotic and early haploid germ cells (Ro et al., 2007). Several of these mRNAs and pre-miRNAs are stored along with other components of the RISC complex in the chromatoid body (CB) (Kotaja et al., 2006). The CB is a male germ-cell-specific RNA storage and processing center located in the cytoplasm of round spermatids just after meiosis (Fawcett et al., 1970). It is functionally analogous to the somatic processing body or P-body (Kotaja et al., 2006; reviewed in Kotaja and Sassone-Corsi, 2007). As such, the CB plays a central

role in post-transcriptional gene control at later stages of spermatogenesis. The CB plays an important role especially midway through spermiogenesis after histones are replaced with protamines and the chromatin is packed in such a manner that transcription does not occur at significant levels (Yu et al., 2003). At this stage of development, cells rely on stored mRNAs and posttranscriptional control of gene expression (Penttila et al., 1995; Yang et al., 2005). Bhattacharyya and colleagues have demonstrated that miRNA-mediated translational repression of target mRNAs is a reversible process, and repressed mRNAs can be released from P-bodies under certain conditions (Bhattacharyya et al., 2006). Kotaja and Sassone-Corsi speculate that a similar process occurs in the CB, whereby stored pre- and post-meiotic transcripts are released from translational repression at a specific time in spermatid maturation (Kotaja and Sassone-Corsi, 2007).

Another important posttranslational mechanism that involves the CB is the turnover or degradation of highly stable transcripts. The mechanism by which miRNAs mediate mRNA decay is by accelerating the removal of the poly(A) tail via deadenylation (Wu et al., 2006; Behm-Ansmant et al., 2006). Recently, Yu and colleagues have shown that miRNAs can facilitate the degradation of target mRNAs by endonucleolytic cleavage (Yu et al., 2005). Specifically, they have found that miR-122a is expressed in spermatids and negatively regulates TNP2 (transition protein 2) expression by cleavage of *Tnp2* mRNA (Yu et al., 2005).

## MIRNA AND FERTILIZATION

Spermatozoa have been shown to contain a wide spectrum of RNAs, including miRNAs that are delivered to the oocyte at fertilization (Ostermeier et al., 2004; Ostermeier et al., 2005; Yan et al., 2008). It is tempting to speculate that spermatozoal miRNAs could be important in the maternal-to-zygote transition during embryogenesis. It is known that miRNAs play a significant role in embryonic development (reviewed in Pasquinelli et al., 2005; Zhao and Srivastava, 2007; Williams, 2008). Interestingly, zebrafish miR-430 is expressed at the onset of zygote transcription and has been shown to target a large set of maternally derived mRNAs (Giraldez et al., 2006). This effectively purges maternal transcripts that may interfere at later stages of embryonic development. Amanai and colleagues studied the influence of a select number of sperm-derived miRNAs on fertilization and found that those miRNAs have very little, if any, influence on the fertilization process (Amanai et al., 2006). They found that miRNA profiles of newly fertilized and unfertilized oocytes were identical, indicating that the oocyte miRNA landscape was not changed (Amanai et al., 2006). It is possible that sperm contain unidentified miRNAs that were not included in the study by Amanai et al. (2006), as miRNA arrays used at the time were from the Sanger Institute miRBase, release 7.0 or 7.1 (<http://microrna.sanger.ac.uk/sequences/>) (Griffiths-Jones et al., 2008). The current Sanger Institute miRBase release 11.0 contains many newly discovered miRNAs that could play a role in fertilization. Furthermore, Rassoulzadegan and colleagues have shown that microinjection of a single miRNA (miR-221 or miR-222) is able to induce

a permanent and heritable epigenetic change in expression of the *kit* gene in early mouse embryos (Rassoulzadegan et al., 2006).

#### **EFFECTS OF RADIATION ON THE MALE GERMLINE**

Based on current statistics, approximately 12% (~20,900) of newly diagnosed cancer patients are people younger than 50 years of age (Canadian Cancer Society, 2008). Among these, approximately 40% (~ 8000 new cases/year) occur in males. Cancer treatment may involve surgery, chemotherapy, radiotherapy, or any combination of the later three. Unfortunately, these treatments may be followed by permanent infertility or impaired spermatogenesis. This section will focus on the effects of radiation on the testes, specifically germ cells of the seminiferous epithelium.

Ionizing radiation (IR) is an effective cancer treatment that targets the DNA of rapidly dividing cancer cells. Male germ cells undergoing spermatogenesis are also rapidly dividing, and consequently, are targets for this therapy. The unique feature of IR as a DNA damaging agent is the wide range of lesions induced by it (reviewed in Frankenber-Schwager, 1990). These lesions include single and double strand breaks (SSBs and DSBs, respectively), as well as a varying complexity of DNA cross links and base damages. The major lesions associated with genotoxic effects of IR exposure are DSBs. As a consequence, the stability of the genome is compromised and manifests itself as an increase in chromosomal aberrations, aneuploidy, micronuclei formation, gene mutation, microsatellite instability, and apoptosis

(reviewed in Morgan, 2003a, b, c). In irradiated germ cells, DNA damage response mechanisms are induced to repair the damage and maintain genomic stability.

Approximately 80% of the testis tissue consists of germ cells at varying stages of spermatogenesis and dramatic differences in chromatin structure (Bellve et al., 1977). Accordingly, these germ cells exhibit stage-dependent variations in radiosensitivity. Of all the cell types present in the seminiferous epithelium, the differentiating type A spermatogonia are the most susceptible to IR effects (Meistrich, 1986; Haines et al., 2002; Cordelli et al., 2003). In mice, these cells undergo six mitotic divisions in 7.4 days before maturing into B type spermatogonia (Meistrich, 1986). The non-proliferating or stem cell type A spermatogonia are more radioresistant than their dividing counterparts (Meistrich, 1986). The effects of IR exposure manifest as a marked decrease in testis weight and a significant loss of primary spermatocytes at higher doses of radiation (Hasegawa et al., 1998; Haines et al., 2002; Cordelli et al., 2003). The magnitude of DNA damage is also dose dependent (Rowley et al., 1974; Haines et al., 2002), as is the time for recovery of normal spermatogenesis (Kangasniemi et al., 1996; Cordelli et al., 2003). At higher doses of radiation, spermatogenesis recovery may take several years, or spermatogenesis may not recover at all, resulting in permanent azoospermia (reviewed in Howell and Shalet, 2005; Magelssen et al., 2006).

Exposure to radiation causes a clear reduction of the proliferating type A spermatogonia, however, radiosensitivity of type B spermatogonia appears to be cell cycle dependent (Henriksen et al., 1996; West and Lahdetie, 2001). Type B spermatogonia in the G2/M phase are radiosensitive and subsequently die by

apoptosis, whereas type B spermatogonia in the G1 phase are radioresistant (West and Lahdetie, 2001). Interestingly, uncommitted or quiescent type A spermatogonia are much more radioresistant than their dividing counterparts, suggesting that the cell cycle phase also influences their radiosensitivity. Joshi and colleagues found that primary spermatocytes and early spermatids are moderately radiosensitive and hence display a very efficient capacity to repair SSBs (Joshi et al., 1990). This efficiency is demonstrated in early spermatids; as within one hour of exposure to 10 Gy IR, 90% of the SSBs were rejoined (Joshi et al., 1990). The sensitivity of spermatids decreases as spermiogenesis progresses, with a peak in sensitivity at mid-stage (Joshi et al., 1990). This corresponds to the time just before histones are replaced with transition proteins and prior to chromatin compaction. Stimulation of SSB repair and maintenance of DNA integrity during spermiogenesis have been shown to involve transition proteins 1 and 2 (TNP1 and TNP2, respectively) (Caron et al., 2001; Kierszenbaum, 2001; Zhao et al., 2004). Thus, spermatid DNA appears to be more susceptible to damage in the absence of these proteins.

Spermatozoa are much less radiosensitive than spermatogonia and spermatids (Joshi et al., 1990; Cordelli et al., 2003). This resistance is thought to be related to a number of factors, including chromatin condensation, altered repair capacity, and decreased nuclear volume (Joshi et al., 1990 and references therein). In addition, cytoplasmic elimination reduces the amount of fluid in spermatozoa (Sprando and Russell, 1987), which would decrease the impact of the indirect component of radiation damage. However, both the level of chromatin condensation and the apparent decreased nuclear volume would concentrate DNA (reviewed in Ward and

Coffey, 1991), thereby making it more susceptible to IR damage (Hawkins, 2005). It is possible that protamines which replace transition proteins on the chromatin at mid to late-stage spermiogenesis exhibit a radioprotective effect and in combination with reduced cytoplasmic volume significantly reduce radiosensitivity of spermatozoa. This hypothesis is compatible with that of Joshi and colleagues, according to which the relatively high abundance of cysteine residues (known radioprotectors) in TP2 are positioned in the minor groove and scavenge radiation-induced radicals (Joshi et al., 1990).

Notwithstanding, male germ cells are more radiosensitive than the majority of the somatic cells.

In somatic cells, IR activates checkpoints that lead to cell cycle arrest in G1, S, and G2/M phases (reviewed in Bartek et al., 2004; Bartek and Lukas, 2007; Callegari and Kelly, 2007). These cell cycle arrests are thought to give cells additional time to repair DNA damage before entering the next mitotic/meiotic phase. West and Lahdetie (2001) found that type B spermatogonia exposure in S-phase results in a small delay in progression through meiosis, however it is not known whether the delay is due to DNA synthesis inhibition or an arrest at another stage. Direct inhibition of DNA synthesis would be expected considering the effect of radiation on somatic cells (Stokes and Michael, 2004; Hurley and Bunz, 2007). Interestingly, primary spermatocytes irradiated in G1 do not undergo cell cycle arrest and progress through meiotic S phase without activating the DNA damage checkpoint (West and Lahdetie, 2001). Haines and colleagues also found that a portion of germ cells with radiation-induced damage survive (Haines et al., 2002).

Consequently, unrepaired DNA damage can be found in round spermatids, spermatozoa and mature sperm cells (West and Lahdetie, 2001; Haines et al., 2001; Haines et al., 2002; Cordelli et al., 2003). Haines and colleagues found that although DNA damage can be found in sperm cells, it is not homogenous; rather there exist two populations carrying high or low DNA damage (Haines et al., 2002). This suggests that a portion of germ cells with radiation-induced damage survive; and it is in agreement with the findings of West and Lahdetie (2001). The observation of a heterogenous population of damaged sperm cells is not unique to radiation and has been observed following exposure to chemical mutagens (Marchetti and Wyrobek, 2008). This suggests that there are subpopulations of germ cells that are either highly sensitive or highly resistant to DNA damage. Haines and colleagues observed DNA damage in the sperm of mice four spermatogenic cycles after irradiation (Haines et al., 2002). This suggests that genome instability within the testes is responsible for the production of damaged sperm and impaired spermatogenesis (Haines et al., 2002).

#### **RADIATION-INDUCED APOPTOSIS**

At low doses of radiation, sperm count drops significantly with no increase in detectable DNA damage (Haines et al., 2002). This suggests that damaged germ cells are eliminated from the seminiferous epithelium, most likely by apoptosis. Indeed, during normal spermatogenesis, apoptosis is essential in regulating germ cell density, and it occurs primarily in A type spermatogonia (reviewed in de Rooij and Grootegoed, 1998; de Rooij, 2001). As type A spermatogonia develop, they remain connected by intercellular bridges that allow communication between germ cells

within a clone (Braun et al., 1989). It is believed that apoptotic signals are transmitted through these cytoplasmic bridges, resulting in chains of interconnected apoptotic spermatogonia (Huckins, 1978). However, in the testes of irradiated mice, spermatogonia undergo apoptosis without affecting other interconnected spermatogonia (Hamer et al., 2003). The mechanisms are unknown, but it suggests that apoptotic pathways triggered by IR are different than those that regulate germ cell density (Knudson et al., 1995; Beumer et al., 2000; Russell et al., 2002).

### **THE RADIATION-INDUCED BYSTANDER EFFECT**

Radiation exposure gives rise to the phenomenon called the bystander effect. It is defined as the ability of cells affected or damaged by radiation to convey damage distress signals to distant bystander naïve cells (Djordjevic, 2000). The bystander effect can manifest itself in many forms, including, but not limited to: delayed cell death, chromosomal instability, deletions, amplifications, mutations, and many other hallmarks of genomic instability (reviewed in Morgan, 2003a, b, c). Bystander effects have been studied in great detail *in vitro*; however, *in vivo* data are somewhat limited. The research, presented herein, focuses on radiation effects in animal models, and *in vivo* bystander effects will be emphasized.

The very first bystander effect investigations were performed at the beginning of the twentieth century. Murphy, whose research interests were devoted to the studies of lymphoid cells, showed morphological changes in lymphoid cells after co-culturing them with serum from radiation-exposed animals (Murphy and 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 (Goh and Sumner, 1968; Hollowell et al, 1968; Pant and Kamada, 1977; Emerit et al, 1994; Emerit et al, 1995). The clastogenic activity was found in plasma of patients receiving a high dose of radiotherapy and individuals who were accidentally exposed to substantial levels of radiation (Goh and Sumner, 1968; Hollowell et al, 1968; Pant et Kamada, 1977; Emerit et al, 1994; Marozik et al, 2007).

By the late 1990s, non-direct effects of radiation, including the bystander effect, have become the subject of study for biologists and radiation oncologists.

Bystander effects have been reported to occur within an exposed organ in rodent models. If the lung base was irradiated, significant molecular and cellular damage was observed in the shielded lung apex (Khan et al, 1998; Khan et al, 2003). It was also shown that if one lung, either right or left, was exposed, there was a marked increase of micronuclei in the unexposed, shielded lung (Khan et al., 1998; Khan et al., 2003). Similar within-the-organ bystander effects were observed during partial liver irradiation in a rodent model (Brooks et al, 1974; Brooks, 2004).

Bystander effects also manifest themselves in the context of an organism in its entirety. Kovalchuk and colleagues have established a model system to study *in vivo* bystander effects and have pioneered research in this field. They found that male mice exhibit a more pronounced bystander effect. The bystander effects discussed below are all exhibited by male animals (Koturbash et al., 2008a). Koturbash and colleagues have demonstrated that radiation exposure to one half of the body leads to elevated

levels of DNA damage in skin tissue of the shielded bystander half (Koturbash et al., 2006a). They also analyzed indicators of DNA repair capacity and found that Rad51 which is involved in homologous recombination (HR) repair was upregulated in the exposed bystander half of the body and remained so 4 days after irradiation (Koturbash et al., 2006a). This suggests an increased capacity to repair DNA DSBs; however, it might also lead to an increase in genomic instability (Richardson et al., 2004).

In further studies, Koturbash and colleagues found that cranial irradiation led to increased levels of DNA damage in the distant shielded spleen (Koturbash et al., 2008b). This damage was paralleled by decreased levels of cellular proliferation and increased levels of apoptosis and p53 expression. This may be interpreted as a sign of a radiation-induced cell-cycle block, whereby p53-dependent cell-cycle arrest allows for DNA repair and/or elimination of unreparable cells. Importantly, these findings were confirmed by highly similar trends in two different strains of mice, indicating a true bystander phenomenon (Koturbash et al., 2008b). Increased levels of DSBs in the bystander spleen were accompanied by a global loss of DNA methylation (Koturbash et al., 2008a). Certain DNA lesions are known to inhibit the activity of DNMTs (Turk et al., 1995; Panayiotidis et al., 2004), and repair processes incorporate cytosine and not methyl-cytosine, which may explain the later observation. Furthermore, this association has been proposed as a mechanism for radiation-induced hypomethylation (Pogribny et al., 2004, 2005; Koturbash et al., 2005).

## **MECHANISMS OF RADIATION-INDUCED BYSTANDER EFFECTS**

Molecular mechanisms that mediate the observed bystander effect are more mysterious than bystander phenomena themselves. A large body of evidence indicates that soluble factors capable of inducing DNA damage, commonly referred to as clastogenic factors, are released into the blood following radiation exposure. Support for this hypothesis comes from the observation that culturing of normal human peripheral blood cells in media containing plasma from irradiated individuals results in a significant increase in chromosomal aberrations in these blood cells (reviewed in Morgan, 2003a; and references therein).

The identity of these blood-borne clastogenic factors remains unknown, and thus far most, if not all, potential clastogenic factors have been identified using *in vitro* systems. The huge diversity of the *in vivo* environment greatly complicates the search for potential bystander signals *in vivo*. In general, two mechanisms have been proposed and reviewed in detail by Lorimore and colleagues (Lorimore et al., 2003). Briefly, one of the mechanisms involves communication and stimulation of p53-dependent damage-signaling pathways via intercellular gap-junctions (Azzam et al., 1998; Azzam et al., 2001), while the other one involves secreted cytokines that are known to increase intracellular levels of reactive oxygen species (ROS) (Lehnert et al., 1997; Lehnert and Goodwin, 1997; Iyer and Lehnert, 2000).

Wright and Coates make an interesting point as to the mechanism of bystander effects; they note that intercellular signaling as well as cytokines and free radical production are all key features of an inflammatory type response and may be protective or damaging depending on the context (reviewed in Wright, 2004; Wright

and Coates, 2006). Koturbash and colleagues observed upregulation of methyl-binding proteins in bystander skin tissue only when the spleen (left side) was in the field of exposure (Koturbash et al., 2006a). Given that the spleen is a central component of the immune system, the later observation is an important finding that merits further investigation into the potential influence of inflammatory responses in mediating bystander effects.

As discussed above, radiation-induced bystander effects occur *in vivo* in distant somatic tissues; yet it is not known if radiation-induced bystander effects occur in the germline. If so, then localized exposures to IR may have serious consequences on male fertility and the heritability of DNA damage in subsequent generations.

#### **RADIATION-INDUCED TRANSGENERATION GENOME INSTABILITY**

Radiation induced DNA damage in the male germline has been associated with reduced rates of fertilization, defective embryonic development, and increased rates of miscarriage and morbidity of the early fetus (Burrue et al., 1997; Muller et al., 1999; Dobrzynska and Czajka, 2005). In addition, genotypic alterations found in un-exposed offspring include chromosome aberrations, micronuclei formation, altered gene expression patterns, and many other hallmarks of genome instability (reviewed in Morgan, 2003a; Dubrova, 2003; Barber and Dubrova, 2006). The manifestation of these alterations is collectively termed transgenerational genome instability. Genotypic alterations found in the non-irradiated progeny of exposed fathers may predispose them to an increased risk of genetic diseases, infertility, and

even cancer (reviewed in Nomura, 2003; Aitken and De Iuliis, 2007). The molecular mechanisms by which this occurs are not known; however, recent publications implicate an epigenetic phenomenon (reviewed in Kovalchuk and Baulch, 2008). The majority of studies on transgenerational effects of radiation exposure use animal models, and evidence of transgenerational effects in humans remains debated (reviewed in Nomura, 2006).

Dubrova and colleagues have made a significant contribution to the current understanding of radiation-induced transgenerational instability (reviewed in Dubrova, 2003; Barber and Dubrova, 2006). The majority of their studies monitor germline mutation using a set of hypervariable expanded simple tandem repeat (ESTR) DNA loci which consist of short tandem repeats (4-6 bp) that exhibit high spontaneous and induced mutation rates (Dubrova et al., 1993; Dubrova et al., 1998; Dubrova et al., 2000; Barber et al., 2000).

Barber and colleagues studied mutation rates of two ESTR loci in the germline of first- and second-generation offspring of irradiated male mice (Barber et al., 2002). They show an increased mutation rate in the germline of F1 offspring of males exposed to radiation at either premeiotic or postmeiotic stages of spermatogenesis. It was shown that the elevated mutation rate continues in the germline of the F2 offspring (Dubrova et al., 2000b; Barber et al., 2002). Furthermore, elevated mutation rates were detected in alleles from both the irradiated fathers and the unexposed mothers, suggesting that genomic destabilization occurs after fertilization and affects both males and females alike (Barber et al., 2002; Barber et al., 2006). The observed transgeneration instability is not unique to one

particular strain of mice; in fact, it has been observed in the F1 and F2 offspring of irradiated males from four different inbred strains of mice (Baulch et al., 2001; Barber et al., 2002; Barber et al., 2006). Further analysis of the unexposed F1 offspring of irradiated males has shown that high ESTR mutation rates correlate with elevated mutations of protein coding genes in somatic tissues, such as the spleen and bone marrow (Barber et al., 2006).

Barber and colleagues have also shown that spontaneous levels of SSBs and DSBs are significantly higher in the unexposed F1 offspring; however, the efficiency of DNA repair was not compromised (Barber et al., 2006). Likewise, Koturbash and colleagues found that DNA DSBs were higher in the thymus of offspring from irradiated fathers (Koturbash et al., 2006b). They also analyzed Rad51 and Ku70 protein levels as indicators of competent homologous recombination (HR) and nonhomologous end joining (NHEJ) repair pathways, respectively. In contrast to the results of Barber and colleagues, they found evidence of a compromised HR repair pathway, indicated by downregulation of Rad51, while NHEJ was unaffected (Koturbash et al., 2006b). This may not necessarily result in a decrease in DNA repair efficiency but may impact the accuracy and quality of DNA repair. Furthermore, changes in expression levels of Rad51, be it up or down, have been associated with genome instability and cancer (Sonoda et al., 1998; Richardson et al., 2004; and reviewed in Thacker, 2005).

Baulch and colleagues studied the F3 offspring of males irradiated at the B type spermatogonial stage and found altered protein kinase activities and protein levels of p53 and p21 (Baulch et al., 2001; Vance et al., 2002). p21 is a target of p53

that arrests or slows cell cycle progression (Xiong et al., 1993). Further investigation including the fourth generation offspring revealed similar changes in signaling kinase activity and protein levels of p53 and p21, although the magnitude and direction of change for each endpoint differed between generations and within generations (Baulch and Raabe, 2005). This finding alone highlights the phenotypic variability observed in the offspring of exposed males.

#### **MECHANISMS OF TRANSGENERATIONAL GENOME INSTABILITY**

The observed increases in mutation rates are transmitted from irradiated males to their unirradiated offspring. The signal or damage transmitted is not known, however, several lines of evidence implicate epigenetic mechanisms in the destabilization of the genome following radiation exposure. First, mutation rates are observed in both somatic and germline tissues of both sexes of offspring equally (Barber et al., 2002; Barber et al., 2006; Koturbash et al., 2006b). Second, elevated mutation rates are persistent and can be transmitted to the second generation offspring with little change, thus eliminating the possibility of an inherited mutation at any specific set of genes (Barber et al., 2002). Third, the signal must be DNA-dependent, as the cytoplasmic component of mature sperm is insignificant in comparison to the cytoplasm contributed by the egg. For this reason, radiation induced free radicals carried by the sperm are unlikely to contribute to DNA damage in the fertilized egg (Dubrova et al., 2000b).

The candidate epigenetic mechanism that occurs in the germline and influences genetic and epigenetic events following fertilization is DNA methylation.

In support of this, Koturbash and colleagues have shown that after paternal radiation exposure, the thymus in the unexposed offspring exhibits a significant loss of global DNA methylation that was paralleled by a significant decrease in the expression of DNMT1, 3a, 3b and MeCP2 (Koturbash et al., 2006b). Studies of Baulch and colleagues have indicated that DNA damage is repaired in irradiated spermatogonia, but subtle heritable chromatin alterations caused by radiation go unrepaired and are subsequently transmitted to later generations (Baulch et al., 2007). Dubrova has also proposed several different epigenetic models that may explain mechanisms behind the observed transgenerational effects (Dubrova, 2003; Barber and Dubrova, 2006).

In one model, DNA methylation changes in the exposed germline influence the methylation status of genes involved in maintaining genome integrity in the fertilized egg (Dubrova, 2003). In support of this hypothesis is the finding that genes involved in DNA repair and cell cycle checkpoints have altered expression patterns in the offspring of irradiated male mice (Baulch and Raabe, 2005). It is also known that exposure to IR causes DNA methylation changes, and that these changes are dose-, sex- and tissue-specific (Pogribny et al., 2004; Kovalchuk et al., 2004; Raiche et al., 2004). Specifically in male mice, it was found that chronic exposure to low-dose radiation caused significant genome wide hypomethylation in muscle tissue (Kovalchuk et al., 2004). No global methylation changes were observed in the liver, but locus specific de novo methylation was observed in the promoter p16<sup>INKa</sup> (Kovalchuk et al., 2004), a tumor suppressor gene involved in promoting cell cycle arrest (reviewed in Ohtani et al., 2004). In contrast, chronic low-dose exposure caused genome wide hypermethylation in the spleen which correlated with a significant

increase in DNMT3a and DNMT3b (Raiche et al., 2004). When exposed to higher acute doses of radiation, the spleen exhibits pronounced genome wide hypomethylation that is paralleled by an increase in DSBs (Pogribny et al., 2004). This suggests that radiation induced genome wide DNA hypomethylation may be DNA repair-related (Pogribny et al. 2004). Global methylation levels of irradiated mouse testes have not been analyzed in detail and require further investigation. Methylation at the H19 locus has been analyzed. It was found that IR can interfere with the imprinting process (Zhu et al., 2006). In their study, Zhu and colleagues found that CpG islands within the ICR of H19 were demethylated at random in sperm of irradiated mice (Zhu et al., 2006). Preliminary studies suggest that these altered methylation patterns can be inherited and maintained in their offspring (Zhu et al., 2006).

In a second model, transgenerational effects may be modulated by the DNA damage response of transcriptionally active germline stages. In support of this, elevated ESTR mutation rates were observed following exposure of spermatogonia and round spermatid stages (Dubrova et al., 2000b; Barber et al., 2002). It is possible that certain classes of RNAs, such as mRNA or miRNA, could be produced in these germ cells and transmitted into the fertilized egg, thereby destabilizing the embryonic genome (Rassoulzadegan et al., 2006). However, transgenerational changes have been observed following exposure of late post-meiotic stages, at which gene transcription is suppressed (Niwa and Kominami, 2001; Shiraishi et al., 2002; Koturbash et al., 2006b; Hatch et al., 2007). That is not to say that irradiation of an active germline stage will not influence the zygote in any way different from that of

irradiation at an inactive stage. In fact, the incidence of dominant lethal mutations induced by radiation is higher in round spermatids and spermatocytes than in late spermatids and spermatogonia (Ehling et al., 1982).

In a third model, radiation-induced epigenetic changes take place after fertilization, whereby radiation damage in the sperm triggers a cascade of epigenetic events in the fertilized egg that result in epigenetic modifications. Maternal factors are known to have a profound influence on the transmission and repair of paternal DNA damage in the fertilized egg (Marchetti et al., 2007). Shimura and colleagues have shown that fertilization with irradiated sperm triggers a p53 dependent S-phase DNA damage checkpoint (Shimura et al., 2002a). DNA synthesis was suppressed in irradiated male pronuclei and unirradiated female pronuclei, indicating a true checkpoint rather than a mechanical block of replication by DNA damage (Shimura et al., 2002a). Moreover, DNA damage in the sperm pronucleus is repaired or modified at this time, and allows for an uninhibited cleavage into a two-cell stage embryo (Shimura et al., 2002b). Paternal DNA damage was shown to alter the expression of some, but not all, DNA repair mechanisms in the early embryo (Harrouk et al., 2000). Marchetti and Wyrobek found that exposure of postmeiotic male germ cells to chemical mutagens resulted in accumulation of heritable DNA damage in mouse sperm (Marchetti and Wyrobek, 2008). Furthermore, the observed DNA damage had no affect on fertilization or development during the first cell cycle after fertilization (Marchetti and Wyrobek, 2008). DNA double strand breaks were produced in the zygote before oocyte DNA repair machinery had a chance to fix the original lesion, most likely during reprogramming of the male pronucleus. It may be the case where

the portion of misrepaired DNA damage in the germline or the fertilized egg contributes more to transgenerational instability than the portion of unrepaired lesions (Joshi et al., 1990).

**Overall, several broad conclusions can be drawn from the existing literature:**

- Radiation exposure is an important DNA damaging agent that affects the male germline, and leads to genome instability in the germline, and to transgeneration genome instability in unexposed offspring of preconception exposed parents;
- Radiation exposure affects directly irradiated cells as well as neighboring shielded naïve cells, giving rise to the ‘bystander effect’ phenomenon;
- Transgeneration genome instability is thought to have an epigenetic nature;
- Epigenetic phenomena, specifically DNA methylation and microRNAome, are important for the maintenance of genome stability in cells, including germ cells.

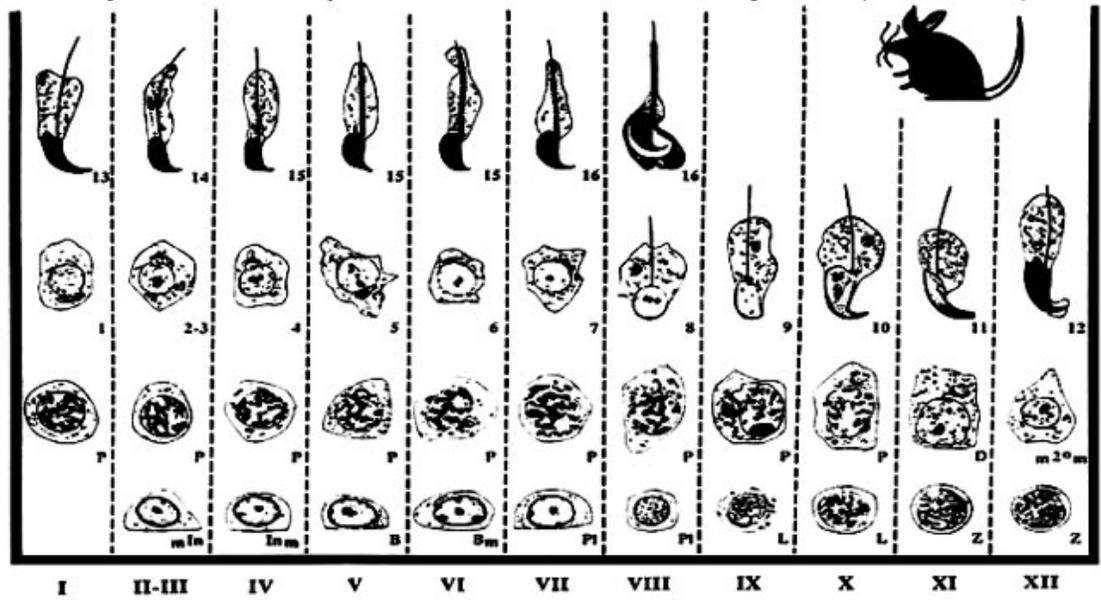
Yet, a lot has to be learned about the exact mechanisms of direct irradiation and indirect bystander radiation on the male germline. The role of DNA damage and its biological repercussion on the germline as well as epigenetic dysregulation in the germline need to be further defined.

## **HYPOTHESIS**

Therefore, the current study was aimed to dissect the role of DNA damage and epigenetic changes in the directly exposed and shielded bystander male germline.

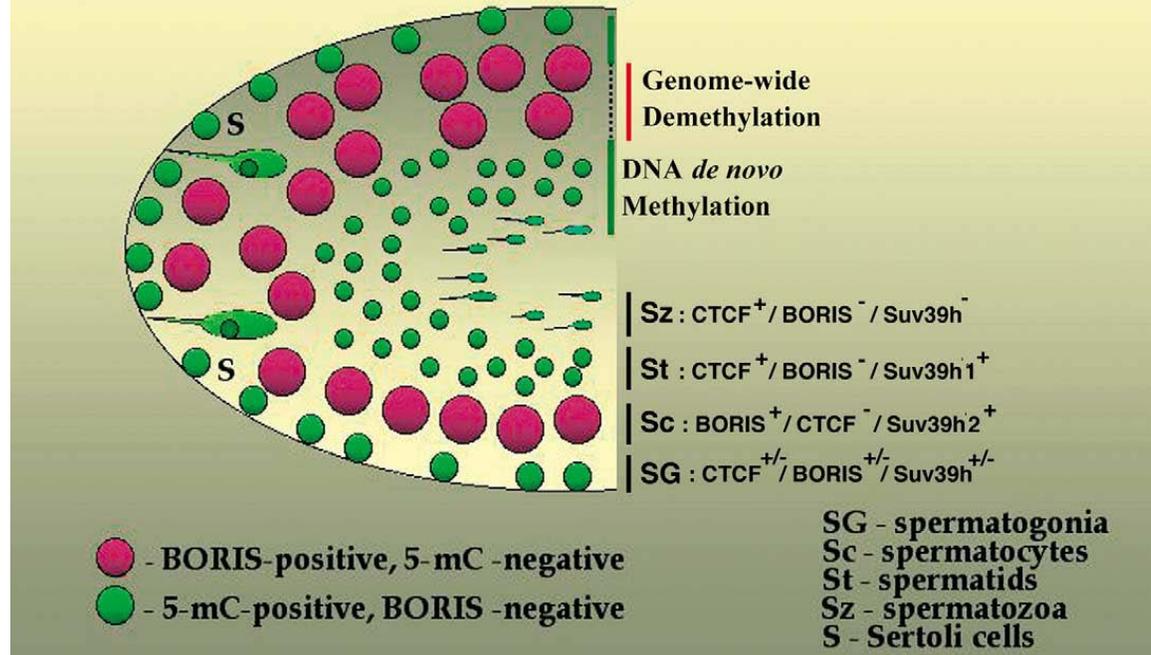
**We hypothesized that radiation exposure would cause DNA damage in the directly exposed and shielded bystander male germline. We predicted that accumulation of DNA damage would be correlated with altered DNA methylation and levels of proteins that regulate DNA methylation in the male germline. We also thought that radiation exposure would dysregulate the microRNAome of male testes tissue and therefore affect a wide range of crucial proteins and thus allow fine-tuning of radiation responses in the germline. We thought that bystander effects would significantly manifest in the shielded male germline.**

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



**Figure 1.0. Diagrammatic representation of the 12 stages in the mouse seminiferous epithelium.** Adapted with permission from: Bustos-Obregon E, Carvallo M, Hartley-Belmar R, Sarabia L, Ponce C. Histopathological and Histometrical Assessment of Boron Exposure Effects on Mouse Spermatogenesis. *Int. J. Morphol.* 2007; 24: 919-925.

Re-setting of genome methylation during male germ cell development is associated with silencing of *CTCF* and activation of *BORIS*



**Figure 1.1. Schematic representation of the expression pattern of BORIS, CTCF, and genome methylation during spermatogenesis.** Adapted with permission from: Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, Rasko JE, Docquier FM, Kistler M, Breen JJ, Zhuang Z, Quitschke WW, Renkawitz R, Klenova EM, Feinberg AP, Ohlsson R, Morse HC 3rd, Lobanenko VV. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. *Proc Natl Acad Sci U S A.* 2002; 99:6806-11. Copyright (2002) National Academy of Sciences, U.S.A.

**CHAPTER 2: DNA DAMAGE-INDUCED UPREGULATION OF MIR-709 IN  
THE GERMLINE DOWNREGULATES BORIS TO PREVENT ABERRANT  
DNA HYPOMETHYLATION**

Chapter 2 has been submitted in its entirety:

Tamma J, Kathiria P, Koturbash I, and Kovalchuk O. DNA damage-induced upregulation of miR-709 in the germline downregulates BORIS to prevent aberrant DNA hypomethylation. *Oncogene* (in review)

## **ABSTRACT**

MicroRNAs as potent regulators of gene expression are involved in spermatogenesis, yet their role in the response of the germline to genotoxic stress is obscure. Here we show that the DNA damage-induced and ATR/Rfx1-mediated increase of miR-709 expression in exposed testes may be a protective mechanism that effectively decreases a cellular level of the Brother of the Regulator of Imprinted Sites (BORIS) to prevent massive aberrant erasure of DNA methylation after radiation exposure.

## **INTRODUCTION**

The male germline is extremely sensitive to genotoxic stressors such as radiation, chemotherapy drugs and herbicides. At the same time, paternal exposure leads to transgenerational genome instability in the progeny (Aitken and De Iuliis, 2007). Molecular mechanisms that mediate transgenerational effects in the paternal germline are not well understood (Aitken and De Iuliis, 2007).

MicroRNAs (miRNAs), small single stranded non-coding RNAs, are potent regulators of gene expression (Ambros, 2004). They are also important for spermatogenesis (Hayashi et al., 2008). Furthermore, our previous studies indicate that cellular microRNA levels are affected by genotoxic stresses (Koturbash et al., 2008c).

Therefore, we hypothesized that genotoxic stress-induced microRNAome dysregulation in the male germline could be a factor affecting genome stability. With this in mind, we studied the miRNA profile of X-ray irradiated mouse testes using the microarray technique.

## **MATERIALS AND METHODS**

### ***Animal exposure***

Mice (mature 60 days old male C57BL/6 animals) were randomly assigned to different treatment groups. Handling and care of animals were performed in accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures were approved by the University of Lethbridge Animal Welfare Committee. Animals were housed in a virus-free facility and given food and water *ad libitum*. The exposed cohort (10 animals) received 2.5Gy (3Gy/min) of X-rays (90kV,

5mA) to the whole body. In our previous studies, this dose led to significant deleterious effects in the progeny (Koturbash et al., 2006b). For the irradiation procedure, animals were placed in small (10x5x6cm) plastic vented containers. These containers limit the movement of animals and insure dose uniformity. Control mice (10 animals) were sham treated. For sham treatment, containers with animals were placed into the irradiator machine, but X-rays were not turned on. Four days (96 hours) after exposure, mice were humanely sacrificed, then testes were sampled and processed for further analysis. The experiment was independently reproduced using 5 animals per control and exposed groups.

#### ***MiRNA microarray expression analysis***

Total RNA was extracted from mouse testes tissue using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. Tissue from 2 animals per group was used for the analysis. The miRNA microarray analysis was performed by LC Sciences (Houston, TX) as described before (Koturbash et al., 2007; 2008c).

#### ***MicroRNA fluorescence in situ hybridization (FISH) using locked nucleic acid (LNA) probes and tyramide signal amplification***

Tissue fixation of miRNA FISH was conducted as recommended (Obernosterer et al, 2007). In brief, testes tissue was pre-fixed for 30 min in 4% PFA at room temperature. After fixation, tissue specimens were placed in PBS with 30% sucrose to minimize freeze-fracture of tissue, then they were incubated overnight at 4°C. Following an incubation, the tissue was placed in the freeze mold, frozen and sectioned into 10µM sections. MicroRNA FISH was conducted in strict accordance

with the protocol published by Silahatoglu et al. (2007) using anti-miR-709 LNA 5'-DIG labeled probe (Exiqon).

### ***Luciferase reporter assay for targeting BORIS-3'-UTR***

For the luciferase reporter experiments, a 3'-UTR segment of BORIS gene corresponding to a region of 995 bps (from 2161 nt through 3156 nt of the total transcript) for BORIS (Acc. # DQ153171) was amplified by PCR from mouse genomic DNA using primers that included an XbaI and SacII tail on the 5' and 3' strand, respectively. Following gel extraction of the PCR product, an A-overhang was added and the product was ligated into pGEM-T (Promega, Madison, WI). Plasmid DNA containing the correct insert was isolated and restricted with both XbaI and SacII restriction endonucleases, and then gel-purified. The amplified 3'-UTR of BORIS contains an XbaI restriction site; therefore, BORIS-3'-UTR was ligated into the pGL3-control vectors (Promega, Madison, WI) by using the XbaI site located immediately downstream of the stop codon of luciferase. The HEK293 cells were transfected with the firefly luciferase UTR-report vector, control Renilla luciferase pRL-TK vector (Promega), transfection controls and precursor miR-709, or miRNAs that do not have binding sites within the 3'-UTR of *BORIS* (miR-127) (Ambion) using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), as previously described (Kovalchuk et al., 2008). Twenty-four hours after transfection, cells were lysed with a 1X passive lysis buffer (Promega) and the activity of both renilla and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions, as previously described (Kovalchuk et al., 2008).

***RNA preparation, reverse transcription and semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)***

Total RNA was extracted from mouse testes tissue using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. The samples were treated with DNase I (Invitrogen) according to manufacturer's instructions and stored at -80°C.

First strand cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario) with the following modifications: 1.5µg of total RNA was mixed with 1µg oligo(dT)<sub>18</sub> primers. The final concentration of cDNA was diluted in half with DEPC water and stored at -20°C.

Semi-quantitative RT-PCR was carried out on a Flexigene PCR machine (Barloworld Scientific Ltd, Staffordshire, United Kingdom) using *Taq* DNA Polymerase (Fermentas, Burlington, Ontario). Each reaction contained 2µl of cDNA, 10pmol of forward and reverse primer, 2mM MgCl<sub>2</sub>, 1x *Taq* Buffer with KCl, and 0.625 units of *Taq* DNA polymerase. Primers for *BORIS* and *β-actin* were previously designed to overlap two exons so as not to amplify genomic DNA. *BORIS* primers: forward: 5'- gagagacagacaagagagaagagaggttgctc-3', reverse: 5'- cctgtgtgggtgttcacatggttctaagaag-3'. *Actin* primers: forward 5'- ctgtgctgtcctgtatgcc - 3', reverse 5'- gtggtggtgaagctgtagcc -3'<sup>7</sup>.

For *Rfx1*, primers were designed to amplify from two exons, with the extension time short enough so as not to amplify genomic DNA. *Rfx1* primers: forward 5' gtattccagccaggtgcagt-3', reverse 5' agtggcagtagagggtgctc -3'. PCR conditions were independently determined for each set of primers such that the

concentration of PCR product was below saturation. Each reaction consisted of an initial denaturation at 94°C for 5 minutes followed by a predetermined number of cycles of a denaturation step at 94°C for 30 seconds, annealing at the respective primer melting temperature for 30 seconds, and an extension at 72°C for 30s. Following a final extension at 72°C for 10 minutes the samples were stored at 4°C. Agarose gel electrophoresis was carried out in TAE buffer using a 2% agarose (EMD Chemicals, Darmstadt, Germany) gel containing Ethidium Bromide. The amplified product was visualized under UV light and quantified using Image J 1.36b software (NIH, [www.rsbl.info.nih.gov/ij](http://www.rsbl.info.nih.gov/ij)) and normalized to  $\beta$ -actin.

### ***Western immunoblotting***

Tissue samples for protein extraction were snap frozen immediately after collecting. Tissues were sonicated in 1% SDS and boiled. Small aliquots (10  $\mu$ l) of extracts were reserved for protein determination using protein assay reagents from BioRad (Hercules, CA). Equal amounts of proteins (20  $\mu$ g) were separated by SDS-polyacrylamide electrophoresis in slab gels of 12% polyacrylamide, made in duplicate and transferred to PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ). The membranes were incubated with antibodies against BORIS (1:200; Abcam), and actin (loading control) (1:2000; Abcam). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and an ECL Plus immunoblotting detection system (GE Healthcare Biosciences). Chemiluminescence was detected by GE ECL Hyperfilm (GE Healthcare Biosciences). The unaltered PVDF membranes were stained with

Coomassie Blue (BioRad), and the intensity of the Mr 50,000 protein band was assessed as an additional loading control.

### ***DNA extraction and DNA methylation analysis***

DNA was extracted from testes using a Qiagen DNAeasy kit (Qiagen), according to the manufacturer's instructions. A well-established radiolabeled [<sup>3</sup>H]dCTP extension assay was employed to evaluate global DNA methylation levels (Pogribny et al., 1999; Tamminga et al., 2008). The assay measures the proportion of CCGG sites without methyl groups on both DNA strands. HpaII is a methylation-sensitive restriction enzyme that cleaves CCGG sequences in the case where internal cytosine residues are unmethylated on both strands. It leaves a 5'-guanine overhang after DNA cleavage. This overhang can be used for the subsequent single nucleotide extension with labeled [<sup>3</sup>H]dCTP. The extent of [<sup>3</sup>H]dCTP incorporation opposite the exposed guanine is directly proportional to the number of cleaved and thus unmethylated CpG sites and inversely proportional to methylation levels (i.e., the higher the methylation, the less [<sup>3</sup>H]dCTP is incorporated). Taking into consideration that the vast majority of frequently occurring HpaII tetranucleotide recognition sequences are constitutively methylated *in vivo*, an increase in cleavage at these sites indicates genome-wide hypomethylation. The assay was performed as described before (Pogribny et al., 1999; Tamminga et al., 2008; Koturbash et al., 2008a). The results were expressed as a percent difference in [<sup>3</sup>H]dCTP incorporation relative to control values.

### ***DNA strand break measurement***

A modification of the random oligonucleotide-primed synthesis (ROPS) assay was used to detect the presence of DNA strand breaks in high molecular weight DNA (Tamminga et al., 2008; Basnakian and James, 1996). The assay is based on the ability of Klenow fragment polymerase to initiate ROPS from re-annealed 3'-OH ends of single-stranded DNA. Briefly, 3'-OH DNA fragments that exist in 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 incorporating radioactively labeled dNTPs. The assay was performed as described before (Pogribny et al., 1999; Koturbash et al., 2008a). The results were expressed as a percent difference in [<sup>3</sup>H]dCTP incorporation relative to control values.

### ***Immunofluorescence***

Paraffin embedding and sectioning were conducted at Central Vet Labs, Edmonton, AB. Cryosectioning was performed as described above. IF staining was conducted on the paraffin sections and on the cryosections sections using anti- $\gamma$ H2AX antibodies (Cell Signaling), anti-BORIS antibodies (Abcam), anti-5-methylcytosine antibodies (Abcam), and anti-ATR antibodies (Santa Cruz Biotechnology) in accordance with the manufacturer's instructions.

### *Statistical analysis*

For the determination of the significance of the difference between the means, the Student's t-test and Bonferroni correction were used. Statistical treatment and plotting of the results were performed using the Excel for Windows XP, and JMP 5.0 software.

### **RESULTS AND DISCUSSION**

Analysis of array data revealed that expression levels of a large number of miRNAs were changed following radiation exposure, and a majority of miRNAs were up-regulated (Fig. 2.0A). MiR-709 was highly abundant in both control and irradiated testes, and a big difference in miR-709 levels was observed between the two groups, as shown by a miRNA microarray and fluorescent in situ (FISH) detection (Fig. 2.0B). Interestingly, miR-709 is also among the most abundant miRNAs in ovaries of newborn mice (Choi et al., 2007). Therefore, it likely has some role in germ cell-specific pathways.

As revealed by *in silico* analysis, putative targets of miR-709 include the 3' UTR region of *BORIS* mRNA. *BORIS* is a testes-specific gene that directs epigenetic reprogramming during male germ cell differentiation (Loukinov et al., 2002). To examine whether or not *BORIS* is indeed functionally targeted by miR-709, the segment of *BORIS*-3'-UTR containing the complementary miR-709 site was cloned into the 3'-UTR of a luciferase reporter system. Figure 2.0C shows that miR-709 inhibited luciferase activity from the construct with a *BORIS*-3'-UTR segment in a concentration-dependent manner (Figure 2.0C), which was not observed in controls

that used unrelated miRNA or scrambled oligonucleotides (supplementary data/ data not shown).

In order to further confirm that miR-709 indeed affects *BORIS in vivo*, we analyzed *BORIS* mRNA and protein levels in testes tissue of control and radiation-exposed mice. We observed a negative correlation between levels of miR-709 and *BORIS* in mouse testes. While the expression of miR-709 was increased in the germline of exposed mice, the levels of *BORIS* transcript (Figure 2.0D) and *BORIS* protein diminished following irradiation (Figure 2.0D, E). The decrease of both transcript and protein suggest that miR-709 may inhibit *BORIS* expression through mRNA cleavage and/or translation inhibition. Cleavage of mRNA targets in the germline has been documented, and it may represent a means of facilitating the turnover of some stable transcripts (Yu et al., 2005). Therefore we concluded that miR-709 partakes in fine-tuning of *BORIS* gene expression at the posttranscriptional level.

Co-localization by combined protein immunohistochemistry (or indirect immunofluorescence) and miRNA FISH analysis further confirmed mutually exclusive patterns of *BORIS* and miR-709 expression in murine testes (Figure 2.0F, G). Cells harboring high levels of miR-709 were negative or very weakly positive for *BORIS* and vice versa (Figure 2.0F, G). In accordance to previous experiments, exposed testes tissue exhibited high levels of miR-709 and low levels of *BORIS*, while patterns of control tissue were reversed (Figure 2.0 F).

*BORIS* is an important regulator of DNA methylation and imprinting, and it itself is regulated by DNA methylation (Loukinov et al., 2002; Renaud et al., 2007;

Woloszynska-Read et al., 2007). Therefore, having seen the significantly altered expression of BORIS in testes of exposed mice, we decided to analyze levels of cytosine DNA methylation in testes tissue. We noted that in exposed testes, in which high expression of miR-709 and low BORIS levels were observed, the levels of DNA methylation were significantly decreased (Fig. 2.0 E, H). It is well-established that in the normal unexposed germline, genome-wide erasure of methylation overlaps extensively with BORIS upregulation, and remethylation of DNA is associated with BORIS silencing (Renaud et al., 2007; Woloszynska-Read et al., 2007). Furthermore, DNA hypomethylation is a proven positive regulator of BORIS expression (Renaud et al., 2007; Woloszynska-Read et al., 2007). This interesting observation points to yet unknown regulation of BORIS in the maintenance of genotoxic stress-exposed germline DNA methylation.

In an attempt to explain the observed decrease of DNA methylation, we further analyzed radiation-induced DNA damage in mouse testes. Indeed, the observed global genome hypomethylation may be a consequence of radiation-induced DNA damage (Koturbash et al., 2005). DNA lesions were previously reported to interfere with methylation ability of DNA methyltransferases (Koturbash et al., 2005; Turk et al., 1995). Furthermore, during repair DNA synthesis, cellular DNA polymerases incorporate cytidine, but not 5-methyl-cytidine. Consequently, the presence and repair of radiation-induced DNA lesions may result in DNA hypomethylation. Based on these considerations, we tried to determine whether radiation exposure indeed induces DNA damage in testes tissue. DNA strand breaks were measured using the modification of a random oligonucleotide primed synthesis

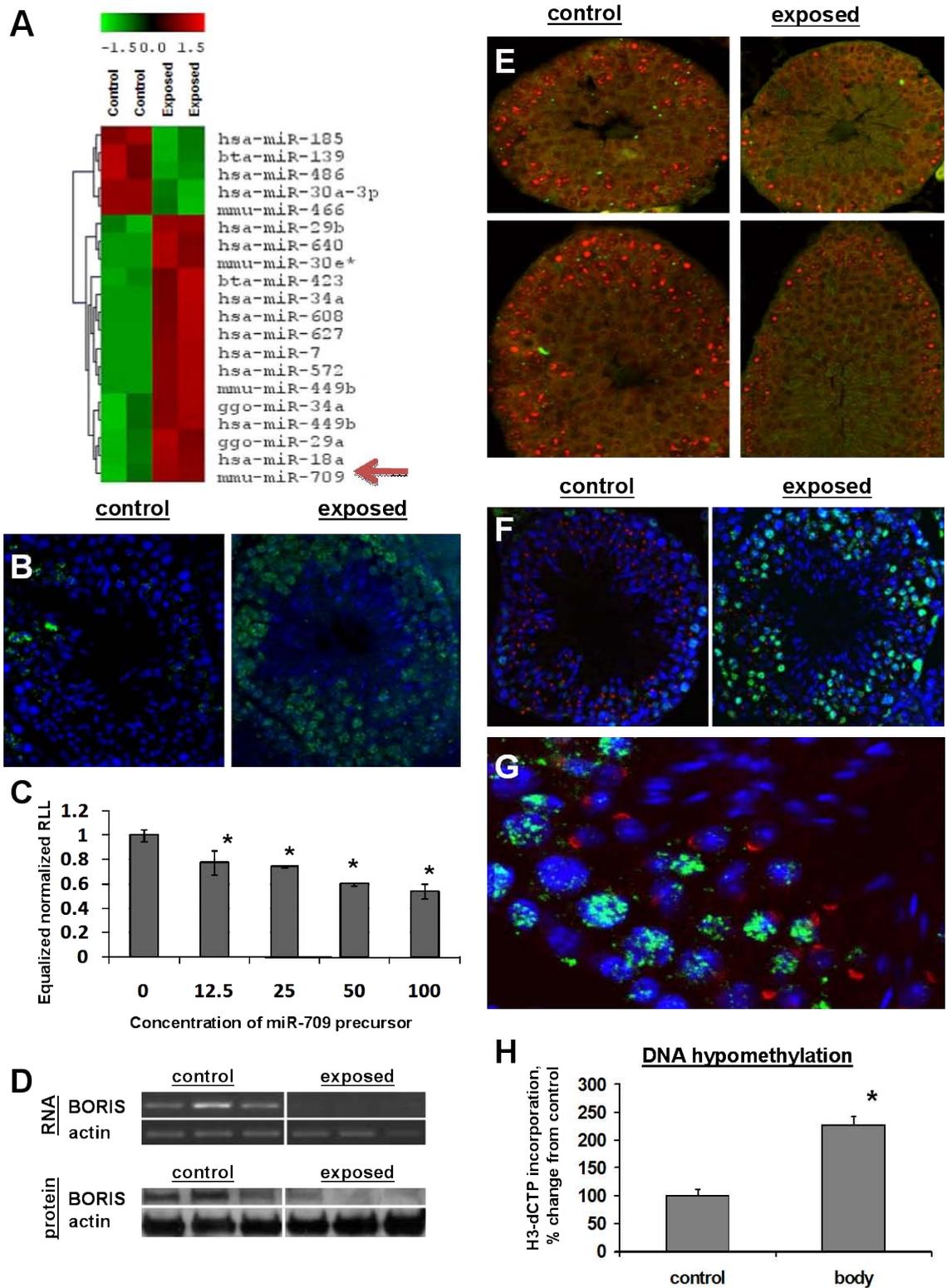
(ROPS) assay (Tamminga et al., 2008). Using this assay, we found that radiation exposure induced a significant 2.2 fold increase in the level of DNA strand breaks in testes of exposed animals (Fig. 2.1A). In parallel, we employed an alternative method to study the levels of DNA damage. The presence of  $\gamma$ H2AX foci, a direct indicator of DNA damage, was assayed using immunohistochemistry (Tamminga et al., 2008). Analysis revealed that radiation exposure led to a noticeable increase in  $\gamma$ H2AX foci in exposed testes (Figure 2.1B). Therefore, elevated DNA damage may result in significant global DNA hypomethylation observed in exposed testes tissue. Hypomethylation should have resulted in the increased BORIS levels which could have led to even more significant hypomethylation and altered resetting of methylation marks in exposed testes. Yet, elevated levels of miR-709 seem to counteract this putative hypomethylation circle.

Having found that significant radiation-induced changes in miR-709 levels were associated with the altered levels of BORIS and DNA methylation, we proceeded to deduce regulation of miR-709 expression *per se*. The location of a particular miRNA gene is an important factor in determining its expression pattern. In the case of intronic miRNAs, there is a high correlation between the expression of miRNA and host genes (Rodriguez et al., 2004). The location of miR-709 is within an intron of the transcription factor Rfx1, which was up regulated in testes of exposed animals (Figure 2.1B). Interestingly, Rfx1 has been implicated in DNA damage recognition, and it is also a downstream target of the DNA damage sensor ATR kinase (Lubelsky et al., 2005). Rfx1 up-regulation might be as follows: ATR is induced by DNA damage caused by IR, then ATR acts on its downstream targets

including Rfx1. Up-regulation of Rfx1 consequentially up-regulates miR-709, which then inhibits the translation of BORIS and affects DNA methylation processes in the germline (Figure 2.1E).

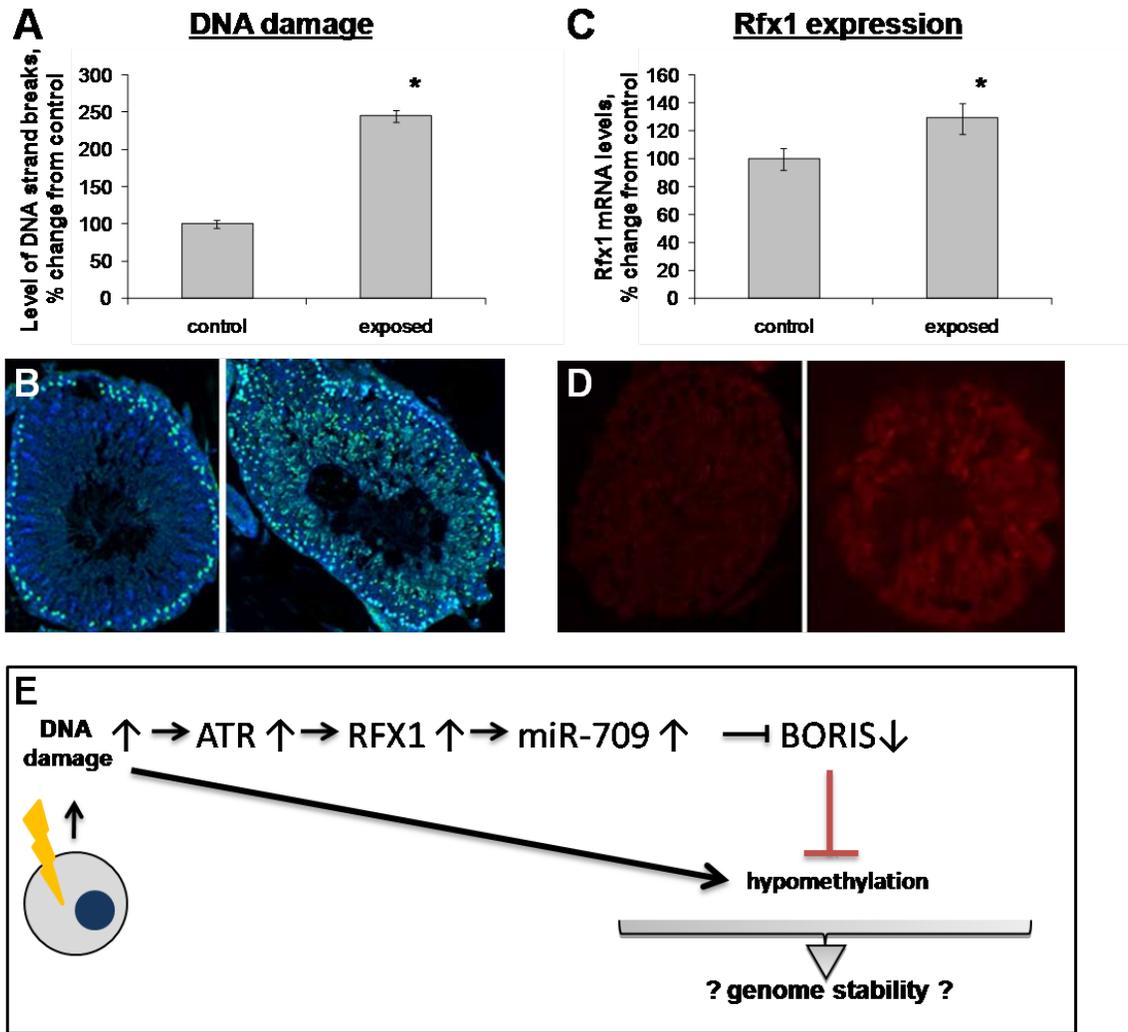
Interestingly, the DNA damage-induced Rfx1 upregulation is usually considered to be a protective event due to its inhibitory effects on the proto-oncogene *c-myc* and proliferating cell nuclear antigen (PCNA) (Chen et al., 2005). Down-regulation of these proteins allows cell cycle arrest and provides critical time for repair. In exposed testes, the level of both Rfx1 targets was significantly decreased (data not shown).

Therefore on the one hand, radiation exposure results in elevated DNA damage as well as global DNA hypomethylation. The observed DNA hypomethylation should result in an increase in BORIS expression (Renaud et al., 2007; Woloszynska-Read et al., 2007). However, it appears as though DNA damage-induced upregulation of miR-709 may be a protective mechanism to decrease cellular levels of BORIS and thereby counteract its induction by radiation-induced DNA hypomethylation. In the later case, DNA damage responses override the normal control mechanisms of BORIS and prevent aberrant DNA hypomethylation, thereby stabilizing the epigenome. Further studies are clearly needed to investigate the mechanisms and biological and evolutionary consequences of deleterious and protective responses of the germline genome and epigenome to genotoxic stress.



**Figure 2.0. Radiation exposure alters microRNAome of mouse testes, leads to increased expression of miR-709, decreased expression of miR-709 target BORIS, and results in global genome hypomethylation.**

**A. Hierarchical clustering of differentially expressed miRNA genes in control and exposed murine testes.** Red denotes high expression levels, whereas green depicts low expression levels. **B. Increased accumulation of miR-709 in exposed mouse testes as determined by fluorescence in situ (FISH) detection of miR-709 in cryosections.** Positive FISH signals are visualized in green (fluorescein isothiocyanate), while blue depicts a diamidino-2-phenylindole (DAPI) nuclear stain. **C. miR-709 directly targets *BORIS*.** Dose-dependent inhibition of *BORIS* expression in the luciferase assay after transfection of HEK293 cells with miR-709. RLU – relative luminescence units; \* -  $p < 0.05$ , Student's t-test with Bonferroni correction. **D. Radiation exposure decreases *BORIS* mRNA and protein levels *in vivo*.** Representative semi-quantitative RT-PCR and western blot. **E. Decreased levels of *BORIS* and 5-methyl-cytosine in testes of exposed animals as detected by immunofluorescence (IF).** Red – a *BORIS* signal, green – a 5 methylcytosine signal. **F. Mutually exclusive expression of miR-709 and *BORIS* in murine testes (IF and FISH analyses).** Control testes exhibit low miR-709 levels and high *BORIS* levels. Exposed samples show high levels of miR-709 and low levels of *BORIS*. Green - miR-709, red – *BORIS*, blue – DAPI. **G. A close-up of miR-709 and *BORIS* co-localization in murine testes.** Cells expressing *BORIS* are negative for miR-709 and vice versa. Green - miR-709, red – *BORIS*, blue – DAPI. **H. DNA hypomethylation in whole mouse testes after irradiation.** Levels of global genome DNA methylation were measured by the *Hpa*II cytosine extension assay. The results were expressed as relative [<sup>3</sup>H]dCTP incorporation/0.5 µg of DNA and percent change with reference to control. Data are presented as mean values ± SD, \* -  $p < 0.05$ , Student's t-test.



**Figure 2.1. Radiation-induced DNA damage responses in the male germline.**  
**A.** Levels of DNA damage in testes tissue of control and exposed animals as studied by the ROPS assay. The data are presented as mean values  $\pm$  SD, \* -  $p < 0.05$ , Student's t-test. **B.** Levels of  $\gamma$ H2AX in testes tissue of control and exposed mice. Green -  $\gamma$ H2AX, blue - DAPI. **C.** Radiation-induced changes in Rfx1 expression. The data are presented as mean values relative to control  $\pm$  SD, \* -  $p < 0.05$ , Student's t-test. **D.** Induction of ATR expression in exposed mouse testes. Red - ATR. **E.** A proposed model of radiation-induced genetic and epigenetic alterations in the mouse germline.

**CHAPTER 3: DNA DAMAGE, DNA HYPOMETHYLATION AND ALTERED  
EXPRESSION OF THE BROTHER OF THE REGULATOR OF IMPRINTED  
SITES (BORIS) IN THE GERMLINE OF RADIATION-EXPOSED MALE  
MICE**

## **ABSTRACT**

Ionizing radiation (IR) is a curative treatment for many human malignancies, an important diagnostic modality, and a pivotal preparative regimen for bone marrow transplantation. On the other hand, IR is a potent damaging agent that can affect a variety of processes in directly exposed cells, in their descendants, and in neighboring un-irradiated naïve ‘bystander’ cells. Accumulation of DNA damage caused by IR in conjunction with disrupted cellular regulation processes can lead to genome instability in the germline, and therefore to transgenerational genome instability in offspring of exposed males. The exact mechanisms of IR-induced genome instability in directly exposed, naïve bystander germ cells still remain obscure, yet accumulating evidence points to the role of DNA damage and DNA methylation changes in genome instability development.

In the current study, we used a well-established murine model to define the role of DNA methylation, DNA damage, as well as two important germline regulators of DNA methylation, BORIS and CTCF, in IR responses of the male germline. Here we report that irradiation leads to a significant accumulation of DNA damage, altered DNA methylation, and dysregulated BORIS expression in the exposed testes tissue. The possible molecular mechanisms and biological consequences of the changes observed are discussed.

## INTRODUCTION

Based on current statistics, approximately 12% (~20,900) of newly diagnosed cancers will occur in patients younger than 50 years of age (Canadian Cancer Society, 2008). Among these, approximately 40% (~ 8000 new cases/year) will occur in males. Fortunately, current advances in treatment regimens, such as radiation therapy (RT), have significantly lowered cancer related mortality rates in men of all ages (Canadian Cancer Society, 2008). As a curative treatment for many human malignancies, ionizing radiation (IR) is an important diagnostic modality, and a pivotal preparative regimen for bone marrow transplantation.

On the other hand, IR is a potent damaging agent that 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 (Amundson and Fornace 2003; Criswell et al. 2003; Fei and El-Deiry 2003; Iliakis et al. 2003; Powell and Kachnic 2003; Andreev et al. 2006; Jeggo and Lobrich 2006; Rodemann and Blaese 2007; Valerie et al. 2007). Most importantly, it is capable of producing DNA damage such as cross linking, nucleotide and base damage, and single and double strand breaks (Little 2000). The accumulation of DNA damage caused by IR in conjunction with disrupted cellular regulation processes can lead to genome instability (Little, 2000; Barcellos-Hoff, 2005; Sowa et al., 2006).

Genome instability is characterized by an increased rate of acquisition of alterations in the genome. It manifests itself as an induction of chromosomal aberrations, aneuploidy, micronuclei, gene mutations and amplifications, microsatellite instability and cell death in the directly exposed cells, their descendents

and the neighboring un-irradiated naïve ‘bystander’ cells (Morgan, 2003a, b; Suzuki et al., 2003).

The exact mechanisms of IR-induced genome instability in the directly exposed and naïve bystander cells are not well understood, yet accumulating evidence points to the epigenetic nature of this phenomenon (Morgan, 2003b; Jirtle and Skinner, 2007; Dubrova, 2003)

Male germ cells undergoing spermatogenesis are rapidly dividing and, consequently, are important IR targets. Genomic instability has been well documented in the exposed male germline and is thought to lead to the transgenerational genome instability in offspring of exposed males (reviewed in Dubrova, 2003; Aitken and De Iuliis, 2007). Similar effects were noted in the shielded bystander germline of animals that were subjected to cranial irradiation while the whole body was protected (Tamminga et al., 2008).

Notwithstanding, the exact mechanisms of IR-induced genome instability in the exposed and bystander male germline need to be further defined, and a lot of recent evidence points to the importance of IR-induced epigenetic alterations, and specifically – DNA methylation changes in its molecular etiology (reviewed in Kovalchuk and Baulch, 2008).

DNA methylation is an important epigenetic mechanism that safeguards genome stability of the cells, regulates gene expression and chromatin structure (Jaenisch and Bird, 2003; Klose and Bird, 2006; Weber and Schubeler, 2007). The altered DNA methylation has deleterious consequences for the germ cells (Aitken and De Iuliis, 2007). DNA methylation patterns are regulated in male germ cells by two

paralogous proteins, CCCTC binding factor (CTCF) and Brother of the Regulator of Imprinted Sites (BORIS) (Loukinov et al., 2002), also known as CTCF-like (CTCFL). CTCF is a nuclear protein that has been implicated in the functional reading of imprinted marks in somatic cells (reviewed in Klenova et al., 2002; Dunn and Davie, 2003). BORIS, on the other hand, can be found in the cytoplasm as well as the nucleus (Loukinov et al., 2002), and has been implicated in the recognition and re-establishment of methylation marks during epigenetic reprogramming in the male germline (Loukinov et al., 2002; Jelinic et al., 2006). The role(s) of BORIS and CTCF in the direct and bystander IR responses of the male germline have not been analyzed.

In the current study we used a well-established murine model to define the roles of DNA methylation, DNA damage, and BORIS and CTCF in the IR responses of male germline. Here we report that irradiation led to significant accumulation of DNA damages, altered DNA methylation and dysregulated BORIS expression in the exposed testes.

## **MATERIALS AND METHODS**

### ***Animal model: irradiation scheme and tissue sampling***

In this study, we analyzed molecular changes in the testes of male C57BL/6 mice following *in vivo* whole body or cranial irradiation exposure. Sixty four sexually mature 55 day old male mice were randomly assigned to different treatment groups. The whole-body exposed cohort (16 animals) received 2.5 Gy of whole-body exposure to X-rays (5cGy/s, 90kV, 5mA). The second cohort (16 animals) received 2.5 Gy of X-ray exposure to the skull only, while the rest of the animals body was

protected by a ~3mm thick lead shield, the same type as used for 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 (16 animals) were sham treated.

Another cohort of 16 animals was exposed to an approximate scatter dose of ~0.02 Gy (Koturbash et al., 2006a, 2007, 2008a). To determine the bystander ventral skin/testes dose resulting from photon scatter within a mouse itself, a Monte Carlo simulation was performed (Koturbash et al., 2006a, 2007, 2008a). 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 ~0.015Gy.

The animals were humanely sacrificed 4 days and 56 days after exposure to measure the initial effects on the germ cell population at the time of exposure and persistent effects which remain after one complete spermatogenic cycle (spermatogonial effects), respectively. Testes tissue was sampled upon sacrifice and processed for further molecular studies. One testis of each animal was immediately frozen in liquid nitrogen and stored at -80°C, while the other one was fixed in 4% paraformaldehyde (J.T. Baker) for approximately 48 hrs at 4°C and embedded in

paraffin. Paraffin embedding and sectioning were conducted at Central Vet Labs, Edmonton, AB, Canada.

### ***Nucleic acid extractions***

Total DNA was prepared from testes tissues using a Qiagen DNAeasy kit (Qiagen), according to the manufacturer's instructions and stored at -20°C. Total RNA was extracted from frozen mouse testes using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions and stored at -80°C.

### ***Cytosine extension assay to detect sequence-specific changes in DNA methylation***

A well established radiolabeled [<sup>3</sup>H]dCTP extension assay was employed to evaluate global DNA methylation levels (Pogribny et al., 1999). The assay measures the proportion of CCGG sites that lost methyl groups on both DNA strands. HpaII is a methylation-sensitive restriction enzyme that cleaves CCGG sequences in the case where internal cytosine residues are unmethylated on both strands. It leaves a 5'-guanine overhang after DNA cleavage. This overhang can be used for the subsequent single nucleotide extension with labeled [<sup>3</sup>H]dCTP. The extent of [<sup>3</sup>H]dCTP incorporation opposite the exposed guanine is directly proportional to the number of cleaved and thus unmethylated CpG sites and inversely proportional to methylation levels (i.e. the higher the methylation, the less [<sup>3</sup>H]dCTP is incorporated). Taking into consideration that the vast majority of frequently occurring HpaII tetranucleotide recognition sequences are constitutively methylated *in vivo*, an increase in cleavage at these sites indicates genome-wide hypomethylation. DNA (0.5 µg) was digested overnight with a 20-fold excess of *HpaII* endonuclease according to the manufacturer's protocol (New England Biolabs, Beverly, MA, USA). A second DNA

aliquot (0.5  $\mu\text{g}$ ) was incubated without restriction enzyme addition and served as a background control. The single nucleotide extension reaction was performed in a 25  $\mu\text{g}$  of DNA, 1xPCR bufferII, 1.0mM  $\text{MgCl}_2$ , 0.25 units of Taq DNA polymerase (Fisher Scientific, Ottawa, ON, USA), [ $^3\text{H}$ ]dCTP (57.4 Ci/mmol) (Perkin-Elmer, Boston, MA, USA) and incubated at 55°C for 1 h, then immediately placed on ice. Duplicate aliquots (25  $\mu\text{l}$ ) from each reaction were placed on Whatman DE-81 ion-exchange filters and washed three times 10 min with gentle agitation with sodium phosphate buffer (0.5M, pH 7.0) at room temperature. The filters were dried and processed by scintillation counting (Beckman Coulter). Background label incorporation was subtracted from enzyme-digested samples and results were expressed as percent change from control (Pogribny et al., 1999, 2004, 2005; Raiche et al., 2004).

#### ***DNA strand break measurement***

A modification of the random oligonucleotide-primed synthesis (ROPS) assay was used to detect the presence of DNA strand-breaks in high molecular weight DNA (Basnakian and James, 1996; Pogribny et al., 2004). The assay is based on the ability of Klenow fragment polymerase to initiate ROPS from the reannealed 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 reassociated by cooling. The resulting random reassociation 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 [<sup>3</sup>H]dCTP (57.4 Ci/mmol) (Perkin-Elmer, Boston, MA), 0.05mM concentrations of each dGTP, dATP, dTTP, 0.6 mM dCTP, 10mM Tris-HCl (pH 7.5), 5mM MgCl<sub>2</sub>, 7.5mM DTT, and 0.5U Klenow polymerase (New England Biolabs, Beverly, MA, USA) 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 12.5mM 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 [<sup>3</sup>H]dCTP incorporation relative to control values.

#### ***BORIS and CTCF reverse transcriptase polymerase chain reaction (RT-PCR)***

Total RNA was extracted from mouse testes tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacture's instructions and stored at -80°C. First strand cDNA was synthesized using the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Burlington, Ontario) with the following modifications: 1.5µg of total RNA was mixed with 1µg oligo(dT)<sub>18</sub> primers. The final concentration of cDNA was diluted in half with DEPC water and stored at -20°C.

Semi-quantitative RT-PCR was carried out on a Flexigene PCR machine (Barloworld Scientific Ltd, Staffordshire, United Kingdom) using *Taq* DNA Polymerase (Fermentas, Burlington, Ontario). Each reaction contained 2µl of cDNA, 10pmol of forward and reverse primer, 2mM MgCl<sub>2</sub>, 1x *Taq* Buffer with KCl, and 0.625 units of *Taq* DNA polymerase. Primers for *BORIS*, *CTCF* and *β-actin* were

previously designed to overlap two exons so as not to amplify genomic DNA (Table 3.0) (Loukinov et al., 2002). PCR conditions were independently determined for each set of primers such that the concentration of PCR product was below saturation. Each reaction consisted of an initial denaturation at 94°C for 5 minutes followed by a predetermined number of cycles (Table 3.0) of a denaturation step at 94°C for 30 seconds, annealing at the respective primer melting temperature for 30 seconds, and an extension at 72°C for 30s. Following a final extension at 72°C for 10 minutes the samples were stored at 4°C. Agarose gel electrophoresis was carried out in TAE buffer using a 2% agarose (EMD Chemicals, Darmstadt, Germany) gel containing Ethidium Bromide. The amplified product was visualized under UV light and quantified using Image J 1.36b software (NIH, [www.rsb.info.nih.gov/ij](http://www.rsb.info.nih.gov/ij)) and normalized to  $\beta$ -actin.

### ***Immunofluorescence***

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 examination, the tissues were assembled into tissue microarrays (TMAs) with 4.5 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](http://www.pantomics.com)). Dual Immunofluorescent (IF) staining was carried out on paraffin embedded TMA sections, 5 $\mu$ m thick, for 5-methylcytosine (1:500, sheep anti-5meC, Abcam) and BORIS (1:100, rabbit anti-BORIS, Abcam). An Alexa Fluor secondary antibody was used for BORIS (1:1000, Alexa Fluor Goat anti-rabbit 546nm, Invitrogen) and a

FITC conjugated anti-sheep antibody was used for 5meC (1:1000, Rabbit anti-sheep FITC). Frozen testis sections were obtained from an additional experiment involving 20 animals and stained for BORIS protein (1:100, rabbit anti-BORIS, Abcam). An Alexa Fluor secondary antibody (1:1000, Alexafluor Donkey anti-rabbit 546nm, Invitrogen) was used and the section was counterstained with 4',6-diamidino-2-phenyl-indole, dihydrochloride (DAPI) (Invitrogen).

### ***Statistical analysis***

For determination of the significance of the difference between the means Student's *t* test for independent variance was used. Statistical analysis and plotting of results were performed using MS Excel for Windows XP software. The results are presented as mean values  $\pm$  standard error of the mean (SEM).

## **RESULTS**

### ***Radiation-induced DNA damage in irradiated and bystander testes tissue of the whole body and cranially exposed mice***

In this study we used a well-established murine model to analyze and compare the effects of direct whole body and localized head-only exposure on the molecular changes in the male germline. To analyze the direct irradiation effects, the animals were exposed to 2.5 Gy of X-rays to the whole body. To simulate the cranial irradiation scenario, another cohort of animals was subjected to a localized 2.5 Gy X-ray exposure to the skull, while the rest of the body was completely protected by a medical grade shield. The lead shielding used for these studies was similar to one used for patients exposed to radiation in the clinic. The same shielding was used in

the published studies on the bystander effect in mouse skin, mouse and rat spleen, and rat testes (Koturbash et al., 2006a, 2007, 2008b). We also considered the fact that even though the shielding of the animal body is complete, the X-rays can be reflected as they pass through the tissue, thus forming a small ‘scatter’ dose in the protected tissue (Koturbash et al., 2006a). To check if a possible small scatter dose could contribute to the generation of bystander effects in testes, we exposed a separate cohort of animals to a scatter dose of ~0.02 Gy calculated according to the Monte Carlo simulation and directly measured by RAD-CHECK<sup>TM</sup> (Koturbash et al., 2006a, 2007, 2008a). Sham treated animals served as a control group.

DNA damage is a major hallmark of radiation exposure and is the most studied molecular endpoint. In our previous studies, we showed a pronounced induction of DNA damage and particularly DNA double-strand breaks (DSBs) in the directly exposed and bystander cells and tissues (Koturbash et al., 2006a; Sedelnikova et al., 2007; Koturbash et al., 2008b). In light of these considerations, we assessed if the X-ray exposure induced DNA damage in whole testes tissue of control, whole body-exposed, head-exposed and scatter-exposed animals 4 days and 56 days after irradiation.

Using a well-established ROPS assay (Basnakian and James, 1996; Pogribny et al., 2004) we found that whole body exposure to 2.5 Gy of X-rays led to a significant ( $p < 0.05$ ) 2.4-fold increase in the level of DNA strand breaks in mouse testes 4 days after irradiation. Head irradiation resulted in a significant ( $p < 0.05$ ) 1.8 fold increase in DNA strand breaks in the shielded testes tissue (Figure 3.0, and Table 3.1). Furthermore, elevated levels (1.5-fold and 1.4-fold in the whole- body and head

exposure groups, respectively;  $p < 0.05$ ) of DNA strand breaks were observed 56 days after exposure (Figure 3.0, and Table 3.1). Even though levels of DNA strand breaks decreased from 4 days to 56 days after exposure, indicating that a portion of DNA lesions was repaired, some lesions persisted for a long period of time. Surprisingly, animal exposure to a scatter dose also led to a small but statistically significant (1.2-fold as compared to the control,  $p < 0.05$ ) increase in the level of DNA strand breaks in the murine testes (Figure 3.0, and Table 3.1). The scatter-dose induced DNA damage was effectively repaired, and no long – term persistence was found 56 days after exposure.

#### ***Altered levels of global DNA methylation in the irradiated testis***

It has been shown previously that the accumulation of DNA damage is accompanied by the loss of DNA methylation (Turk et al., 1995; Panayiotidis et al., 2004; Pogribny et al., 2004), and the direct exposure and bystander radiation effects may be epigenetically mediated (Morgan, 2003a, b; Mothersill and Seymour, 2004; Koturbash et al., 2007, 2008a; Wright and Coates, 2006). DNA methylation is an important epigenetic mechanism that safeguards genome stability of the cells, regulates gene expression and chromatin structure (Jaenisch and Bird, 2003; Klose and Bird, 2006; Weber and Schubeler, 2007). The altered DNA methylation has deleterious consequences for the germ cells (Aitken and De Iuliis, 2007). In the present study we examined DNA methylation changes in murine testes following an acute 2.5Gy whole body or head-only X-ray exposure. To assess the changes in DNA

methylation, we used the well-established and sensitive cytosine extension assay (Pogribny et al., 1999; Pogribny et al., 2004; Raiche et al., 2004).

An acute exposure of 2.5Gy X-irradiation led to a significant (2.3 fold,  $p < 0.05$ ) decrease in global DNA methylation levels in the testes 4 days after exposure (Figure 3.1). No significant changes were observed in global DNA methylation levels in the testes of head or scatter exposure groups (Figure 3.1). Further more, global DNA methylation levels for all exposure groups returned to control levels 56 days after X-irradiation (Figure 3.1).

***Irradiation alters mRNA levels of BORIS, but not CTCF in the exposed testes tissues***

Radiation-induced alterations in gene expression are an important contributing factor in radiation-induced genome instability and may have an impact on transgenerational genome instability.

In previous experiments, it has been found that paternal IR exposure led to profound epigenetic dysregulation in the progeny (Koturbash et al., 2006b). Given the role that BORIS and CTCF play in epigenetic reprogramming of the paternal germline, we hypothesized that BORIS dysregulation upon IR exposure may lead to the epigenetic dysregulation in the germline and, in the future, possibly the fertilized egg. Thus, we analyzed the expression levels of BORIS and CTCF in the male germline upon radiation exposure.

BORIS mRNA levels were significantly decreased (15%;  $p < 0.05$ ) 4 days after whole-body exposure; however, mRNA levels return to control values 56 days after

radiation exposure (Figure 3.2). No significant changes in BORIS mRNA levels were observed in the head or scatter exposure groups 4 or 56 days after X-irradiation (Figure 3.2). CTCF expression was not altered by radiation exposure in any of the groups at either time point (data not shown).

Western blot analysis for mouse BORIS protein failed to produce convincing results with the commercially available antibody. This finding is not unique to this study and has been reported previously (Woloszynska-Read et al., 2007). For this reason we opted for a qualitative analysis of BORIS expression by IF staining.

On paraffin embedded tissue sections we found that BORIS was, as reported previously, expressed in spermatocytes (Loukinov et al., 2002). However, in addition to expression in spermatocytes, we found BORIS expression in spermatogonia and Leydig cells (Figure 3.3). This finding has also been reported in paraffin embedded tissue by Hoffmann and colleagues, who found that BORIS appears to be exclusively expressed in the cytoplasm of spermatogonia and Leydig cells (Hoffmann et al., 2006). In the current study on paraffin embedded tissue, co-localization of BORIS with a fluorescent nuclear stain, such as DAPI, was not done and as such exclusive nuclear or cytoplasmic expression cannot be confirmed with certainty. In any case, there is an apparent decrease in BORIS protein in the seminiferous tubules of mice exposed to whole-body X-irradiation. This decrease was not observed in any of the other exposure groups, which correlates with the RT-PCR results of BORIS mRNA expression (Figure 3.2).

In a follow-up study involving a group of 20 animals exposed according to the same scheme and sacrificed 4 days after exposure we used the same BORIS antibody

as well as DAPI to stain frozen testis tissue sections. On these sections we found that BORIS is indeed expressed exclusively in the spermatocyte population with no apparent staining in spermatogonia or Leydig cells (Figure 3.4). Further more, the majority of BORIS protein is found in the cytoplasm with only a few cells displaying nuclear localization of BORIS (Figure 3.4). The latter findings are supportive of the observations of Loukinov and colleagues who found exclusive expression in spermatocytes and that 10-30% of BORIS was nuclear with the remaining fraction being cytoplasmic (Loukinov et al., 2002).

## **DISCUSSION**

Approximately 5% of newly diagnosed cancers will occur in patients less than 40 years of age, of which ~ 3100 will be males in the most productive years of their life (Canadian Cancer Society, 2008). While the current advances in treatment regimens, such as radiotherapy, have significantly lowered cancer related mortality rates in men of all ages (Canadian Cancer Society, 2008), one major quality-of-life issue in cancer survivors is the ability to produce healthy progeny.

In fact, un-exposed offspring of irradiated fathers exhibit a wide variety of genotypic alterations including chromosome aberrations, micronuclei formation, altered gene expression patterns and many other hallmarks of transgeneration genome instability (reviewed in Morgan, 2003a; Dubrova, 2003; Barber and Dubrova, 2006). The genotypic alterations may predispose the offspring of the exposed fathers to an increased risk of genetic diseases, infertility and even cancer (reviewed in Nomura, 2003; Aitken and De Iuliis, 2007). The molecular mechanism(s) by which this occurs

is not known, however, recent studies implicate accumulation of DNA damage and epigenetic DNA methylation changes in the paternal germline in the molecular etiology of transgenerational genome instability (Koturbash et al., 2006b).

Testis tissue is one of the most radiosensitive tissues and may be damaged by direct irradiation or from scattered radiation during treatment of adjacent tissues. Furthermore, it can also be damaged by the enigmatic bystander signals originating from the exposed body parts during localized irradiation (Tamminga et al., 2008). Recovery of spermatogenesis after irradiation is dependent on the dose and may take anywhere from 9 months to 5 years or more in humans. The presence of DNA damage in mature sperm induced by radiation may have serious consequences in the fertilized oocyte.

To that end, we studied the effects of localized and whole-body radiation exposure on the germline of male mice. Previous studies have shown a strong interrelationship between genetic and epigenetic changes in exposed tissue (Pogribny et al., 2004, 2005; Koturbash et al., 2005). Here in the male germline, we find that irradiation causes a significant increase in global levels of DNA strand breaks that are paralleled by a significant increase in global DNA hypomethylation. Pogribny and colleagues propose that radiation-induced global genome DNA hypomethylation is DNA repair-related (Pogribny et al., 2004). Several mechanisms may contribute to this phenomenon including altered methyltransferase activity or expression (Turk et al., 1995; Panayiotidis et al., 2004; Raiche et al., 2004; Pogribny et al., 2005), or the activation of DNA repair pathways that incorporate cytosine, instead of

methylcytosine, at break sites. Clearly, further studies are needed to explain the cause(s) of genome wide DNA hypomethylation following radiation exposure.

In the irradiated thymus, DNA damage is also paralleled by significant DNA hypomethylation (Koturbash et al., 2005). However, one month after exposure DNA damage was repaired while DNA methylation changes persisted (Koturbash et al., 2005). In contrast, in irradiated testes tissues we found that DNA damage was persistent while changes to global DNA methylation levels were not. These data correlate with the previously established finding of the tissue specificity of radiation-induced epigenetic changes (Raiche et al., 2004). The molecular basis of the DNA damage persistence needs to be further dissected.

Radiation-induced DNA damage in sperm cells may trigger a cascade of epigenetic events in the fertilized egg that result in aberrant epigenetic modifications. Shimura and colleagues have shown that fertilization with irradiated sperm triggers a p53 dependent S-phase DNA damage checkpoint (Shimura et al., 2002a). Paternal DNA damage was shown to alter the expression of some, but not all, of the DNA repair mechanisms in the early embryo (Harrouk et al., 2000). It may be the case where the portion of misrepaired DNA damage in the germline or the fertilized egg contributes more to transgenerational instability than the portion of unrepaired lesions (Joshi et al., 1990).

The acquisition of DNA methylation patterns in mammals is very complex and involves the erasure, establishment, and maintenance of DNA methylation. In male germ cells, DNA methylation patterns are regulated by two paralogous proteins, CTCF and BORIS (Loukinov et al., 2002). The expression of BORIS, followed by

CTCF, takes place with the erasure and re-establishment of methylation marks, respectively (Loukinov et al., 2002). Dysregulation of BORIS upon IR exposure may lead to the epigenetic dysregulation in the germline and possibly the fertilized egg. We found that expression of BORIS, but not CTCF, is altered by radiation exposure. BORIS mRNA and protein levels are decreased in the germline of whole body exposed animals. This suggests that BORIS may be involved in response to radiation-induced DNA damage in the germline while CTCF is not. It is known that radiation exposure causes a significant loss of primary spermatocytes (Hasegawa et al., 1998; Haines et al., 2002; Cordelli et al., 2003), which are the only cells that normally express BORIS (Loukinov et al., 2002). Yet, it is unlikely that we are measuring the effects of germ cell apoptosis as CTCF is re-expressed in spermatids and did not change in exposed tissues.

The radiation-induced decrease of BORIS expression seen in parallel with the radiation-induced hypomethylation in the testes tissue of the whole-body exposed mice is an interesting and unexpected finding. It is well-established that in the normal unexposed germline the genome-wide erasure of methylation overlaps extensively with BORIS upregulation, and that remethylation of DNA is associated with BORIS silencing (Renaud et al., 2007; Woloszynska-Read et al., 2007). Furthermore, DNA hypomethylation is a proven positive regulator of BORIS expression (Renaud et al., 2007; Woloszynska-Read et al., 2007). This interesting observation points to a yet unknown role/regulation of BORIS in the maintenance of the genotoxic stress-exposed germline DNA methylation.

We predict that radiation-induced decrease of BORIS expression may be a protective mechanisms aimed to prevent aberrant global hypomethylation. Therefore, on the one hand radiation exposure results in elevated DNA damage and global DNA hypomethylation. Hypomethylation may increase BORIS expression and facilitate the further aberrant erasure of methylation patterns. On the other hand, silencing of BORIS by some yet unknown mechanisms may prevent the massive improper DNA hypomethylation. Further studies are clearly needed to deduce radiation-induced BORIS regulation and the biological and evolutionary repercussions of BORIS expression in the exposed germline.

A curious finding of the current study is the discrete localization of BORIS protein in the cytoplasm of primary spermatocytes. The localization did not change upon irradiation. In all groups BORIS appears to concentrate close to the nucleus, and may be the early CB. The CB functions as an RNA processing and storage center for mRNA, pre-miRNA as well as components of the RNA-induced silencing complex (RISC) (Kotaja et al., 2006; and reviewed in Kotaja and Sassone-Corsi, 2007). Components of the CB appear in the cytoplasm of primary spermatocytes but the CB is not fully compact until the round spermatid stage (Kotaja et al., 2006). It is interesting to note that BORIS contains a zinc finger domain capable of binding single-strand DNA and RNA (Klenova et al., 2002). Further studies are in progress to find out which proteins, if any, share this localization.

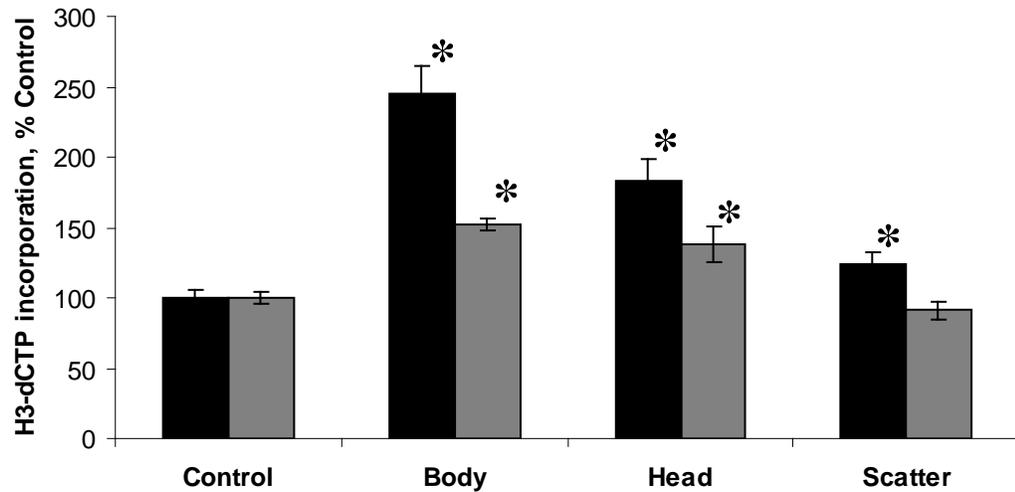
Perhaps one of the most intriguing findings of the current study is that localized exposure to radiation can influence genetic endpoints in the distant germline. Bystander effects are well characterized *in vitro* and have been the topic of

much debate *in vivo* (reviewed in Morgan, 2003a, b, c). Previous studies have shown that increased levels of DSBs in the bystander spleen were accompanied by a global loss of DNA methylation (Koturbash et al., 2008a). Similarly, in bystander rat testes, exposure to 20Gy of X-rays led to significant accumulation of DNA damage and DNA hypomethylation. However here, in bystander testes of mice exposed to 2.5 Gy of X-rays DNA damage is not accompanied by global DNA hypomethylation. This may be due to some species specificity, and, most importantly, dose response specificity of the germline bystander effect.

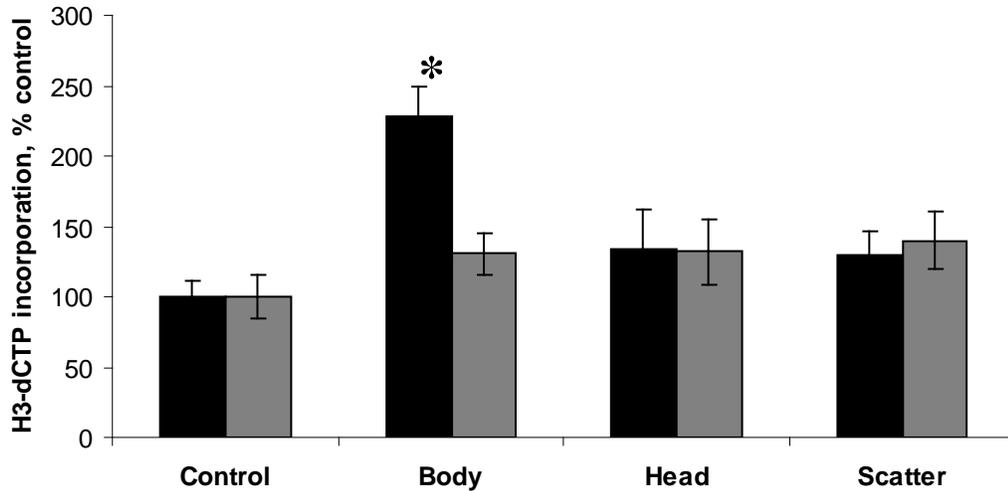
In addition, BORIS is not dysregulated in the bystander mouse testis, further implicating BORIS solely in the direct radiation-induced DNA damage pathway. The molecular mechanisms that mediate the observed bystander effects remain unknown at this time. A large body of evidence indicates that soluble factors capable of inducing DNA damage, commonly referred to as clastogenic factors, are released into the blood following radiation exposure. The identity of these blood-borne clastogenic factors remains unknown. It is interesting to note that radiation-induced DNA damage is persistent in bystander testes, a finding that requires further verification but may have serious implications in the treatment modalities of male cancer patients. The later finding is not all that surprising as clastogenic factors are known to be very persistent, or continuously generated, and can be found years after radiation exposure (Morgan, 2003b; and references therein).

Radiation-induced damage to sperm cells or their progenitors may affect the fertilized egg and interfere with epigenetic reprogramming and thus result in subsequent changes in the embryo. We noted that radiation-induced DNA damage in

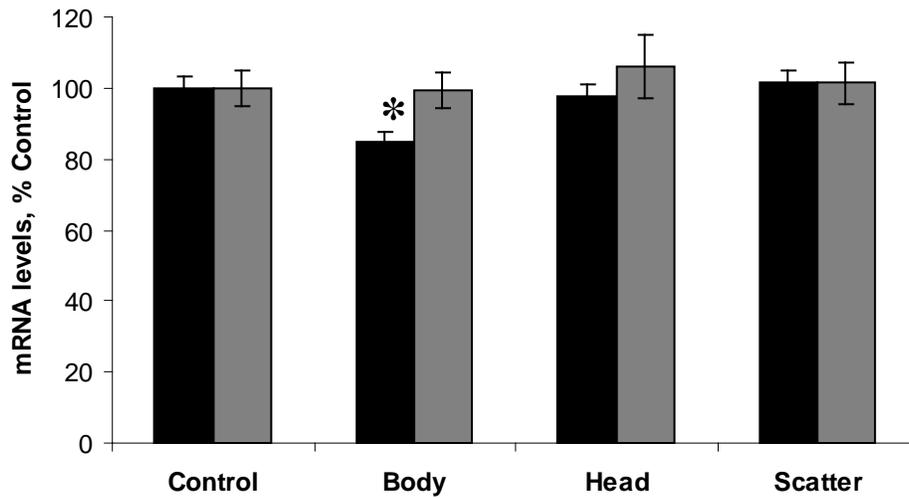
the exposed testes is accompanied by global DNA hypomethylation and that DNA damage persists after one complete round of spermatogenesis. This indicates that the genomic integrity of the spermatogonia may be compromised and may take a significant amount of time to completely recover. Localized exposures to IR also induced a significant and persistent increase in DNA damage. Therefore, the localized exposure that affects the shielded bystander germline may also have serious consequences on male fertility and the heritability of DNA damage in subsequent generations.



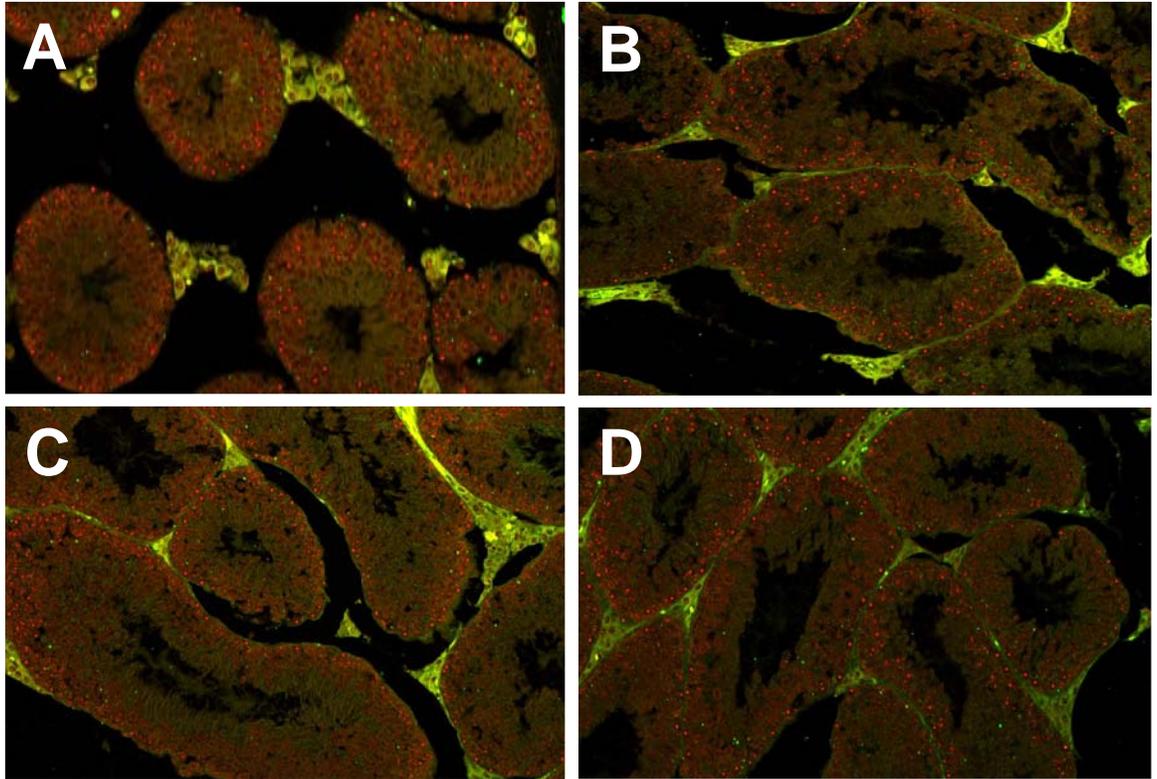
**Figure 3.0. Levels of DNA strand breaks in murine testes following an acute exposure to 2.5Gy of X rays.** DNA damage (levels of stand breaks) was measured using the ROPS assay 4 days (early effects) and 56 days (persistent effects) following whole-body, head, or scatter irradiation (see Materials and Methods). The results are presented as mean values  $\pm$  SEM, n=8; \*p <0.05. Black bars – 4 days post exposure, and Grey bars – 56 days post exposure.



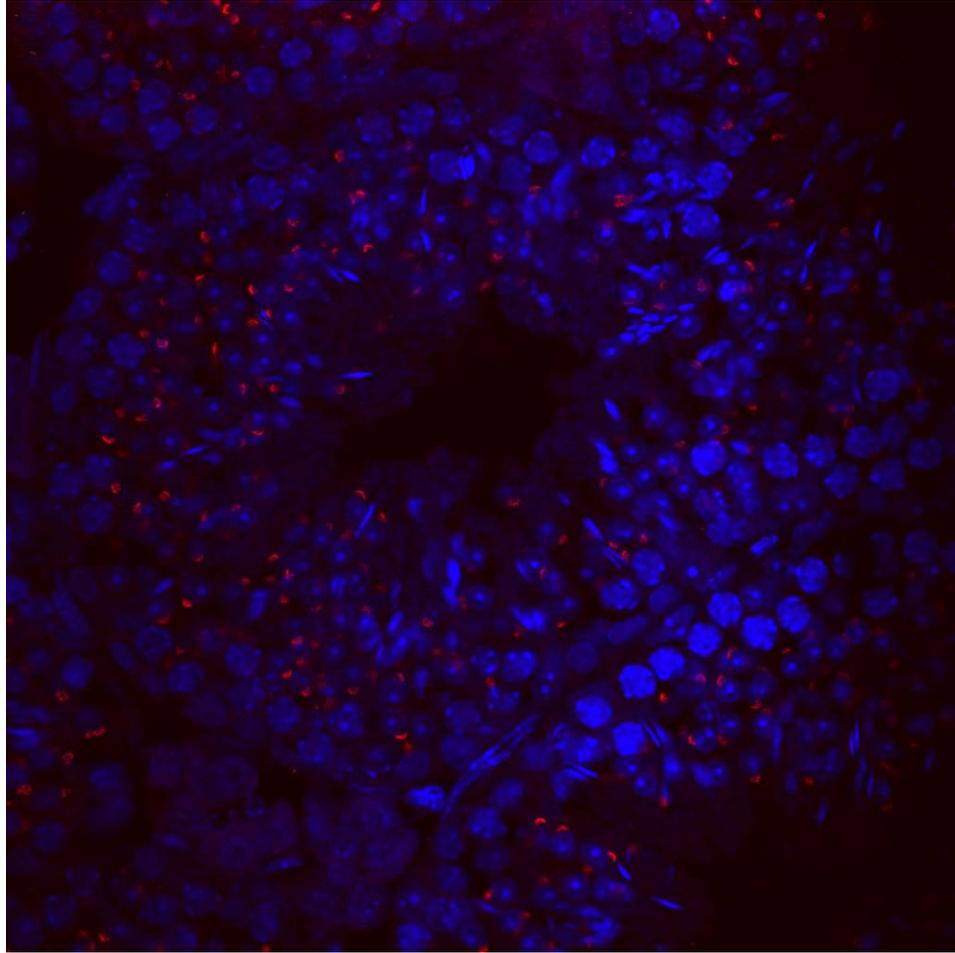
**Figure 3.1. Effects of an acute 2.5Gy of X-rays on global DNA methylation levels in testis tissue.** The levels of DNA methylation were measured using the cytosine extension assay 4 days (early effects) and 56 days (persistent effects) following whole-body, head, or scatter irradiation (see Materials and Methods). The results are presented as mean values  $\pm$  SD, n=8; \*p <0.05. Black bars – 4 days post exposure, and Grey bars – 56 days post exposure.



**Figure 3.2. Expression levels of BORIS mRNA in murine testes tissues following an acute exposure to 2.5Gy of X-rays.** Transcript levels for BORIS were measured using RT-PCR with primers designed specifically to amplify cDNA (Loukinov et al., 2002). The results are presented as mean values  $\pm$  SEM, n=8; \*p <0.05. Black bars – 4 days post exposure, and Grey bars – 56 days post exposure (see Materials and Methods).



**Figure 3.3. Radiation-induced hypomethylation and altered expression of BORIS protein in testis tissue of whole-body exposed mice.** Immunofluorescent staining of paraffin embedded testis for BORIS and 5meC in control (A), body (B), head (C), and scatter (D) exposure groups. Red - BORIS protein, and Green – methylcytosine.



**Figure 3.4. Immunofluorescent localization of BORIS protein in the spermatocytes population of the mouse testis.** Characteristic BORIS (Red) expression pattern in a seminiferous tubule of a frozen section of the mouse testis, counter stained with DAPI (Blue).

**Table 3.0** RT-PCR conditions for BORIS, CTCF, and  $\beta$ -actin

Gene	Sense	Primer Sequence 5' to 3'	Tm (°C)	Cycles	Amplicon
BORIS	Forward	gagagacagacaagagagaagagaggtgctc	69.5	37	361bp
	Reverse	cctgtgtgggtgttcacatggttcctaagaag	69.5		
CTCF	Forward	tcgcaagtggacacccaaatc	62.5	32	176bp
	Reverse	gaaccattcaggggaaaagc	62.5		
Actin	Forward	ctgtgctgtccctgtatgcc	64.5	24	196bp
	Reverse	gtggtggtgaagctgtagcc	64.5		

**Table 3.1** Induction of DNA damage in testis tissue following 2.5Gy X-ray exposure

Days post exposure	Exposure Group		
	Body	Head	Scatter
4 Days	<b>2.4</b>	<b>1.8</b>	<b>1.2</b>
56 Days	<b>1.5</b>	<b>1.4</b>	0.9

DNA strand breaks were measured by the ROPS assay. Data are presented as fold change compared to control. Statistically significant ( $p < 0.05$ ) values are in bold.

**CHAPTER 4: PATERNAL CRANIAL RADIATION EXPOSURE INDUCES  
DISTANT BYSTANDER DNA DAMAGE AND ALTERES DNA  
METHYLATION IN THE SHIELDED GERMLINE**

Published in its entirety in:

Tamma J, Koturbash I, Baker M, Kutanzi K, Kathiria P, Pogribny IP, Sutherland RJ, Kovalchuk O. Paternal cranial irradiation induces distant bystander DNA damage in the germline and leads to epigenetic alterations in the offspring. *Cell Cycle*. 2008, 7:1238-45.

## **ABSTRACT**

It is now well accepted that parental whole body irradiation causes transgenerational genome and epigenome instability in the offspring. The majority of human exposures to radiation, such as therapeutic and diagnostic irradiation, are localized and focused. The potential of localized body-part exposures to affect the germline and thus induce deleterious changes in the progeny has not been studied. To investigate whether or not the paternal cranial irradiation can exert deleterious changes in the protected germline, we studied the accumulation of DNA damage in the shielded testes tissue. Here we report that the localized paternal cranial irradiation results in a significant accumulation of unrepaired DNA lesions and loss of global DNA methylation in sperm cells.

## INTRODUCTION

Currently it is well accepted that the pre-conception whole-body radiation exposure poses a significant threat to the progeny of irradiated parents by inducing DNA damage to sperm cells (Aitken and De Iuliis, 2007).

Damaged sperm cells may affect fertilization and embryo development by causing numerous harmful phenotypic and genotypic effects in the offspring (Aitken and De Iuliis, 2007). Phenotypic effects include a decreased fertility and variety of teratogenic effects. Genotypic alterations comprise the increased mutation rates and elevated frequencies of chromosome aberrations, micronuclei formation, altered gene expression and many other signs of a transgeneration genome instability (as reviewed by Nomura, 2003; Dubrova, 2003a; Barber and Dubrova, 2006; Hamada et al., 2007; Morgan and Sowa, 2007). Such genotypic alterations may predispose the progeny of irradiated parents to an increased risk of genetic diseases, infertility and cancer (Dubrova, 2003; Barber and Dubrova, 2006; Mohr et al., 1999).

The majority of studies on the germline and transgeneration radiation effects have analyzed the consequences of the parental whole body irradiation; however, such exposure types are relatively rare. In contrast, the localized body-part exposures occur very frequently during the radiation diagnostics and therapy. One third of people alive today are likely to get cancer, and more than half will receive some form of radiotherapeutic treatment (American Cancer Society, 2003). Amongst the radiotherapy procedures, brain irradiation is among the most widespread, since brain tumors are the second most common cancers in the young adults of reproductive age (Huff, 2007; Lyons and Vora, 2007). The recent advances in radiation therapy have

led to the increased treatment success and patient survival rates. Yet, the potential of the localized parental exposures to affect the germline and thus induce genome instability and deleterious changes in the progeny needs to be addressed.

This is especially important since the exposed cells may communicate the damage to the distant unexposed cells and tissues resulting in genome instability (Morgan, 2003a, b; Morgan and Sowa, 2007). This phenomenon is widely known as a bystander effect and is very well studied *in vitro* using cell culture models (Morgan and Sowa, 2007; Mothersill and Seymour, 2004). Recent studies have shown that bystander effects also operate *in vivo*, and that body part or cranial irradiation may exert deleterious changes in the distant protected somatic tissues (Koturbash et al., 2006a, 2007, 2008b). However, the potential of distant bystander damage to the germline has never been studied.

To investigate if paternal cranial irradiation can exert deleterious changes in the protected germline, we studied the accumulation of DNA damage and changes in DNA methylation in the shielded testes tissue. Here for the first time, we show that bystander effects indeed exist *in vivo* in the testes tissue upon the localized cranial exposure. We report that the localized paternal cranial irradiation results in a significant accumulation of unrepaired DNA damage and loss of global DNA methylation in the sperm cells.

## **MATERIALS AND METHODS**

### ***Animal exposure***

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

The cranial-exposed cohort (10 animals) received 20 Gy (3Gy/min) of X-rays (90kV, 5mA) to the hippocampal area of the skull, applied as two doses of 10Gy in two consecutive days. The rest of the animal body was protected by a ~3 mm thick lead shield, the same type as used for the human body protection in diagnostic radiology and as previously published (Koturbash et al., 2006a, 2007, 2008b). The protection of shielded ‘bystander’ tissue was complete, as verified by careful dosimetry using the RAD-CHECK™ monitor (Nuclear Associates div. of Victoreen, Inc, Carle Place, NY). Control rats (10 animals) were sham treated. For the sham treatment the animals were placed into the irradiator machine and completely shielded by lead. No radiation leakage through the shield occurred, as verified by dosimetry using the RAD-CHECK™ monitor. Another cohort of 10 animals was exposed to an approximate scatter dose of ~0.0012Gy, as determined by a Monte Carlo simulation and directly measured using the RAD-CHECK™ monitor.

Seven days after exposure, the rats were humanly sacrificed, testes were sampled upon the sacrifice and processed for a further analysis.

### ***DNA extraction***

DNA was extracted from testes tissues using a Qiagen DNAeasy kit (Qiagen), according to the manufacturer's instructions.

### ***DNA methylation analysis***

To monitor changes in DNA methylation, we employed the well-established cytosine extension assay, which measures the proportion of unmethylated CCGG sites in the genomic DNA. The assay was performed as described before (Pogribny et al., 2004; Pogribny et al., 1999; Raiche et al., 2004).

### ***DNA strand break measurement***

DNA strand breaks were detected using a modification of the random oligonucleotide-primed synthesis (ROPS) (Basnakian and James, 1996) assay, as described previously (Koturbash et al., 2008a).

### ***DNA damage analysis – histone $\gamma$ H2AX foci accumulation***

The testes 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 (Sedelnikova et al., 2007; Sedelnikova et al., 2003; Rogakou et al., 1998). Foci were counted by eye in a blinded fashion by two independent investigators. At least 100 cells (with an equal representation of each of the germ cell types) from the studied tissue of each animal were examined as recommended. The examinations were

repeated 3 times. The data are presented as a mean number of foci per cell  $\pm$  a standard error of the mean.

### ***Immunocytochemistry***

The testes 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 ATM, ATR and DNA-PK (Santa Cruz Biotechnology) and counter-stained with DAPI in accordance with the manufacturer's instructions.

### ***Immunohistochemistry***

Paraffin embedding and sectioning were conducted at Central Vet Labs, Edmonton, AB. IHC staining for  $\gamma$ H2AX was using anti- $\gamma$ H2AX antibodies (Cell Signalling) in accordance with the manufacturer's instructions.

### ***Sperm cell extraction and sperm DNA preparation***

Sperm DNA was prepared using procedures similar to those described by Yauk et al., 2002. Briefly, caudal epididymus was finely chopped in 1 ml of PBS, filtered and centrifuged. The sperm pellet was re-suspended in 1 $\times$ SSC, and somatic cells were lysed by addition of SDS to 0.15%. The lysate was centrifuged, and the resulting sperm pellet was re-suspended in 1 ml 0.2 $\times$ SSC, 1% SDS, 1 M 2-mercaptoethanol and digested with 200  $\mu$ g ml<sup>-1</sup> proteinase K (Sigma) for 1 h at 37 °C. The differential lysis of germline versus somatic cells was monitored by the

phase-contrast microscopy; all somatic cells appeared to be eliminated prior to the release of the sperm DNA. DNA was recovered after phenol/chloroform extraction by ethanol precipitation and re-dissolved in 5 mM Tris–HCl (pH 7.5).

### *Statistical analysis*

For the determination of the significance of the difference between the means, the Student's t-test with the Bonferroni correction for multiple comparisons was used. Statistical treatment and plotting of the results were performed using the Excel for Windows XP, and JMP 5.0 software.

## **RESULTS**

### *Induction of bystander effect in the testes tissue of rats subjected to cranial irradiation*

To simulate the brain irradiation scenario, we developed an animal model whereby the sexually mature Long Evans male rats were subjected to a localized 20 Gy X-ray exposure to the hippocampal area of the skull, while the rest of the body was completely protected by a medical grade shield. The lead shielding used for these studies was similar to one used for patients exposed to radiation in the clinic. The same shielding was used in the published studies on the bystander effect in mouse skin and in mouse and rat spleen (Koturbash et al., 2006a, 2007, 2008c).

We also considered the fact that even though the shielding of the animal body is complete, the X-rays can be reflected as they pass through the tissue, thus forming a small 'scatter' dose in the protected tissue (Koturbash et al., 2006a). To check if a

possible small scatter dose could contribute to the generation of bystander effects in testes, we exposed a separate cohort of animals to a scatter dose of ~0.0012Gy calculated according to the Monte Carlo simulation and directly measured by RAD-CHECK™ (Koturbash et al., 2006a, 2007, 2008b). Sham treated animals served as a control group.

#### ***Accumulation of DNA damage in the testes tissue of cranially exposed rats***

In our previous studies, we showed a pronounced induction of DNA damage and particularly DNA double-strand breaks (DSBs) in the bystander cells and tissues (Koturbash et al., 2006a; Sedelnikova et al., 2007; Koturbash et al., 2008b). In the light of these considerations, we studied if the cranial X-ray exposure induces DNA damage in the shielded whole testes tissue of control, cranial-exposed and scatter-exposed animals one week after irradiation. DNA strand breaks were measured using a modification of the random oligonucleotide primed synthesis (ROPS) assay (Basnakian and 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 incorporating radioactively labeled dNTPs. Therefore, the ROPS assay detects the physical DNA damage. Using

this assay we found that cranial exposure resulted in a significant ( $p < 0.05$ , Student's t-test with Bonferroni correction) ~1.6 fold accumulation of DNA strand breaks in the rat whole testes tissue that contains cells at different stages of spermatogenesis (Figure 4.0A).

***Bystander-mediated formation of  $\gamma$ H2AX foci in the testes of cranially exposed rats***

In order to further investigate the distribution of bystander DNA damage through the different stages of spermatogenesis, we employed an alternative approach for analyzing the occurrence of DSBs. We analyzed the presence of  $\gamma$ H2AX foci using the immunohistochemical (IHC) analysis. H2AX is a member of the H2A histone subfamily that is phosphorylated by ATM, ATR or DNA-PK at S139 ( $\gamma$ H2AX) as one of the earliest cellular responses to DSBs occurrence (Sedelnikova et al., 2007; Sedelnikova et al., 2003; Rogakou et al., 1998; Burdak-Rothkamm et al., 2007).  $\gamma$ H2AX accumulates in the nucleus in the vicinity of DSBs forming the  $\gamma$ H2AX foci. The number of DSBs correlates directly with the levels of  $\gamma$ H2AX foci which appear within minutes of radiation exposure. Notwithstanding, numerous studies have shown that a subset of DNA breaks and  $\gamma$ H2AX foci persist for many hours and even days (Rothkamm et al., 2003). The  $\gamma$ H2AX IHC was performed on the fresh touch-printed whole testes tissue and on the paraffin embedded testes and epididymis samples. This made it possible to quantify the  $\gamma$ H2AX foci levels in the whole tissue and to dissect the changes at various stages of spermatogenesis. We found that 1 week after cranial exposure the bystander  $\gamma$ H2AX foci formation was significantly increased, if evaluated in the whole testes tissue of head-exposed rats

(~3 times over control,  $p < 0.05$ , Figure 4.0B). No changes were observed in the testes tissue of the scatter-exposed rats.

While studying the distribution of the foci between the germ cells at different spermatogenesis stages, we noted that the most pronounced changes in the levels of  $\gamma$ H2AX foci were seen in the spermatogonia and spermatocytes (~3.5 times over control,  $p < 0.05$ ), and in elongating spermatids (~2.5 times over control,  $p < 0.05$ ), but not in the fully elongated spermatids and mature spermatozoa (Figure 4.0C-P).

### ***Cranial irradiation leads to unrepaired damage in the mature spermatozoa***

The fact that we did not detect  $\gamma$ H2AX foci in the mature spermatozoa constitutes an interesting and important finding. This could mean that spermatozoa have not suffered a bystander-mediated damage. Alternatively, mature spermatozoa that are known to be transcriptionally and translationally inactive probably lacked the activity of ATM, ATR or DNA-PK kinases that are capable of phosphorylating H2AX, and thus  $\gamma$ H2AX foci were not formed. Based on the crucial role of  $\gamma$ H2AX in DNA repair (Fillingham et al., 2006), the DNA strand breaks in the mature sperm cells might be left unrepaired.

To test for this, we extracted the spermatozoa and analyzed the levels of DNA damage in them using the ROPS assay that detects the presence of DNA lesions. The analysis revealed that the mature spermatozoa of the head-exposed animals had the significantly ( $p < 0.05$ ) elevated levels of DNA strand breaks (~1.6 fold as compared to control and scatter-exposed animals) (Figure 4.1A).

To test if the lack of the bystander-induced  $\gamma$ H2AX foci formation in the transcriptionally and translationally inactive mature spermatozoa was indeed due to the absence of ATM, ATR and DNA-PK expression we analyzed the levels of the aforementioned proteins using the fresh tissue touch-prints. We did not observe any ATM, ATR or DNA-PK expression in the mature sperm cells (Figure 4.1B-D). Therefore we concluded that the bystander-induced DNA damage was formed but not repaired in the mature sperm cells.

#### ***Loss of DNA methylation in the testes of cranially exposed rats***

It has been shown previously that the accumulation of DNA damage is accompanied by the loss of DNA methylation (Pogribny et al., 2004; Turk et al., 1995; Panayiotidis et al., 2004), and the bystander effects X-irradiation may be epigenetically mediated (Morgan, 2003a, b; Mothersill and Seymour, 2004; Koturbash et al., 2007, 2008; Wright and Coates, 2006). DNA methylation is an important epigenetic mechanism that safeguards genome stability of the cells, regulates gene expression and chromatin structure (Weber and Schubeler, 2007; Klose and Bird, 2006; Jaenish and Bird, 2003). The altered DNA methylation has deleterious consequences for the sperm cells (Aitken and De Iuliis, 2007). To test if changes in DNA methylation were observed in the bystander testes tissue, the level of global cytosine methylation was measured in the DNA extracted from the whole testes of un-irradiated, head-exposed and scatter-exposed animals 1 week after exposure. For this purpose, we employed the well-established HpaII-based cytosine extension assay which measures the proportion of unmethylated CCGG sites in

genomic DNA (Pogribny et al., 1999, 2004; Raiche et al., 2004; Koturbash et al., 2007). Using this approach, we found that cranial exposure to 20 Gy of X-rays led to a significant ( $p < 0.05$ ) ~1.3 fold decrease in the global DNA methylation in the bystander shielded testes tissue 1 week post-treatment (decreased methylation appears as an increase in 3H-dCTP incorporation; Figure 4.2A). No significant changes were observed in the testes tissue of the scatter-exposed animals (Figure 4.2A).

Additionally, we extracted DNA from the mature sperm cells. We found that the mature sperm cells of animals subjected to cranial exposure exhibited a significant ( $p < 0.05$ ) loss of global DNA methylation (Figure 4.2B). This loss of DNA methylation may be a result of elevated DNA damage levels (Turk et al., 1995; Panayiotidis et al., 2004).

## **DISCUSSION**

Approximately 5% of human live births today have a birth defect, de novo genetic disease or chromosomal abnormality (Wyrobek et al., 2007). Even having this knowledge, we still have little understanding of the impact of various genotoxic agents on the germline and thus on the progeny.

Of special concern is the lack of knowledge about the effects of the localized body part exposures that occur during radiation therapy. With cancer rates increasing in people of child-bearing age, the problem of potential deleterious effects of radiation treatment on the progeny of exposed patients becomes dramatic.

The effects of the preconception whole-body radiation exposure are well-documented to cause significant genome destabilization and carcinogenesis in the

unexposed progeny (Nomura, 2003; Dubrova 2003; Barber and Dubrova, 2006; Koturbash et al., 2006b). Knowing that radiation may affect distant bystander naïve cells, the potential of radiation to influence the distant protected germline cannot be excluded (Morgan and Sowa, 2007; Mothersill and Seymour, 2004).

In this study using an *in vivo* rat model, we have pioneered in revealing that cranial paternal exposure results in a very profound accumulation of DNA damage and loss of global DNA methylation in the germline completely shielded by a medical-grade shield. Specifically, we found that preconception cranial radiotherapy-like exposure leads to the pronounced accumulation of DNA strand breaks and DNA methylation changes in the mature sperm cells.

The increased level of DNA damage and decreased DNA methylation in the bystander sperm cells of rats subjected to cranial irradiation constitutes a novel and important finding. The deleterious effects of paternal irradiation are believed to be linked to the unrepaired damage to the sperm DNA. Sperm cells that harbor DNA damage and alter DNA methylation levels can affect the fertilization and embryo development and cause numerous deleterious effects within the offspring (Aitken and De Iuliis, 2007). Specifically, DNA damage and altered DNA methylation levels in the sperm cells may result in the altered epigenetic reprogramming after fertilization, and therefore may lead to the altered DNA methylation in the offspring.

DNA methylation is known to maintain genome stability and promote normal development and proliferation (Weber and Schubeler, 2007; Klose and Bird, 2006; Jaenisch and Bird, 2003). The altered DNA methylation is linked to the increased mutation rates and cancer (Weber and Schubeler, 2007; Klose and Bird, 2006;

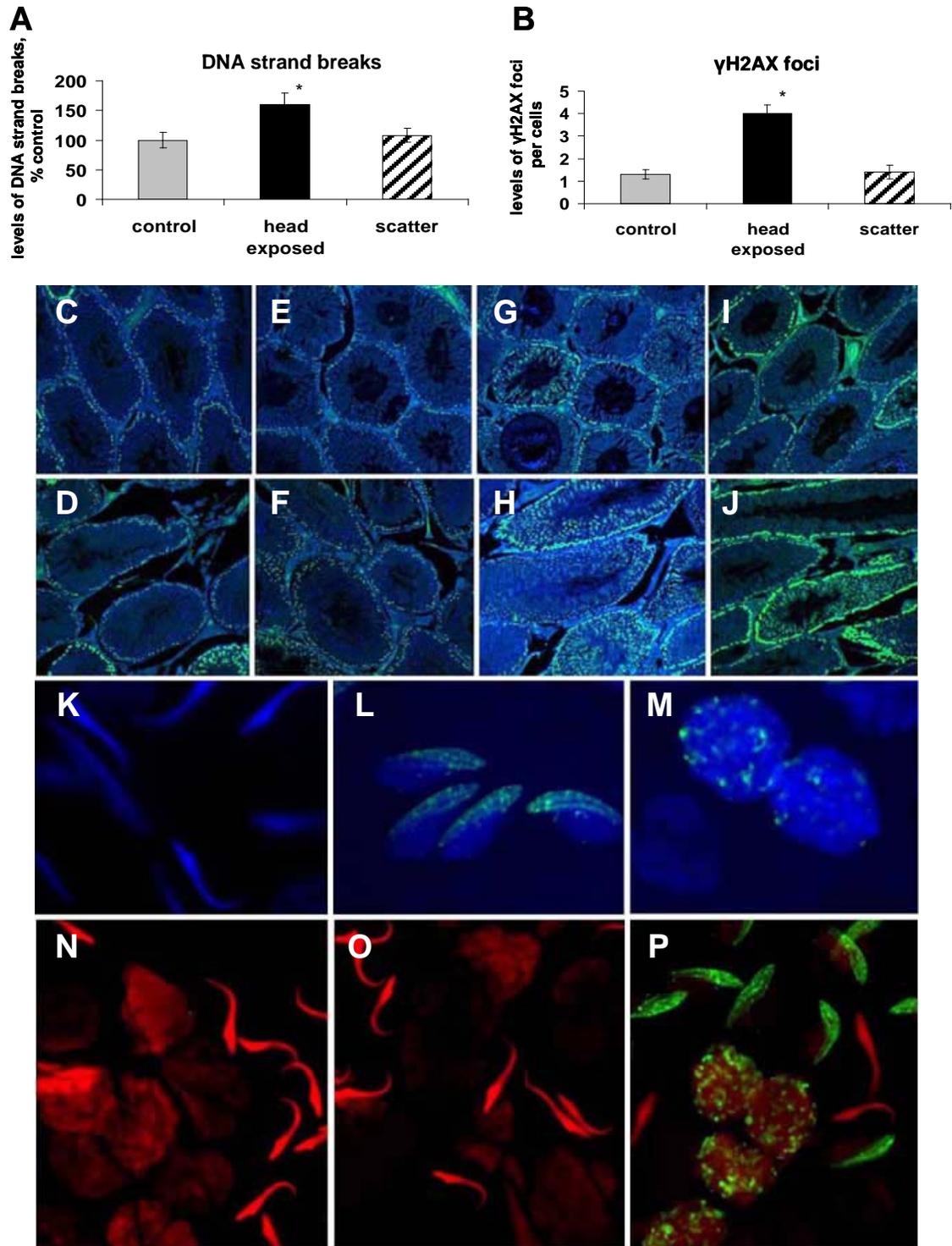
Jaenisch and Bird, 2003; Ehrlich, 2006), including hematological malignancies (Galm et al., 2006; Toyota and Issa, 2005).

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

Importantly, the germline effect shown here were not induced by the scatter irradiation, they constituted the real bystander effect.

Now the exact nature of a bystander signal needs to be determined. Most importantly, the mechanisms of the signal spread have to be identified. One plausible explanation is that the signal stems from the exposed blood cells and spreads via blood. Indeed, during the cranial exposure, a certain amount of blood cells is in the exposure field. The exposed cells can later undergo apoptosis or necrosis, thus releasing a variety of soluble factors that are small enough to cross the germline barrier. These can be cytokines or small regulatory RNAs. Both molecules were previously suggested to be putative bystander signals (Koturbash et al., 2007; Iyer et al., 2000). Interestingly, IR exposure was previously shown to result in the release of enigmatic soluble clastogenic factors into the circulating blood of the exposed individuals. These factors were proven to induce chromosome damage in the cultured cells (Hollowell and Littlefield, 1968; Pant and Kamada, 1977; Marozik et al., 2007). The bystander and clastogenic factors may have similar nature, yet their exact molecular identity still needs to be defined. However, notwithstanding what precisely the signal is and how it can damage the germline, the current study is the first to

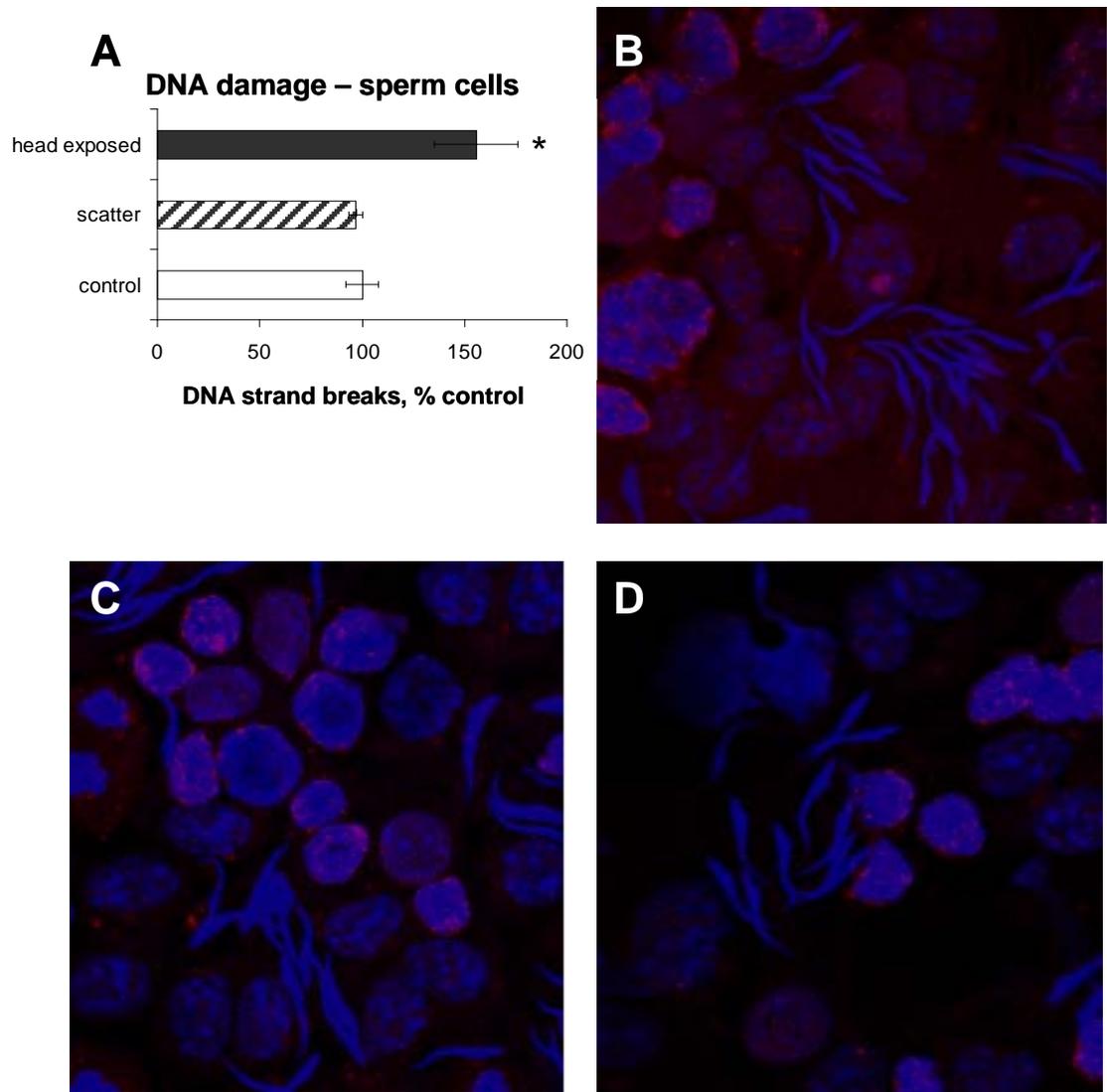
provide solid evidence that the localized cranial irradiation can damage the completely shielded germline.



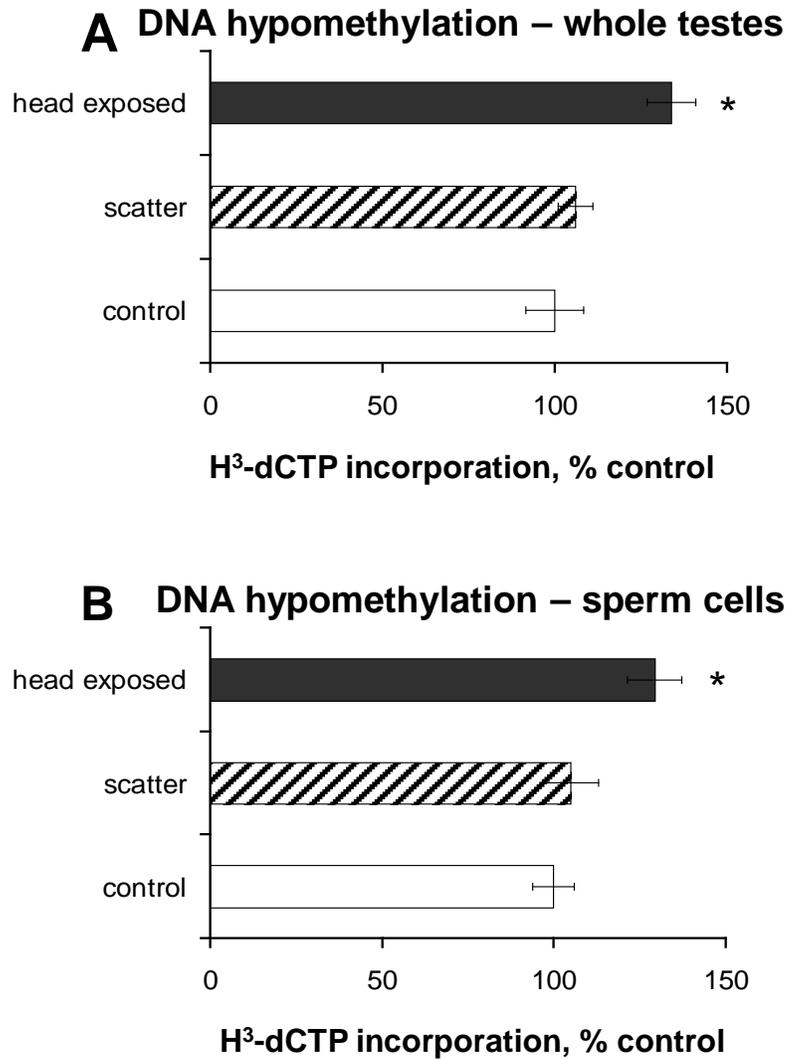
**Figure 4.0. Bystander-induced accumulation of DNA damage in the testes tissue of control, head-exposed and scattered dose exposed animals as studied by the ROPS assay and by the  $\gamma$ H2AX foci formation.**

**A.** Induction of DNA damage upon cranial radiation exposure in testes tissue as studied by the ROPS assay. Data are presented as mean values  $\pm$  SD, \* -  $p < 0.05$ ,

Student's t-test with Bonferroni correction for multiple comparison. **B.** Levels of  $\gamma$ H2AX foci in the testes cells of the control, head-exposed and scatter exposed rats. Data are presented as a number of  $\gamma$ H2AX foci per cell, mean values  $\pm$  SEM, n=400; \* -  $p < 0.05$ , Student's t-test with Bonferroni correction. **C.**  $\gamma$ H2AX foci in the control epididymis tissue. **D.**  $\gamma$ H2AX foci in the control testes tissue. **E.**  $\gamma$ H2AX foci in the epididymis tissue of the scatter-exposed animals. **F.**  $\gamma$ H2AX foci in the testes tissue of the scatter-exposed animals. **G** and **H.**  $\gamma$ H2AX foci in the epididymis tissue of the head-exposed animals. **I** and **J.**  $\gamma$ H2AX foci in the testes tissue of the head-exposed animals. **K.** Absence of  $\gamma$ H2AX foci in fully elongated spermatids and spermatozoa. **L.**  $\gamma$ H2AX foci in condensing spermatids, step 10. **M.**  $\gamma$ H2AX foci in pachytene spermatocytes and condensing spermatids. **N.**  $\gamma$ H2AX foci in the touch-printed testes tissue of the control animals. **O.**  $\gamma$ H2AX foci in the touch-printed testes tissue of the scatter-exposed animals. **P.**  $\gamma$ H2AX foci in the touch-printed testes tissue of the head-exposed animals.



**Figure 4.1 Bystander-mediated DNA damage in the mature sperm cells of cranially exposed rats.** **A.** Levels of DNA damage in the mature and fully condensed sperm cells of the control, head-exposed and scatter exposed animals as studied by the ROPS assay. Data are presented as mean values  $\pm$  SD, \* -  $p < 0.05$ , Student's t-test with Bonferroni correction. **B.** Lack of the ATM expression in the mature sperm cells, red-ATM, blue – DAPI. **C.** ATR levels in the mature sperm cells, red - ATR, blue – DAPI. **D.** DNA-PK expression in the mature sperm cells, red-DNA-PK, blue – DAPI.



**Figure 4.2. DNA hypomethylation in the whole rat testes and mature sperm cells after the localized cranial irradiation.** Levels of the global genome DNA methylation were measured by the HpaII cytosine extension assay. The results were expressed as a relative [3H]dCTP incorporation/1  $\mu$ g of DNA and as a percent change from control. Data are presented as mean values  $\pm$  SD, \* -  $p < 0.05$ , Student's t-test with Bonferroni correction. **A.** Whole testes. **B.** Mature sperm cells.

## **GENERAL DISCUSSION AND CONCLUSIONS**

Based on current incidence rates, almost 45% of men will develop cancer during their lifetime (Canadian Cancer Society, 2008). Even though cancer is primarily a disease of the aged, it frequently occurs in males of reproductive age. Fortunately, current advances in treatment regimens, such as radiation therapy (RT), have significantly lowered cancer mortality rates in men of all ages (Canadian Cancer Society, 2008). As a curative treatment and diagnostic modality for many human malignancies, ionizing radiation (IR) continues to be used in the clinical setting. However, IR is a potent damaging agent that can affect a variety of processes in directly exposed cells, in their descendents, and in neighboring, naïve unirradiated bystander cells.

Testis tissue is perhaps one of the most radiosensitive tissues. It can be damaged by direct irradiation or by scattered irradiation during treatment of adjacent tissues. Furthermore, the germline can also be damaged by enigmatic bystander signals originating from exposed body parts during localized irradiation (Tamminga et al., 2008).

Exposure to IR leads to genomic instability in the germline, and further to transgenerational genome instability in unexposed offspring of preconceptionally exposed parents.

The results presented here attempt to define, at least in part, molecular consequences of direct and indirect irradiation for the male germline. Particular emphasis is placed on epigenetic events in the germline, as the latter have been

recently implicated in transgeneration genome instability (reviewed in Kovalchuk and Baulch, 2008).

DNA methylation and miRNA are two important epigenetic mechanisms that safeguard genome stability of cells and regulate gene expression and chromatin structure. Altered DNA methylation and microRNAome have deleterious consequences for germ cells and may contribute to transgeneration genome instability. Likewise, accumulation of DNA damage caused by IR in conjunction with disrupted cellular regulation processes may also contribute to genome instability in the germline.

The aim of the current study is to dissect the role of DNA damage and epigenetic changes in the directly exposed and shielded bystander male germline.

**Three major findings of this thesis are:**

- 1) Exposure to IR leads to a significant accumulation of DNA damage that is accompanied by loss of global DNA methylation and microRNAome dysregulation in exposed rodent (murine) testes.
- 2) Localized cranial irradiation results in a significant accumulation of unrepaired DNA lesions and loss of global DNA methylation in the rodent (rat) germline. This effect was dose-dependent and less pronounced after lower doses of radiation in the murine germline.
- 3) A damage-induced and ATR/Rfx1-mediated increase of miR-709 expression in exposed testes may be a protective mechanism that effectively decreases a cellular level of the Brother of the Regulator of

Imprinted Sites (BORIS) to prevent massive aberrant erasure of DNA methylation after radiation exposure.

## **FUTURE DIRECTIONS**

The findings presented in this thesis characterize only a fraction of the biological consequences of IR exposure for the male germline. As mentioned, deleterious and protective responses of the germline genome and epigenome to genotoxic stress require further in-depth evaluation. Similarly, further studies are required to understand mechanisms of germline bystander effects and nature of bystander signals. The following are suggestions for future studies:

- 1) The current study characterized the role of a single miRNA in the germline of irradiated male mice. Further studies are required to define the role of the entire microRNAome in radiation response of the male germline. Furthermore, bystander tissues are known to exhibit altered levels of microRNAs and changes in expression levels of enzymes involved in microRNA maturation and microRNA-mediated translational inhibition (Koturbash et al., 2008c). Testes also exhibit a bystander microRNAome response; however, analysis is in the preliminary stages and requires further substantiation (Tamminga et al., unpublished). Another important question needs to be answered - is miRNA dysregulation a cause or a consequence of a bystander response?
- 2) The exact nature of the bystander signal needs to be determined. Most importantly, mechanisms of bystander signal spread have to be identified. A

plausible explanation is that the signal stems from exposed blood cells and spreads via blood. Indeed, during cranial exposure, a certain amount of blood cells are present in the exposure field. The exposed cells can later undergo apoptosis or necrosis, thus releasing a variety of soluble factors that are small enough to cross the germline barrier. The candidate molecules are numerous and may include reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Azzam et al., 2003; Lyng et al., 2002; Lyng et al., 2000; Mothersill and Seymour, 1998), short RNAs (Koturbash et al., 2007, 2008c), Ca<sup>2+</sup> ions (Lyng et al., 2006; Lyng et al., 2002; Lyng et al., 2000) and cytokines (Facoetti et al., 2006; Bonner, 2003; Iyer and Lehnert., 2000). It needs to be further established if anti-inflammatory responses mounted by the host immune system contribute to bystander signaling and bystander responses.

- 3) Normal and complete spermatogenesis is dependent on the hypothalamic-pituitary-gonadal (HPG) axis functioning normally. The HPG axis involves complex interactions between endocrine cells of the testes, such as the Leydig and Sertoli cells, and the pituitary gland. In the current study, we did not analyze the impact of radiation damage on the HPG axis and on the reported bystander effect in the shielded bystander germline. Radiation damage to the pituitary may affect the secretion of hormones critical for normal levels of sperm production. The question whether it is clastogenic in nature still needs to be answered.

- 4) Perhaps the most important problem remaining is the combined contribution of the observed germline DNA damage, hypomethylation and gene dysregulation to transgeneration genome instability. Dissecting molecular events that follow fertilization by radiation-damaged sperm would contribute significantly to better understanding of transgeneration genome instability. Further evaluation of the progeny of irradiated males would help uncover how epigenetic dysregulation in the exposed male germline influence destabilization of the genome of unexposed offspring.

## REFERENCES

- Aitken RJ, De Iuliis GN. Origins and consequences of DNA damage in male germ cells. *Reprod Biomed Online*. 2007; 14:727-33
- Amanai M, Brahmajosyula M, Perry AC. A restricted role for sperm-borne microRNAs in mammalian fertilization. *Biol Reprod*. 2006; 75:877-84
- American Cancer Society. *Cancer facts and figures 2003*. Atlanta (GA): American Cancer Society; 2003. p. 1-52
- Amundson SA, Fornace AJ Jr. Monitoring human radiation exposure by gene expression profiling: Possibilities and pitfalls. *Health Phys*. 2003; 85:36-42
- Andreev SG, Eidelman YA, Salnikov IV, Khvostunov IK. Mechanistic modelling of genetic and epigenetic events in radiation carcinogenesis. *Radiat Prot Dosimetry*. 2006; 122:335-9
- Azzam EI, de Toledo SM, Gooding T, Little JB. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiat Res*. 1998; 150:497-504.
- Azzam EI, de Toledo SM, Little JB. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. *Proc Natl Acad Sci U S A*. 2001; 98:473-8
- Azzam EI, de Toledo SM, Little JB. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. *Oncogene*. 2003; 22:7050-7
- Barber R, Plumb M, Smith AG, Cesar CE, Boulton E, Jeffreys AJ, Dubrova YE. No correlation between germline mutation at repeat DNA and meiotic crossover in male mice exposed to X-rays or cisplatin. *Mutat Res*. 2000; 457:79-91
- Barber R, Plumb MA, Boulton E, Roux I, Dubrova YE. Elevated mutation rates in the germ line of first- and second-generation offspring of irradiated male mice. *Proc Natl Acad Sci U S A*. 2002; 99:6877-82
- Barber RC, Dubrova YE. The offspring of irradiated parents, are they stable? *Mutat Res*. 2006; 598:50-60
- Barber RC, Hickenbotham P, Hatch T, Kelly D, Topchiy N, Almeida GM, Jones GD, Johnson GE, Parry JM, Rothkamm K, Dubrova YE. Radiation-induced transgenerational alterations in genome stability and DNA damage. *Oncogene*. 2006; 25:7336-42.

- Barcellos-Hoff MH. Integrative radiation carcinogenesis: interactions between cell and tissue responses to DNA damage. *Semin Cancer Biol.* 2005; 15:138-48
- Bartek J, Lukas C, Lukas J. Checking on DNA damage in S phase. *Nat Rev Mol Cell Biol.* 2004; 5:792-804
- Bartek J, Lukas J. DNA damage checkpoints: from initiation to recovery or adaptation. *Curr Opin Cell Biol.* 2007; 19:238-45
- Basnakian AG, James SJ. Quantification of 3'OH DNA breaks by random oligonucleotide-primed synthesis (ROPS) assay. *DNA Cell Biol.* 1996; 15:255-62
- Baulch JE, Li MW, Raabe OG. Effect of ATM heterozygosity on heritable DNA damage in mice following paternal F0 germline irradiation. *Mutat Res.* 2007; 616:34-45
- Baulch JE, Raabe OG, Wiley LM. Heritable effects of paternal irradiation in mice on signaling protein kinase activities in F3 offspring. *Mutagenesis.* 2001; 16:17-23
- Baulch JE, Raabe OG. Gamma irradiation of Type B spermatogonia leads to heritable genomic instability in four generations of mice. *Mutagenesis.* 2005; 20:337-43
- Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. *Genes Dev.* 2006; 20:1885-98
- Bellvé AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J Cell Biol.* 1977; 74:68-85
- Bernstein E, Allis CD. RNA meets chromatin. *Genes Dev.* 2005; 19:1635-55
- Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB, De Rooij DG. Apoptosis regulation in the testis: involvement of Bcl-2 family members. *Mol Reprod Dev.* 2000; 56:353-9
- Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell.* 2006; 125:1111-24
- Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev.* 2002; 16:6-21
- Bonner WM. Low-dose radiation: thresholds, bystander effects, and adaptive responses. *Proc Natl Acad Sci U S A.* 2003; 100:4973-5

Braun RE, Behringer RR, Peschon JJ, Brinster RL, Palmiter RD. Genetically haploid spermatids are phenotypically diploid. *Nature*. 1989; 337:373-6

Brooks AL, Retherford JC, McClellan RO. Effect of <sup>239</sup>PuO<sub>2</sub> particle number and size on the frequency and distribution of chromosome aberrations in the liver of the Chinese hamster. *Radiat Res*. 1974; 59:693-709

Brooks AL. Evidence for 'bystander effects' in vivo. *Hum Exp Toxicol*. 2004; 23:67-70

Brown RS. Zinc finger proteins: getting a grip on RNA. *Curr Opin Struct Biol*. 2005; 15:94-8

Burdak-Rothkamm S, Short SC, Folkard M, Rothkamm K, Prise KM. ATR-dependent radiation-induced gamma H2AX foci in bystander primary human astrocytes and glioma cells. *Oncogene*. 2007; 26:993-1002

Burrueel VR, Raabe OG, Wiley LM. In vitro fertilization rate of mouse oocytes with spermatozoa from the F1 offspring of males irradiated with 1.0 Gy <sup>137</sup>Cs gamma-rays. *Mutat Res*. 1997; 381:59-66

Callegari AJ, Kelly TJ. Shedding light on the DNA damage checkpoint. *Cell Cycle*. 2007; 6:660-6

Canadian Cancer Society/National Cancer Institute of Canada. *Canadian Cancer Statistics 2008*, Toronto, Canada; 2008. p. 1-106

Caron N, Veilleux S, Boissonneault G. Stimulation of DNA repair by the spermatidal TP1 protein. *Mol Reprod Dev*. 2001; 58:437-43

Chen PY, Meister G. microRNA-guided posttranscriptional gene regulation. *Biol Chem*. 2005; 386:1205-18

Chen T, Tsujimoto N, Li E. The PWWP domain of Dnmt3a and Dnmt3b is required for directing DNA methylation to the major satellite repeats at pericentric heterochromatin. *Mol Cell Biol*. 2004; 24:9048-58

Chuang JC, Jones PA. Epigenetics and microRNAs. *Pediatr Res*. 2007; 61:24R-29R

Chung SS, Sung W, Wang X, Wolgemuth DJ. Retinoic acid receptor alpha is required for synchronization of spermatogenic cycles and its absence results in progressive breakdown of the spermatogenic process. *Dev Dyn*. 2004; 230:754-66

Cordelli E, Fresegna AM, Leter G, Eleuteri P, Spanò M, Villani P. Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation. *Radiat Res*. 2003; 160:443-51

Criswell T, Leskov K, Miyamoto S, Luo G, Boothman DA. Transcription factors activated in mammalian cells after clinically relevant doses of ionizing radiation. *Oncogene*. 2003; 22:5813-27

D'Arcy V, Abdullaev ZK, Pore N, Docquier F, Torrano V, Chernukhin I, Smart M, Farrar D, Metodiev M, Fernandez N, Richard C, Delgado MD, Lobanenkov V, Klenova E. The potential of BORIS detected in the leukocytes of breast cancer patients as an early marker of tumorigenesis. *Clin Cancer Res*. 2006; 12:5978-86

Davis TL, Yang GJ, McCarrey JR, Bartolomei MS. The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. *Hum Mol Genet*. 2000; 9:2885-94

de Rooij DG, Grootegoed JA. Spermatogonial stem cells. *Curr Opin Cell Biol*. 1998; 10:694-701

de Rooij DG. Proliferation and differentiation of spermatogonial stem cells. *Reproduction*. 2001; 121:347-54

Djordjevic B. Bystander effects: a concept in need of clarification. *Bioessays*. 2000; 22:286-90

Dobrzyńska MM, Czajka U. Male-mediated developmental toxicity in mice after 8 weeks' exposure to low doses of X-rays. *Int J Radiat Biol*. 2005; 81:793-9

Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. *Genes Dev*. 2004; 18:504-11

Dubrova YE, Jeffreys AJ, Malashenko AM. Mouse minisatellite mutations induced by ionizing radiation. *Nat Genet*. 1993; 5:92-4

Dubrova YE, Plumb M, Brown J, Boulton E, Goodhead D, Jeffreys AJ. Induction of minisatellite mutations in the mouse germline by low-dose chronic exposure to gamma-radiation and fission neutrons. *Mutat Res*. 2000; 453:17-24

Dubrova YE, Plumb M, Brown J, Fennelly J, Bois P, Goodhead D, Jeffreys AJ. Stage specificity, dose response, and doubling dose for mouse minisatellite germ-line mutation induced by acute radiation. *Proc Natl Acad Sci U S A*. 1998; 95:6251-5

Dubrova YE, Plumb M, Gutierrez B, Boulton E, Jeffreys AJ. Transgenerational mutation by radiation. *Nature*. 2000b; 405:37

Dubrova YE. Radiation-induced transgenerational instability. *Oncogene*. 2003; 22:7087-93

Dunn KL, Davie JR. The many roles of the transcriptional regulator CTCF. *Biochem Cell Biol.* 2003; 81:161-7

Ehling UH, Favor J, Kratochvilova J, Neuhäuser-Klaus A. Dominant cataract mutations and specific-locus mutations in mice induced by radiation or ethylnitrosourea. *Mutat Res.* 1982; 92:181-92

Ehrlich M. Cancer-linked DNA hypomethylation and its relationship to hypermethylation. *Curr Top Microbiol Immunol.* 2006; 310:251-74

Emerit I, Levy A, Cernjavski L, Arutyunyan R, Oganessian N, Pogosian A, Mejlumian H, Sarkisian T, Gulkandian M, Quastel M, et al. Transferable clastogenic activity in plasma from persons exposed as salvage personnel of the Chernobyl reactor. *J Cancer Res Clin Oncol.* 1994; 120:558-61

Emerit I, Oganessian N, Sarkisian T, Arutyunyan R, Pogosian A, Asrian K, Levy A, Cernjavski L. Clastogenic factors in the plasma of Chernobyl accident recovery workers: anticlastogenic effect of Ginkgo biloba extract. *Radiat Res.* 1995; 144:198-205

Facoetti A, Ballarini F, Cherubini R, Gerardi S, Nano R, Ottolenghi A, Prise KM, Trott KR, Zilio C. Gamma ray-induced bystander effect in tumour glioblastoma cells: a specific study on cell survival, cytokine release and cytokine receptors. *Radiat Prot Dosimetry.* 2006; 122:271-4

Fawcett DW, Eddy EM, Phillips DM. Observations on the fine structure and relationships of the chromatoid body in mammalian spermatogenesis. *Biol Reprod.* 1970; 2:129-53

Fei P, El-Deiry WS. P53 and radiation responses. *Oncogene.* 2003; 22:5774–5783

Fillingham J, Keogh MC, Krogan NJ. GammaH2AX and its role in DNA double-strand break repair. *Biochem Cell Biol.* 2006; 84:568-77

Frankenberg-Schwager M. Induction, repair and biological relevance of radiation-induced DNA lesions in eukaryotic cells. *Radiat Environ Biophys.* 1990; 29:273-92

Galm O, Herman JG, Baylin SB. The fundamental role of epigenetics in hematopoietic malignancies. *Blood Rev.* 2006; 20: 1-13

Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ, Schier AF. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science.* 2006; 312:75-9

Goh K, Sumner H. Breaks in normal human chromosomes: are they induced by a transferable substance in the plasma of persons exposed to total-body irradiation?

Radiat Res. 1968; 35:171-81

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. NAR 2008; 36: D154-D158

Greenbaum MP, Ma L, Matzuk MM. Conversion of midbodies into germ cell intercellular bridges. Dev Biol. 2007; 305:389-96

Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005; 123:631-40

Grootegeod JA, Siep M, Baarends WM. Molecular and cellular mechanisms in spermatogenesis. Baillieres Best Pract Res Clin Endocrinol Metab. 2000; 14:331-43

Haines GA, Hendry JH, Daniel CP, Morris ID. Germ cell and dose-dependent DNA damage measured by the comet assay in murine spermatozoa after testicular X-irradiation. Biol Reprod. 2002; 67:854-61

Haines GA, Hendry JH, Daniel CP, Morris ID. Increased levels of comet-detected spermatozoa DNA damage following in vivo isotopic- or X-irradiation of spermatogonia. Mutat Res. 2001; 495:21-32

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA. Epigenetic reprogramming in mouse primordial germ cells. Mech Dev. 2002; 117:15-23

Hamada N, Matsumoto H, Hara T, Kobayashi Y. Intercellular and intracellular signaling pathways mediating ionizing radiation-induced bystander effects. J Radiat Res. 2007; 48:87-95

Hamer G, Roepers-Gajadien HL, Gademan IS, Kal HB, De Rooij DG. Intercellular bridges and apoptosis in clones of male germ cells. Int J Androl. 2003; 26:348-53

Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004; 18:3016-27

Harrouk W, Codrington A, Vinson R, Robaire B, Hales BF. Paternal exposure to cyclophosphamide induces DNA damage and alters the expression of DNA repair genes in the rat preimplantation embryo. Mutat Res. 2000; 461:229-41

Hasegawa M, Zhang Y, Niibe H, Terry NH, Meistrich ML. Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice. Radiat Res. 1998; 149:263-70

- Hatch T, Derijck AA, Black PD, van der Heijden GW, de Boer P, Dubrova YE. Maternal effects of the scid mutation on radiation-induced transgenerational instability in mice. *Oncogene*. 2007; 26:4720-4
- Hawkins RB. The influence of concentration of DNA on the radiosensitivity of mammalian cells. *Int J Radiat Oncol Biol Phys*. 2005; 63:529-35
- Hayashi K, Chuva de Sousa Lopes SM, Kaneda M, Tang F, Hajkova P, Lao K, O'Carroll D, Das PP, Tarakhovsky A, Miska EA, Surani MA. MicroRNA Biogenesis Is Required for Mouse Primordial Germ Cell Development and Spermatogenesis. *PLoS ONE*. 2008; 3:e1738
- Hecht NB. Molecular mechanisms of male germ cell differentiation. *BioEssays*. 1998; 20:555-61
- Henriksen K, Kulmala J, Toppari J, Mehrotra K, Parvinen M. Stage-specific apoptosis in the rat seminiferous epithelium: quantification of irradiation effects. *J Androl*. 1996; 17:394-402
- Hess RA, Schaeffer DJ, Eroschenko VP, Kenn JE. Frequency of the stages in the cycle of the seminiferous epithelium in the rat. *Biol Reprod*. 1990; 43:517-24
- Hess RA. Spermatogenesis, Overview. *Encyclopedia of Reproduction*. 1999; 4: 539-45
- Hiura H, Obata Y, Komiyama J, Shirai M, Kono T. Oocyte growth-dependent progression of maternal imprinting in mice. *Genes Cells*. 2006; 11:353-61
- Hoffmann MJ, Müller M, Engers R, Schulz WA. Epigenetic control of CTCFL/BORIS and OCT4 expression in urogenital malignancies. *Biochem Pharmacol*. 2006; 72:1577-88
- Hollowell JG, Littlefield LG. Chromosome damage induced by plasma of x-ray patients: an indirect effect of X-ray. *Proc Soc Exp Biol Med*. 1968; 129:240-3
- Holstein AF, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. *Reprod Biol Endocrinol*. 2003; 1:107
- Hong JA, Kang Y, Abdullaev Z, Flanagan PT, Pack SD, Fischette MR, Adnani MT, Loukinov DI, Vatolin S, Risinger JI, Custer M, Chen GA, Zhao M, Nguyen DM, Barrett JC, Lobanenkov VV, Schrupp DS. Reciprocal binding of CTCF and BORIS to the NY-ESO-1 promoter coincides with derepression of this cancer-testis gene in lung cancer cells. *Cancer Res*. 2005; 65:7763-74
- Howell SJ, Shalet SM. Spermatogenesis after cancer treatment: damage and recovery. *J Natl Cancer Inst Monogr*. 2005: 12-7

- Huckins C. Spermatogonial intercellular bridges in whole-mounted seminiferous tubules from normal and irradiated rodent testes. *Am J Anat.* 1978; 153:97-121
- Huff JS. 2007. Neoplasms, brain. <http://www.emedicine.com/emerg/topic334.htm>
- Hurley PJ, Bunz F. ATM and ATR: components of an integrated circuit. *Cell Cycle.* 2007; 6:414-7
- Iliakis G, Wang Y, Guan J, Wang H. DNA damage checkpoint control in cells exposed to ionizing radiation. *Oncogene.* 2003; 22:5834-47
- Itoh M, Terayama H, Naito M, Ogawa Y, Tainosho S. Tissue microcircumstances for leukocytic infiltration into the testis and epididymis in mice. *J Reprod Immunol.* 2005; 67:57-67
- Iyer R, Lehnert BE, Svensson R. Factors underlying the cell growth-related bystander responses to alpha particles. *Cancer Res.* 2000; 60:1290-8
- Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet.* 2003; 33:245-54
- Jeggo P, Lobrich M. Radiation-induced DNA damage responses. *Radiat Prot Dosimetry.* 2006; 122:124-7
- Jelinic P, Stehle JC, Shaw P. The testis-specific factor CTCFL cooperates with the protein methyltransferase PRMT7 in H19 imprinting control region methylation. *PLoS Biol.* 2006; 4:e355
- Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. *Nat Rev Genet.* 2007; 8:253-62
- Johnson L, Mckenzie KS, Snell JR. Partial wave in human seminiferous tubules appears to be a random occurrence. *Tissue Cell.* 1996; 28:12 7-36
- Johnson L. A new approach to study the architectural arrangement of spermatogenic stages revealed little evidence of a partial wave along the length of human seminiferous tubules. *J Androl.* 1994; 15:435-41
- Joshi DS, Yick J, Murray D, Meistrich ML. Stage-dependent variation in the radiosensitivity of DNA in developing male germ cells. *Radiat Res.* 1990; 121:274-81
- Kangasniemi M, Huhtaniemi I, Meistrich ML. Failure of spermatogenesis to recover despite the presence of a spermatogonia in the irradiated LBNF1 rat. *Biol Reprod.* 1996; 54:1200-8

- Kerjean A, Dupont JM, Vasseur C, Le Tessier D, Cuisset L, Paldi A, Jouannet P, Jeanpierre M. Establishment of the paternal methylation imprint of the human H19 and MEST/PEG1 genes during spermatogenesis. *Hum Mol Genet.* 2000; 9:2183-7
- Khan MA, Hill RP, Van Dyk J. Partial volume rat lung irradiation: an evaluation of early DNA damage. *Int J Radiat Oncol Biol Phys.* 1998; 40:467-76
- Khan MA, Van Dyk J, Yeung IW, Hill RP. Partial volume rat lung irradiation; assessment of early DNA damage in different lung regions and effect of radical scavengers. *Radiother Oncol.* 2003; 66:95-102
- Kierszenbaum AL. Transition nuclear proteins during spermiogenesis: unrepaired DNA breaks not allowed. *Mol Reprod Dev.* 2001; 58:357-8
- Klenova EM, Morse HC 3rd, Ohlsson R, Lobanenko VV. The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer. *Semin Cancer Biol.* 2002; 12:399-414
- Klose RJ, Bird AP. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci.* 2006; 31:89-97
- Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science.* 1995; 270:96-9
- Kotaja N, Bhattacharyya SN, Jaskiewicz L, Kimmins S, Parvinen M, Filipowicz W, Sassone-Corsi P. The chromatoid body of male germ cells: similarity with processing bodies and presence of dicer and microRNA pathway components. *Proc Natl Acad Sci U S A.* 2006; 103:2647-52
- Kotaja N, Sassone-Corsi P. The chromatoid body: a germ-cell-specific RNA-processing centre. *Nat Rev Mol Cell Biol.* 2007; 8:85-90
- Koturbash I, Baker M, Loree J, Kutanzi K, Hudson D, Pogribny I, Sedelnikova O, Bonner W, Kovalchuk O. Epigenetic dysregulation underlies radiation-induced transgenerational genome instability in vivo. *Int J Radiat Oncol Biol Phys.* 2006b; 66:327-30
- Koturbash I, Boyko A, Rodriguez-Juarez R, McDonald RJ, Tryndyak VP, Kovalchuk I, Pogribny IP, Kovalchuk O. Role of epigenetic effectors in maintenance of the long-term persistent bystander effect in spleen in vivo. *Carcinogenesis.* 2007; 28:1831-8
- Koturbash I, Kutanzi K, Hendrickson K, Rodriguez-Juarez R, Kogosov D, Kovalchuk O. Radiation-induced bystander effects in vivo are sex specific. *Mutat Res.* 2008a; 642:28-36

Koturbash I, Loree J, Kutanzi K, Koganow C, Pogribny I, Kovalchuk O. 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.* 2008b; 70:554-62

Koturbash I, Pogribny I, Kovalchuk O. Stable loss of global DNA methylation in the radiation-target tissue--a possible mechanism contributing to radiation carcinogenesis? *Biochem Biophys Res Commun.* 2005; 337:526-33

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

Koturbash I, Zemp FJ, Kutanzi K, Luzhna L, Loree J, Kolb B, Kovalchuk O. Sex-specific microRNAome deregulation in the shielded bystander spleen of cranially exposed mice. *Cell Cycle.* 2008c; 7:1658-67

Kouprina N, Noskov VN, Pavlicek A, Collins NK, Schoppee Bortz PD, Ottolenghi C, Loukinov D, Goldsmith P, Risinger JI, Kim JH, Westbrook VA, Solomon G, Sounders H, Herr JC, Jurka J, Lobanenkov V, Schlessinger D, Larionov V. Evolutionary diversification of SPANX-N sperm protein gene structure and expression. *PLoS ONE.* 2007; 2:e359

Kovalchuk O, Baulch JE. Epigenetic changes and nontargeted radiation effects--is there a link? *Environ Mol Mutagen.* 2008; 49:16-25

Kovalchuk O, Burke P, Besplug J, Slovack M, Filkowski J, Pogribny I. Methylation changes in muscle and liver tissues of male and female mice exposed to acute and chronic low-dose X-ray-irradiation. *Mutat Res.* 2004; 548:75-84

La Salle S, Mertineit C, Taketo T, Moens PB, Bestor TH, Trasler JM. Windows for sex-specific methylation marked by DNA methyltransferase expression profiles in mouse germ cells. *Dev Biol.* 2004; 268:403-15

La Salle S, Oakes CC, Neaga OR, Bourc'his D, Bestor TH, Trasler JM. Loss of spermatogonia and wide-spread DNA methylation defects in newborn male mice deficient in DNMT3L. *BMC Dev Biol.* 2007; 7:104

La Salle S, Trasler JM. Dynamic expression of DNMT3a and DNMT3b isoforms during male germ cell development in the mouse. *Dev Biol.* 2006; 296:71-82

Lehnert BE, Goodwin EH, Deshpande A. Extracellular factor(s) following exposure to alpha particles can cause sister chromatid exchanges in normal human cells. *Cancer Res.* 1997; 57:2164-71

Lehnert BE, Goodwin EH. A new mechanism for DNA alterations induced by alpha particles such as those emitted by radon and radon progeny. *Environ Health Perspect.* 1997; 105:1095-101

Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet.* 2002; 3:662-73

Little JB. Radiation carcinogenesis. *Carcinogenesis.* 2000; 21:397-404

Lorimore SA, Coates PJ, Wright EG. Radiation-induced genomic instability and bystander effects: inter-related nontargeted effects of exposure to ionizing radiation. *Oncogene.* 2003; 22:7058-69

Loukinov DI, Pugacheva E, Vatolin S, Pack SD, Moon H, Chernukhin I, Mannan P, Larsson E, Kanduri C, Vostrov AA, Cui H, Niemitz EL, Rasko JE, Docquier FM, Kistler M, Breen JJ, Zhuang Z, Quitschke WW, Renkawitz R, Klenova EM, Feinberg AP, Ohlsson R, Morse HC 3rd, Lobanenko VV. BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma. *Proc Natl Acad Sci U S A.* 2002; 99:6806-11

Lyng FM, Maguire P, McClean B, Seymour C, Mothersill C. The involvement of calcium and MAP kinase signaling pathways in the production of radiation-induced bystander effects. *Radiat Res.* 2006; 165:400-9

Lyng FM, Seymour CB, Mothersill C. Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: a possible mechanism for bystander-induced genomic instability? *Radiat Res.* 2002; 157:365-70

Lyng FM, Seymour CB, Mothersill C. Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *Br J Cancer.* 2000; 83:1223-30

Lyons MK, Vora SA. Brain tumors: current issues in diagnosis and management. *Semin Neurol.* 2007; 27:312-24

Magelssen H, Brydøy M, Fosså SD. The effects of cancer and cancer treatments on male reproductive function. *Nat Clin Pract Urol.* 2006; 3:312-22

Marchal R, Chicheportiche A, Dutrillaux B, Bernardino-Sgherri J. DNA methylation in mouse gametogenesis. *Cytogenet Genome Res.* 2004; 105:316-24

Marchetti F, Essers J, Kanaar R, Wyrobek AJ. Disruption of maternal DNA repair increases sperm-derived chromosomal aberrations. *Proc Natl Acad Sci U S A.* 2007; 104:17725-9

- Marchetti F, Wyrobek AJ. DNA repair decline during mouse spermiogenesis results in the accumulation of heritable DNA damage. *DNA Repair (Amst)*. 2008; 7:572-81
- Marcon E, Babak T, Chua G, Hughes T, Moens PB. miRNA and piRNA localization in the male mammalian meiotic nucleus. *Chromosome Res*. 2008; 16:243-60
- Marozik P, Mothersill C, Seymour CB, Mosse I, Melnov S. Bystander effects induced by serum from survivors of the Chernobyl accident. *Exp Hematol*. 2007; 35: 55-63
- Mayer W, Niveleau A, Walter J, Fundele R, Haaf T. Demethylation of the zygotic paternal genome. *Nature*. 2000; 403:501-2
- Meistrich ML. Critical components of testicular function and sensitivity to disruption. *Biol Reprod*. 1986; 34:17-28
- Mohr U, Dasenbrock C, Tillmann T, Kohler M, Kamino K, Hagemann G, Morawietz G, Campo E, Cazorla M, Fernandez P, Hernandez L, Cardesa A, Tomatis L. Possible carcinogenic effects of X-rays in a transgenerational study with CBA mice. *Carcinogenesis*. 1999; 20:325-32
- Morgan WF, Sowa MB. Non-targeted bystander effects induced by ionizing radiation. *Mutat Res*. 2007; 616:159-64
- Morgan WF. Is there a common mechanism underlying genomic instability, bystander effects and other nontargeted effects of exposure to ionizing radiation? *Oncogene*. 2003c; 22:7094-9
- Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects in vivo, clastogenic factors and transgenerational effects. *Radiat Res*. 2003a; 159:581-96
- Morgan WF. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. *Radiat Res*. 2003b; 159:567-80
- Mothersill C, Seymour CB. Mechanisms and implications of genomic instability and other delayed effects of ionizing radiation exposure. *Mutagenesis*. 1998; 13:421-6
- Mothersill C, Seymour CB. Radiation-induced bystander effects--implications for cancer. *Nat Rev Cancer*. 2004; 4:158-64
- Mruk DD and Cheng CY. Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. *Endocr Rev*. 2004; 25:747-806

- Müller WU, Streffer C, Wojcik A, Niedereichholz F. Radiation-induced malformations after exposure of murine germ cells in various stages of spermatogenesis. *Mutat Res.* 1999; 425:99-106
- Murphy JB, Morton JJ. The Lymphocyte as a Factor in Natural and Induced Resistance to Transplanted Cancer. *Proc Natl Acad Sci U S A.* 1915; 1:435-7
- Niwa O, Kominami R. Untargeted mutation of the maternally derived mouse hypervariable minisatellite allele in F1 mice born to irradiated spermatozoa. *Proc Natl Acad Sci U S A.* 2001; 98:1705-10
- Nomura T. Transgenerational carcinogenesis: induction and transmission of genetic alterations and mechanisms of carcinogenesis. *Mutat Res.* 2003; 544:425-32
- Nomura T. Transgenerational effects of radiation and chemicals in mice and humans. *J Radiat Res (Tokyo).* 2006; 47: B83-97
- Oakes CC, La Salle S, Smiraglia DJ, Robaire B, Trasler JM. Developmental acquisition of genome-wide DNA methylation occurs prior to meiosis in male germ cells. *Dev Biol.* 2007; 307:368-79
- Ohtani N, Yamakoshi K, Takahashi A, Hara E. The p16INK4a-RB pathway: molecular link between cellular senescence and tumor suppression. *J Med Invest.* 2004; 51:146-53
- Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. *Nat Genet.* 1997; 17:275-6
- Ostermeier GC, Goodrich RJ, Moldenhauer JS, Diamond MP, Krawetz SA. A suite of novel human spermatozoal RNAs. *J Androl.* 2005; 26:70-4
- Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA. Reproductive biology: delivering spermatozoan RNA to the oocyte. *Nature.* 2004; 429:154
- Oswald J, Engemann S, Lane N, Mayer W, Olek A, Fundele R, Dean W, Reik W, Walter J. Active demethylation of the paternal genome in the mouse zygote. *Curr Biol.* 2000; 10:475-8
- Panayiotidis MI, Rancourt RC, Allen CB, Riddle SR, Schneider BK, Ahmad S, White CW. Hyperoxia-induced DNA damage causes decreased DNA methylation in human lung epithelial-like A549 cells. *Antioxid Redox Signal.* 2004; 6:129-36
- Pant GS, Kamada N. Chromosome aberrations in normal leukocytes induced by the plasma of exposed individuals. *Hiroshima J Med Sci.* 1977; 26:149-54

Parreira GG, Melo RC, Russell LD. Relationship of sertoli-sertoli tight junctions to ectoplasmic specialization in conventional and en face views. *Biol Reprod.* 2002; 67:1232-41

Parsons WB, Watkins CH, Pease GL, Childs DS. Changes in sternal marrow following roentgen-ray therapy to the spleen in chronic granulocytic leukemia. *Cancer.* 1954; 7:179-89

Pasquinelli AE, Hunter S, Bracht J. MicroRNAs: a developing story. *Curr Opin Genet Dev.* 2005; 15:200-5

Penttilä TL, Yuan L, Mali P, Höög C, Parvinen M. Haploid gene expression: temporal onset and storage patterns of 13 novel transcripts during rat and mouse spermiogenesis. *Biol Reprod.* 1995; 53:499-510

Petersen C, Soder O. The sertoli cell--a hormonal target and 'super' nurse for germ cells that determines testicular size. *Horm Res.* 2006; 66:153-61

Pogribny I, Koturbash I, Tryndyak V, Hudson D, Stevenson SM, Sedelnikova O, Bonner W, Kovalchuk O. Fractionated low-dose radiation exposure leads to accumulation of DNA damage and profound alterations in DNA and histone methylation in the murine thymus. *Mol Cancer Res.* 2005; 3:553-61

Pogribny I, Raiche J, Slovack M, Kovalchuk O. Dose-dependence, sex- and tissue-specificity, and persistence of radiation-induced genomic DNA methylation changes. *Biochem Biophys Res Commun.* 2004; 320:1253-61

Pogribny I, Yi P, James SJ. A sensitive new method for rapid detection of abnormal methylation patterns in global DNA and within CpG islands. *Biochem Biophys Res Commun.* 1999; 262:624-8

Powell SN, Kachnic LA. Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation. *Oncogene.* 2003; 22:5784-91

Presutti C, Rosati J, Vincenti S, Nasi S. Non coding RNA and brain. *BMC Neurosci.* 2006; 7:S5

Price TM, Murphy SK, Younglai EV. Perspectives: the possible influence of assisted reproductive technologies on transgenerational reproductive effects of environmental endocrine disruptors. *Toxicol Sci.* 2007; 96:218-26

Raiche J, Rodriguez-Juarez R, Pogribny I, Kovalchuk O. Sex- and tissue-specific expression of maintenance and de novo DNA methyltransferases upon low dose X-irradiation in mice. *Biochem Biophys Res Commun.* 2004; 325:39-47

- Rassoulzadegan M, Grandjean V, Gounon P, Vincent S, Gillot I, Cuzin F. RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. *Nature*. 2006; 441:469-74
- Renaud S, Pugacheva EM, Delgado MD, Braunschweig R, Abdullaev Z, Loukinov D, Benhattar J, Lobanekov V. Expression of the CTCF-paralogous cancer-testis gene, brother of the regulator of imprinted sites (BORIS), is regulated by three alternative promoters modulated by CpG methylation and by CTCF and p53 transcription factors. *Nucleic Acids Res*. 2007; 35:7372-88
- Richardson C, Stark JM, Ommundsen M, Jasin M. Rad51 overexpression promotes alternative double-strand break repair pathways and genome instability. *Oncogene*. 2004; 23:546-53
- Risinger JI, Chandramouli GV, Maxwell GL, Custer M, Pack S, Loukinov D, Aprelikova O, Litzi T, Schrupp DS, Murphy SK, Berchuck A, Lobanekov V, Barrett JC. Global expression analysis of cancer/testis genes in uterine cancers reveals a high incidence of BORIS expression. *Clin Cancer Res*. 2007; 13:1713-9
- Ro S, Park C, Sanders KM, McCarrey JR, Yan W. Cloning and expression profiling of testis-expressed microRNAs. *Dev. Biol*. 2007; 311:592-602
- Robertson KD, Jones PA. DNA methylation: past, present and future directions. *Carcinogenesis*. 2000; 21:461-7
- Rodemann HP, Blaese MA. Responses of normal cells to ionizing radiation. *Semin Radiat Oncol*. 2007; 17:81-8
- Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. *Genome Res*. 2004; 14:1902-10
- Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem*. 1998; 273:5858-68
- Rogaev EI. Small RNAs in human brain development and disorders. *Biochemistry (Mosc)*. 2005; 70:1404-7
- Rothkamm K, Lobrich M. Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. *Proc Natl Acad Sci U S A*. 2003; 100:5057-62
- Rougier N, Bourc'his D, Gomes DM, Niveleau A, Plachot M, Paldi A, Viegas-Péquignot E. Chromosome methylation patterns during mammalian preimplantation development. *Genes Dev*. 1998; 12:2108-13

Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK, Khochbin S. Establishment of male-specific epigenetic information. *Gene*. 2005; 345:139-53

Rowley MJ, Leach DR, Warner GA, Heller CG. Effect of graded doses of ionizing radiation on the human testis. *Radiat Res*. 1974; 59:665-78

Russell LD, Chiarini-Garcia H, Korsmeyer SJ, Knudson CM. Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. *Biol Reprod*. 2002; 66:950-8

Santos F, Hendrich B, Reik W, Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Dev Biol*. 2002; 241:172-82

Schulze W. Evidence of a wave of spermatogenesis in human testis. *Andrologia*. 1982; 14:200-7

Sedelnikova OA, Nakamura A, Kovalchuk O, Koturbash I, Mitchell SA, Marino SA, Brenner DJ, Bonner WM. DNA double-strand breaks form in bystander cells after microbeam irradiation of three-dimensional human tissue models. *Cancer Res*. 2007; 67:4295-302

Sedelnikova OA, Pilch DR, Redon C, Bonner WM. Histone H2AX in DNA damage and repair. *Cancer Biol Ther*. 2003; 2:233-5

Shimura T, Inoue M, Taga M, Shiraishi K, Uematsu N, Takei N, Yuan ZM, Shinohara T, Niwa O. p53-dependent S-phase damage checkpoint and pronuclear cross talk in mouse zygotes with X-irradiated sperm. *Mol Cell Biol*. 2002a; 22:2220-8

Shimura T, Toyoshima M, Taga M, Shiraishi K, Uematsu N, Inoue M, Niwa O. The novel surveillance mechanism of the Trp53-dependent s-phase checkpoint ensures chromosome damage repair and preimplantation-stage development of mouse embryos fertilized with x-irradiated sperm. *Radiat Res*. 2002b; 158:735-42

Shiraishi K, Shimura T, Taga M, Uematsu N, Gondo Y, Ohtaki M, Kominami R, Niwa O. Persistent induction of somatic reversions of the pink-eyed unstable mutation in F1 mice born to fathers irradiated at the spermatozoa stage. *Radiat Res*. 2002; 157:661-7

Shivdasani RA. MicroRNAs: regulators of gene expression and cell differentiation. *Blood*. 2006; 108:3646-53

Sonoda E, Sasaki MS, Buerstedde JM, Bezzubova O, Shinohara A, Ogawa H, Takata M, Yamaguchi-Iwai Y, Takeda S. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. *EMBO J*. 1998; 17:598-608

Sowa M, Arthurs BJ, Estes BJ, Morgan WF. Effects of ionizing radiation on cellular structures, induced instability and carcinogenesis. *EXS*. 2006; 293-301

Sprando RL, Russell LD. Comparative study of cytoplasmic elimination in spermatids of selected mammalian species. *Am J Anat*. 1987; 178:72-80

Stokes MP, Michael WM. A novel replication arrest pathway in response to DNA damage. *Cell Cycle*. 2004; 3:126-7

Suzuki K, Ojima M, Kodama S, Watanabe M. Radiation-induced DNA damage and delayed induced genomic instability. *Oncogene*. 2003; 22:6988-93

Tammaing J, Koturbash I, Baker M, Kutanzi K, Kathiria P, Pogribny IP, Sutherland RJ, Kovalchuk O. Paternal cranial irradiation induces distant bystander DNA damage in the germline and leads to epigenetic alterations in the offspring. *Cell Cycle*. 2008; 7:1238-45

Tang G. siRNA and miRNA: an insight into RISCs. *Trends Biochem Sci*. 2005; 30:106-14

Tawa R, Kimura Y, Komura J, Miyamura Y, Kurishita A, Sasaki MS, Sakurai H, Ono T. Effects of X-ray irradiation on genomic DNA methylation levels in mouse tissues. *J Radiat Res (Tokyo)*. 1998; 39:271-8

Thacker J. The RAD51 gene family, genetic instability and cancer. *Cancer Lett*. 2005; 219:125-35

Timmons PM, Rigby PW, Poirier F. The murine seminiferous epithelial cycle is pre-figured in the Sertoli cells of the embryonic testis. *Development*. 2002; 129:635-47

Toyota M, Issa JP. Epigenetic changes in solid and hematopoietic tumors. *Semin Oncol*. 2005; 32:521-30

Turk PW, Laayoun A, Smith SS, Weitzman SA. DNA adduct 8-hydroxyl-2'-deoxyguanosine (8-hydroxyguanine) affects function of human DNA methyltransferase. *Carcinogenesis*. 1995; 16:1253-5

Ueda T, Abe K, Miura A, Yuzuriha M, Zubair M, Noguchi M, Niwa K, Kawase Y, Kono T, Matsuda Y, Fujimoto H, Shibata H, Hayashizaki Y, Sasaki H. The paternal methylation imprint of the mouse H19 locus is acquired in the gonocyte stage during foetal testis development. *Genes Cells*. 2000; 5:649-59

Ulaner GA, Vu TH, Li T, Hu JF, Yao XM, Yang Y, Gorlick R, Meyers P, Healey J, Ladanyi M, Hoffman AR. Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. *Hum Mol Genet*. 2003; 12:535-49

Valerie K, Yacoub A, Hagan MP, Curiel DT, Fisher PB, Grant S, Dent P. Radiation-induced cell signaling: Inside-out and outside-in. *Mol Cancer Ther.* 2007; 6:789–801

Vance MM, Baulch JE, Raabe OG, Wiley LM, Overstreet JW. Cellular reprogramming in the F3 mouse with paternal F0 radiation history. *Int J Radiat Biol.* 2002; 78:513-26

Vatolin S, Abdullaev Z, Pack SD, Flanagan PT, Custer M, Loukinov DI, Pugacheva E, Hong JA, Morse H 3rd, Schrupp DS, Risinger JI, Barrett JC, Lobanekov VV. Conditional expression of the CTCF-paralogous transcriptional factor BORIS in normal cells results in demethylation and derepression of MAGE-A1 and reactivation of other cancer-testis genes. *Cancer Res.* 2005; 65:7751-62

Ward WS, Coffey DS. DNA packaging and organization in mammalian spermatozoa: comparison with somatic cells. *Biol Reprod.* 1991; 44:569-74

Warnecke PM, Mann JR, Frommer M, Clark SJ. Bisulfite sequencing in preimplantation embryos: DNA methylation profile of the upstream region of the mouse imprinted H19 gene. *Genomics.* 1998; 51:182-90

Weber B, Stresemann C, Brueckner B, Lyko F. Methylation of human microRNA genes in normal and neoplastic cells. *Cell Cycle.* 2007; 6:1001-5

Weber M, Schubeler D. Genomic patterns of DNA methylation: targets and function of an epigenetic mark. *Curr Opin Cell Biol.* 2007; 19:273-80

Webster KE, O'Bryan MK, Fletcher S, Crewther PE, Aapola U, Craig J, Harrison DK, Aung H, Phutikanit N, Lyle R, Meachem SJ, Antonarakis SE, de Kretser DM, Hedger MP, Peterson P, Carroll BJ, Scott HS. Meiotic and epigenetic defects in Dnmt3L-knockout mouse spermatogenesis. *Proc Natl Acad Sci U S A.* 2005; 102:4068-73

West A, Lähdetie J. X-irradiation--induced changes in the progression of type B spermatogonia and preleptotene spermatocytes. *Mol Reprod Dev.* 2001; 58:78-87

Williams AE. Functional aspects of animal microRNAs. *Cell Mol Life Sci.* 2008; 65:545-62

Woloszynska-Read A, James SR, Link PA, Yu J, Odunsi K, Karpf AR. DNA methylation-dependent regulation of BORIS/CTCF expression in ovarian cancer. *Cancer Immun.* 2007; 7:21

Wright EG, Coates PJ. Untargeted effects of ionizing radiation: implications for radiation pathology. *Mutat Res.* 2006; 597:119-32

Wright EG. Commentary on radiation-induced bystander effects. *Hum Exp Toxicol*. 2004; 23:91-4

Wu L, Fan J, Belasco JG. MicroRNAs direct rapid deadenylation of mRNA. *Proc Natl Acad Sci U S A*. 2006; 103:4034-9

Wyrobek AJ, Mulvihill JJ, Wassom JS, Malling HV, Shelby MD, Lewis SE, Witt KL, Preston RJ, Perreault SD, Allen JW, Demarini DM, Woychik RP, Bishop JB. Assessing human germ-cell mutagenesis in the Postgenome Era: a celebration of the legacy of William Lawson (Bill) Russell. *Environ Mol Mutagen*. 2007; 48:71-95

Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. *Nature*. 1993; 366:701-4

Yan W, Morozumi K, Zhang J, Ro S, Park C, Yanagimachi R. Birth of mice after intracytoplasmic injection of single purified sperm nuclei and detection of messenger RNAs and MicroRNAs in the sperm nuclei. *Biol Reprod*. 2008; 78:896-902

Yang J, Medvedev S, Reddi PP, Schultz RM, Hecht NB. The DNA/RNA-binding protein MSY2 marks specific transcripts for cytoplasmic storage in mouse male germ cells. *Proc Natl Acad Sci U S A*. 2005; 102:1513-8

Yauk CL, Dubrova YE, Grant GR, Jeffreys AJ. A novel single molecule analysis of spontaneous and radiation-induced mutation at a mouse tandem repeat locus. *Mutat Res*. 2002; 500:147-56

Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. *Science*. 2004; 304:594-6

Yu Z, Guo R, Ge Y, Ma J, Guan J, Li S, Sun X, Xue S, Han D. Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. *Biol Reprod*. 2003; 69:37-47

Yu Z, Raabe T, Hecht NB. MicroRNA Mirn122a reduces expression of the posttranscriptionally regulated germ cell transition protein 2 (Tnp2) messenger RNA (mRNA) by mRNA cleavage. *Biol Reprod*. 2005; 73:427-33

Zendman AJ, Ruiter DJ, Van Muijen GN. Cancer/testis-associated genes: identification, expression profile, and putative function. *J Cell Physiol*. 2003; 194:272-88

Zhao M, Shirley CR, Hayashi S, Marcon L, Mohapatra B, Suganuma R, Behringer RR, Boissonneault G, Yanagimachi R, Meistrich ML. Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis*. 2004; 38:200-13

Zhao Y, Srivastava D. A developmental view of microRNA function. *Trends Biochem Sci.* 2007; 32:189-97

Zhu B, Huang X, Chen J, Lu Y, Chen Y, Zhao J. Methylation changes of H19 gene in sperms of X-irradiated mouse and maintenance in offspring. *Biochem Biophys Res Commun.* 2006; 340:83-9