RADIATION-INDUCED DEREGULATION OF PiRNA PATHWAY PROTEINS:

A POSSIBLE MOLECULAR MECHANISM UNDERLYING

TRANSGENERATIONAL EPIGENOMIC INSTABILITY

Matthew Merrifield
Bachelor of Science, University of Lethbridge, 2008

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

MASTER OF SCIENCE

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

ABSTRACT

PiRNAs and their Piwi family protein partners are part of a germline specific epigenetic regulatory mechanism essential for proper spermatogenesis, silencing of transposable elements, and maintaining germline genome integrity, yet their role in the response of the male germline to genotoxic stress is unknown.

Ionizing radiation (IR) is known to cause transgenerational genome instability that is linked to carcinogenesis. Although the molecular etiology of IR-induced transgenerational genomic instability is not fully understood, it is believed to be an epigenetically mediated phenomenon. IR-induced alterations in the expression pattern of key regulatory proteins involved in the piRNA pathway essential for paternal germline genome stability may be directly involved in producing epigenetic alterations that can impact future generations.

Here we show whole body and localized X-irradiation leads to significant altered expression of proteins that are necessary for, and intimately involved in, the proper functioning of the germline specific piRNA pathway in mice and rats. In addition we found that IR-induced alterations to piRNA pathway protein levels were time and dose dependent.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my parents, Keith and Ghislaina, for their endless support and encouragement.

To Dr. Olga Kovalchuk I would like to express my great appreciation for the enormous opportunity to develop my professional skills as a student in her laboratory. The experience and skill set I have acquired with this opportunity will be invaluable in all my future endeavors.

I would also like to thank my committee members, Dr. Theresa Burg, Dr. Sergio Pellis, for their support and guidance throughout my time at the University of Lethbridge. I would also like to thank my external examiner for the time and travel commitment to visit the University of Lethbridge for my defence.

Last, but not least, I would like to thank my many colleagues from both Kovalchuk laboratories for their friendship, guidance, and contribution to the following work. Special thanks to Rocio Rodriguez Juarez, and Juilian St. Hilaire for their immense technical support and contributions.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

10A - adenine at the tenth nucleotide position of a piRNA
1U - uridine at the first nucleotide of a piRNA
5meC - 5-methylcytosine
AGO2 - argonaute 2
Bp - base pair
BSA - bovine serum albumin
C57BL/6 - C57 black 6 inbred strain of lab mouse
CpG - cytosine and guanine separated by a phosphate
DAPI - 4', 6-diamidino-2-phenylindole
DNA - deoxyribonucleic acid
DNMT - DNA methyltransferase
dpc - days post-coitum
dpp - days post-partum
DSB - double strand DNA break
dsDNA - double stranded DNA
dsRNA - double stranded RNA
eIF4E - eukaryotic translation initiation factor 4E
ESTR - expanded simple tandem repeat loci
F1 - first filial generation
F2 - second filial generation
Gy - gray (radiation unit)
H2AX - histone H2AX
γH2AX - phosphorylated histone H2AX
H4 - histone 4
HMG - high-mobility group domain
IAP - intracisternal A-particle
IF - immunofluorescent
Ig - immunoglobulin
IHC - immunohistochemistry
IR - ionizing radiation
KIF17b - kinesin motor protein
LET - linear energy transfer
LINE-1 - long interspersed nucleotide element 1
MeCP2 - methyl CpG binding protein 2
Mep50 - methylosome protein 50
miRNA - micro RNA
miRNP - miRNA ribonucleoprotein complex
mRNA - messenger RNA
nt - nucleotide
ORF - open reading frame
PBS - phosphate buffered saline
PFA - paraformaldehyde
PGS - primordial germ cell
piRNA - Piwi-interacting RNA

Piwi - P-element induced wimpy testis protein
pre-miRNA - precursor micro RNA
pri-miRNA - primary micro RNA
Prmt - protein arginine methyltransferase
PTGS - post transcriptional gene silencing
PVDF - polyvinylidene fluoride
rcf - relative centrifugal force
RISC - RNA-induced silencing complex
RNA - ribonucleic acid
RNP - ribonucleoprotein
SCE - sister chromatid exchange
sDMA - symmetrical dimethylarginine
SDS - sodium dodecyl sulfate
SEM - standard error of the mean
SINE B2 - short interspersed elements B2
SSB - single strand DNA break
TE - transposable element
TGS - transcriptional gene silencing
TMA - tissue microarray
UTR - untranslated region

CHAPTER 1: GENERAL INTRODUCTION

1.1 RADIATION CARCINOGENESIS

Life as we know it has always evolved in an environment subjected to ionizing radiation (IR) exposure. Humans are of no exception, as we are exposed to IR via the air we breathe, the food we eat, and from the sky above us (cosmic rays), as well as the ground we walk on. However, it is becoming increasingly common for humans to not only be exposed to natural or "background" levels of IR, but also to man-made sources of radiation. As civilization continues to progress, and radiation continues to be an integral part of modern life, the amount of man-made radiation exposure will increasingly add to natural background exposure levels. Owing to a marked increase in accessibility, and an introduction of innovative techniques that utilize IR, the number of people that receive chronic and/or acute exposures to radiation via occupational, diagnostic, or treatment-related modalities, is progressively rising. Due in part to this, in recent years, much attention has been devoted to elucidate the biological responses and mechanisms underlying human exposure to IR.

The majority of primary data on radiation-induced cancers in humans come predominantly from atomic bomb and nuclear accident survivors, as well as the medically exposed. A number of studies on survivors of the atomic bomb attacks on Japan by the U.S.A., demonstrated a greatly increased incidence of various cancers among survivors (Folley et al., 1952; Watanabe et al., 1972; Wakabayashi et al., 1983; Carmichael et al., 2003). Some of the largest accidentally exposed cohorts of people are currently available in the territory of

the former USSR, which includes exposure groups from major industrial accidents, such as the approximately 30,000 people who live near the Mayak nuclear facility in the southern Ural Mountains in Russia, the 1986 Chernobyl catastrophe, as well as from nuclear weapons testing in Kazakhstan (Dubrova, 2003). The cohort of people in the Mayak region that were chronically exposed to IR demonstrated an increased incidence of leukemia, slightly lower than the rates experienced by atomic bomb survivors (Kossenko, 1996; Shilnikova et al., 2003). The 1986 Chernobyl accident, which was undoubtedly one of the most catastrophic disasters in the history of the nuclear industry, led to a significant elevation in the rates of various cancers ranging from thyroid carcinomas (Bogdanova et al., 2006; Likhtarov et al., 2006; Williams, 2006), to leukemia and lymphomas (Gluzman et al., 2005; Balonov, 2007), breast cancers (Pukkala et al., 2006; Prysyazhnyuk et al., 2007), as well as bladder cancers (Romanenko et al., 2000; Morimura et al., 2004). In addition, elevated cancer and mutation rates were also reported in people living near the Semipalatisk nuclear test site in Kazakhstan (Salomaa et al., 2002; Tanaka et al., 2006).

IR is now universally accepted as a severe DNA damaging agent, which can lead to serious consequences, including cancer (Little, 1999). As mentioned, even though IR is a well-known genotoxic agent and human carcinogen, it is also widely used to effectively diagnose and treat cancer (Little, 1999, 2000; Pollack et al., 2000; Roof et al., 2003; Potter et al., 2006; Erven and Van Limbergen, 2007). Since 1902 when the first radiation-induced cancer was reported (Little, 2000, 2003), and almost one hundred years after radiation was used for the first time to

treat tumors (Gramegna, 1909), it still remains the number one diagnostic and treatment tool for the majority of cancers (Pollack et al., 2000; Roof et al., 2003; De Potter et al., 2006; Erven and Van Limbergen, 2007). While modern cancer radiation therapy has indeed led to increased patient survival rates, the risk of treatment-related deleterious effects, including secondary cancers, is becoming a growing clinical problem (Leone et al., 1999). Relatively recent findings suggest that even fairly low doses of IR, such as those used in diagnostic procedures (e.g., X-ray or Computer Tomography), can lead to the development of radiation-induced cancers (Preston-Martin et al., 1989; Morin et al., 2000; Brenne and Hall, 2004; Liu et al., 2004). This risk of developing secondary treatment-related cancers is even more pronounced in children and young adults who received either diagnostic or therapeutic exposure to IR (Hildreth et al., 1989; Infante-Rivard et al., 2000; Hall, 2002; Shu et al., 2002; Kleinerman, 2006). In addition to children and young adults being more susceptible to secondary cancers related to IR exposure, they also represent a special high-risk group for other possible secondary effects, due to the reality that they are, or will soon be able to, reproduce. Parental exposure to radiation from nuclear reprocessing plants, as well as through diagnostics, has been documented to result in a significant increase in the risk of leukemia and congenital malformations in their children (Shiono et al., 1980; Shu at al., 1988; Gardner et al., 1990; Nomura 1993, Dickenson and Parker, 2002). Consequently, a major quality of life issue faced by young people who are exposed to radiation, especially young cancer patients and survivors, is not only an increased risk of secondary cancer development, but also

the ability to produce healthy offspring. With the continual increase in the number of individuals being subjected to acute/chronic whole body, as well as localized exposure to IR, it is becoming critically important to understand the full range of its biological effects.

1.2 CELLULAR EFFECTS OF DIRECT RADIATION EXPOSURE

IR has the ability to affect a variety of processes within 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; Jeggo and Lobrich, 2006; Rodemann and Blaese, 2007; Valerie et al., 2007). The ability of IR to damage DNA by inducing a wide range of lesions is one of its most important and unique features that impacts biological processes in mammalian cells (reviewed in Frankenber-Scwager, 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. It has historically been accepted that incorrectly repaired DSBs are the principle lesion of importance regarding mutagenesis, as well as many other biological effects of IR (Goodhead, 1994; Ward, 1995; Little, 2000). As a consequence of this damage not being correctly repaired, deleterious genetic changes, such as mutations and chromosomal aberrations, can be acquired at the initial sites of damage (Little, 2006). The accumulation of DNA damage caused by IR in conjunction with disrupted cellular regulation processes can lead to carcinogenesis (Little, 2000;

Barcellos-Hoff, 2005; Sowa et al., 2006). To date, many studies have assessed the adverse impact of exposure to IR on human health in terms of mutation induction in somatic cells, using both *in vitro* and *in vivo* systems (Barber and Dubrova, 2006). As a result of these and other findings, it is now known that the cellular effects and carcinogenic potential of radiation is not limited to what has historically been accepted. The historical assumption was, therefore, that the biological effects of radiation including cytotoxicity, mutation, and malignant transformations would occur in the exposed cells themselves as a consequence of direct DNA damage. It has now become apparent that this may not always be the case, as the biological effects associated with IR exposure can be induced in cells at delayed times after exposure and in cells that did not receive direct irradiation (Morgan, 2003a, b). While the historical viewpoint data are still invaluable in providing information regarding health monitoring and risk assessment for directly exposed cells, they may significantly underestimate deleterious biological effects associated with IR exposure as they offer very little information about the possible delayed and/or non-targeted genetic effects.

1.3 DELAYED AND NON-TARGETED IR EFFECTS

1.3.1 Genomic Instability

The significance of long-term or so-called "delayed effects," of exposure to IR has recently been becoming more evident. It has long been thought that the main factor contributing to the negative biological effects of radiation in mammalian cells, such as chromosomal aberrations, mutations, and cell death, is

the result of DNA damage in directly exposed cells; that is, residual damage that has not been repaired by the metabolic processes in the exposed cell (Little, 1998). This paradigm has largely been challenged in recent years, mostly originating from the results of numerous *in vitro* studies that demonstrated the existence of delayed effects of IR exposure (reviewed in Morgan, 2003a). These delayed effects can manifest in the unexposed progeny of irradiated cells for many cell divisions (and up to 4 years) after the initial insult (Morgan, 2003a). The all-encompassing term given to this phenomenon is "radiation-induced genomic instability," which is used to describe the increased rate of the acquisition of alterations in the genome. Experimentally, genomic instability is observed when a cell is irradiated, then clonally expanded, and the progeny is examined genetically. As mentioned, radiation-induced genomic instability is observed generations after the initial exposure, and a number of studies have shown that this occurs at a high frequency (Limoli et al., 1999, 2000). Multiple genetic endpoints have been utilized to evaluate radiation-induced genomic instability in a number of *in vitro* systems, which include, but are not limited to, chromosomal aberrations, ploidy changes, micronucleus formation, gene mutations, and amplifications, as well as increased microsatellite/ESTR mutation rates and delayed cell death (Morgan, 2003a, b, c; Huang et al., 2003; Suzuki et al., 2003). There are a number of pathways that are implicated in the initiation and perpetuation of radiation-induced genomic instability (Kaplan et al., 1997). The relative amount of contribution of the different pathways primarily depends upon

the genetic background of the irradiated cell or organism (Paquette and Little, 1994; Watson et al., 1997), as well as the type of radiation (Limoli et al., 2000).

A number of different *in vitro* systems that have been studied have demonstrated a high frequency of IR-induced genomic instability by means of examining the various endpoints (as described above) that are associated with IR-induced genomic instability (reviewed in Morgan, 2003a). Although some speculation has been raised regarding what is actually being observed and the combined significance of these observations, the prevailing hypothesis is that IR exposure destabilizes the genome, thus initiating a cascade of genomic events that increases the rate of mutation and chromosomal change in the progeny of that irradiated cell (Morgan, 2003a). It has long been speculated that the development of genomic instability can facilitate the process of cancer initiation and/or progression (Cheng and Loeb, 1993), and indeed, the loss of genomic stability is believed to be a hallmark of many cancers, as well as an important prerequisite for cancer formation (Goldberg, 2003; Little, 2003; Loeb et al., 2003). Therefore, the general assumption is that there is a link between the induction of IR-induced genomic instability and cancer, due to an increase in the accumulation of multiple genetic events within a cell that ultimately enhance radiation-induced carcinogenesis. This assumption is also supported by epidemiological studies' findings, which suggest that some types of radiation-induced cancers may follow a relative risk model, in which IR exposure enhances the rate at which cancers develop, instead of inducing a specific cohort of new tumors (Little, 2000). The demonstration of IR-induced genomic instability in somatic cell culture systems

has greatly increased interest in research concerning the potential long-term effects for exposure. One such area that this has undoubtedly expanded to is the potential long-term effects associated with germline IR exposure and the transmission of adverse effects (e.g. genomic instability) to future generations.

1.3.2 Transgenerational Effects

The *in vitro* data, as mentioned above, have provided overwhelming evidence for delayed IR-effects being manifested in the progeny of irradiated cells (i.e. genomic instability) for many divisions, which may ultimately enhance the carcinogenic potential of these cells. Moreover, they point out that genomic instability could also be induced in the irradiated germline, and therefore may be transmitted to future generations. If this is the case, then the offspring of irradiated parents may be genetically unstable, resulting in a plethora of transgenerational effects, such as elevated mutations rates and a predisposition to cancer. Many publications have indeed characterized a wide variety of phenotypic traits observed in the offspring of irradiated parents, implicating increased mutation rates. Such studies have been reinforced through the use of various molecular techniques used to assess transgenerational genomic instability. Here, I will briefly outline some of the main "classical" publications that have mainly analyzed hereditary phenotypic alterations associated with parental exposure. This will be followed by the chief molecular and genetic studies/techniques that have backed these finding by demonstrating genomic instability in the progeny of irradiated parents (i.e. transgenerational genomic instability).

The first evidence for a transgenerational effect associated with IR exposure was demonstrated by Luning and colleagues, where elevated rates of dominant lethal mutations (early and late embryonic death) were observed following the intraperitonial injection of male mice with a plutonium salt solution (Luning et al., 1976). Accordingly, an increase in dominant lethality was not only found from the germ line of directly irradiated male mice, but also from the germline of their non-exposed, first-generation mice (F1). The offspring of irradiated male mice have also been shown to be reproductively challenged, exhibiting decreased fertilization rates for both *in vivo* and *in vitro* fertilization (Lyon et al., 1964; Burruel et al., 1997), as well as increased levels of prenatal mortality for the F2 generation (Pils et al., 1999). An increase in teratogenic effects was also shown, as the number of malformed F2 fetuses was significantly higher in the paternally exposed group compared to the control (Pils et al., 1999). Nomura (1982 and 2003) not only demonstrated that paternal irradiation leads to an increase in malformations in the progeny of irradiated parents but also a significant increase in the incidence of cancer in these offspring. Several additional transgenerational studies also found a significant increase in cancer incidence among the offspring of paternally irradiated mice following secondary exposure to known carcinogens (Nomura 1983; Lord et al., 1998; Hoys et al., 2001). The predisposition of the offspring of IR-exposed fathers to cancer has been investigated in human populations, where the data obtained have mainly been inconclusive (Roman et al., 1999; McKinney et al., 2003); however, two independent studies have shown a clustering of extremely high leukemia rates in

children whose fathers had been exposed to radiation after working at a nuclear processing plant in the town of Sullafield (Gardner et al., 1990; Dickinson and Parker 2002).

Adding to the classical evidence of transgenerational impacts such as those mentioned above, the majority of recent data have arisen from various molecular techniques used to characterize genotypic alterations in unexposed offspring. Mainly, the genotypic alterations found in the progeny of irradiated parents have included chromosomal aberrations, micro nuclei formation, increased microsatellite/ESTR mutations, and altered gene expression patterns, which are all hallmarks of genomic instability (reviewed in Dubrova, 2003; Morgan, 2003a; Barber and Dubrova, 2006). The manifestation of such alterations has, therefore, collectively been termed transgenerational genome instability. Dubrova and colleagues have made a significant contribution to the current understanding of radiation-induced transgenerational genome instability by pioneering the investigation of transgenerational mutation rates within repetitive sequences of the genome (Dubrova et al., 2003). These repetitive sequences were initially termed minisatellites, but are now known as expanded simple tandem repeat (ESTR) loci, because they are extended (500-16 000 bp) stretches of relatively short (4-6 bp) repeats that are less stable than true minisatellites, which generally consist of longer (6-100 bp) repeats (Dobrova, 2003b). Barber and colleagues studied mutation rates of two ESTR loci in the germline of F1 and F2 offspring of male mice exposed at either the premeiotic or postmeiotic stages of spermatogenesis (Barber et al., 2002). They found an increased mutation rate in

the germline of F1 offspring, which was similarly maintained in the germline of the F2 offspring, for both pre/post-meiotic germ cell exposure groups. Furthermore, the elevated mutation rates were seen in all three of the mice strains they studied, and within each strain, male and female offspring (both F1 and F2) of irradiated fathers equally demonstrated elevated mutation rates (Barber et al., 2002). Further analysis of the unexposed F1 progeny showed that high ESTR mutation rates were observed along with elevated mutations in protein coding genes in germline, as well as in somatic tissues, such as spleen and bone marrow (Barber et al., 2006). The analysis of mutation rates in genomic repeat elements has also been applied to study transgenerational IR effects in human populations, namely in individuals living in the vicinity of the Chernobyl reactor accident and nuclear test sites (Semipalatinsk, Kazakhstan) (Dubrova et al., 1996, 2002). In all of these studies, they found an increase in the mutation rate among the progeny of the exposed parents. Taken together, these data support the hypothesis that exposure to IR can induce germline genomic instability that may predispose future generations to an increase risk of genetic diseases, infertility, and even cancer.

1.3.3 Bystander Effects

In addition to genome instability and transgeneration effects, the paradigm of genetic alterations being restricted to directly hit cells has also been challenged by numerous observations in which cells that were not directly transversed by the IR, but were either in the neighborhood of irradiated cells or exposed to factors produced by irradiated cells, exhibited responses similar to those of the directly

irradiated cells (Morgan, 2003a, b; Morgan and Sowa, 2005). Such "non-targeted" effects are collectively regarded as radiation-induced 'bystander' effects; accordingly, naïve cells exhibiting these responses are commonly called 'bystander cells.'

Evidence supporting the phenomenon of the bystander effect has been demonstrated in studies performed as early as the beginning of the twentieth century. Murphy and Morton, whose research interests were devoted to the study of lymphoid cells, showed morphological changes in lymphoid cells after culturing them with serum from radiation-exposed animals (Murphy and Morton, 1915). Additionally, in 1954, Parsons and colleagues reported the presence of soluble "clastogenic" factors in the circulating blood of patients who underwent radiotherapy (Parsons et al., 1954). Clastogenic factors are known for their ability to induce chromosome damage in cultured cells (Goh and Sumner, 1968; Hollowell et al., 1968., Emerit et al., 1994, 1995). Such clastogenic activity has also been demonstrated in the plasma from patients who received high dose radiotherapy, and from individuals accidentally exposed to radiation from the Chernobyl accident (Goh and Sumner, 1968; Pant and Kamada, 1977; Emerit et al., 1994, 1995); however, the term "bystander effect" was, in fact, not coined until the 1990's, when it was adopted from the gene therapy literature, where it was used to describe the killing of several tumor cell types after targeting only one type of cell within a heterogeneous population (Freeman et al., 1993). Direct studies of bystander effects have most widely been done *in vitro,* and the most common experimental model used to study it has generally involved the exposure

of monolayer cultures to very low fluences of α-particles, such that only a small fraction of the total cell population is hit by a particle (Nagasawa and Little, 1992; Little, 2000). In the initial report of this phenomenon, an enhanced frequency of sister chromatid exchanges (SCE) was observed in up to 50% of the cell population, when only 0.1-1% had been traversed by radiation (Nagasawa and Little, 1992). In the late 1990's, there was resurgence in the interest and awareness of radiation-induced bystander effects, due largely to the development of charged-particle microbeam irradiators (Folkard et al., 1997). The microbeam is capable of putting an exact number of particles through specific subcellular compartments of a defined number of cells in a particular radiation environment (Folkard et al., 1997; Randers-Pehrson et al., 2001). The most convincing demonstration of the bystander effect has employed this technique, demonstrating that not only nuclear, but even cytoplasmic irradiation can have genetic consequences, both of which can be manifested in bystander cells (Wu et al., 1999).

Since then, a variety of cell culture studies have, indeed, demonstrated radiation-induced bystander effects with different endpoints being observed depending on the type of cells receiving/producing the bystander signal, as well as the type of radiation (Lorimore et al., 2003; Morgan 2003a). Some, but not all, of these endpoints are detrimental to the cell. Similar to genomic instability, bystander effects are measured by the induction of gross chromosomal rearrangements, chromosome aberrations, sister chromatid exchanges, deletions, duplications, mutations, amplifications, and cell death (Kovalchuk and Baulch,

2008, and references therein). Bystander effects such as these have also been demonstrated in three-dimensional tissue models (Perssaud et al., 2005), and in reconstructed human tissue models (Belyakov et al., 2005; Sedelnikova et al., 2007). As a result, bystander effects are accepted as a ubiquitous consequence of radiation exposure (Mothersill and Seymour, 2004). By the nature of their occurrence, bystander effects can be grouped into two separate, but not necessarily mutually exclusive, mechanisms for the transfer of a signal from irradiated cells to naive cells. One mechanism of the bystander effect is gap-junction communication-mediated, and is based on the ability of intercellular gap junctions some type of signal from irradiated to non-irradiated cells (Bishayee et al., 2001; Azzam et al., 2003a, 2003b; Shao et al., 2003; Suzuki and Tsuruoka, 2004;). The other proposed mechanism is known as medium-mediated bystander effects, and is based on the ability of irradiated cells to secrete certain factors into the medium that are then received by non-irradiated cells (Zhou et al., 2002; Yang et al., 2005; Liu et al., 2006; Lyng et al., 2006b; Maguire et al., 2007). Although candidate signaling molecules are numerous, current literature suggests key players include reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Mothersill and Seymour, 1988; Lyng et al., 2000; Lyng et al., 2002; Azzam et al., 2003), short RNAs (Koturbash et al., 2007, Kovalchuk and Baulch, 2008), Ca^{2+} ions (Lyng et al., 2000, 2002, 2006), and cytokines (Facoetti et al., Bonner, 2003; Iyer and Lennert., 2000).

As previously mentioned, the occurrence of bystander effects *in vivo* had long been suggested ever since it was shown that exposure to radiation produces

"clastogenic" factors in the circulating blood of exposed animals and humans (Murphy and Morton, 1915; Parsons et al., 1954; Emerit et al., 1994, 1995). Nevertheless, compared to data from cell culture studies, the conclusive data on somatic, and especially germ cell, bystander effects *in vivo* are rather scarce (Goldberg and Lehnert, 2002; Hall, 2003; Koturbash et al., 2006a, b, 2007; Mothersill et al., 2007; Tamminga et al., 2008). However, there is accumulating evidence for IR-induced bystander effects *in vivo*. Bystander effects have been shown to occur within the exposed organ in rodent models. When only the base of the lung was irradiated, significant molecular and cellular damage was observed in the shielded lung apex (Khan et al., 1998, 2003). It was also shown that when one lung was exposed there was a marked increase of micronuclei in the unexposed shielded lung (Khan et al., 1998, 2003). Similar intra-organ bystander effects were observed in a rodent model that underwent partial liver irradiation (Brooks et al., 1974; Brooks, 2004). Not surprisingly, bystander effects also manifest themselves in the context of an organism in its entirety. Koturbash and colleagues have established a murine model system to study *in vivo* bystander effects, and have pioneered research in this field. To analyze the role of epigenetic changes associated with radiation-induced bystander effects *in vivo*, they developed a mouse model, whereby half of an animal's body was exposed to radiation, while the other half was protected by a medical grade shield (Koturbash et al., 2006a). They confirmed the existence of somatic bystander effects, by showing that X-ray exposure to one side of an animal's body caused profound changes in the unexposed bystander portion of the body (Koturbash et al., 2006a,

2007). They also found that male mice exhibit a more pronounced bystander effect. It has recently been shown for the first time that localized cranial exposure causes an *in vivo* bystander response, not only in somatic tissue but in the male germline as well (Tamminga et al., 2008). In addition, they showed that bystander damage to the germline caused by localized cranial radiation had transgenerational consequences, causing profound epigenetic dysregulation in the unexposed progeny (Tamminga et al., 2008).

A plethora of information available in the literature from *in vitro* studies, as well as compelling data from 3D tissue models and whole organisms, has provided convincing evidence for the existence of IR-induced bystander, as well as transgenerational effects, both of which have been linked to the phenomenon of IR-induced genomic instability. Notwithstanding are the underlying molecular mechanisms that lead to their development; however, there is strong evidence for a common underlying molecular mechanism linking these phenomena. Again, this is most compellingly evident in the commonality of the end points observed for these phenomena (i.e. of genomic instability). A high frequency of induction and persistence of IR-induced genomic instability, as well as a non-Mendelian mode of inheritance of transgenerational effects suggests an epigenetic based mechanism (Wiley et al., 1997; Lorimore et al., 2003; Morgan, 2003a, b; Nagar et al., 2003; Barber, 2006; Kaup et al., 2006; Wright and Coates, 2006; Kovalchuk and Baulch, 2008).

1.4 EPIGENETICS AND IR EXPOSURE

Epigenetic alterations are meiotically heritable and mitotically stable alterations in gene expression with no change in DNA sequence, which include DNA methylation, histone modifications, and RNA-associated silencing (Jaenisch and Bird, 2003).

1.4.1 DNA Methylation

DNA methylation was the first epigenetic alteration identified, and is the most widely studied epigenetic mechanism. In mammals, DNA is methylated at the carbon 5 of cytosine residues to form 5-methyl-cytosines, (5meC), which is established by the *de novo* DNA methyltransferases (DNMT3a, DNMT3b, and DNMT3L), and subsequently maintained by DNMT1 (Robertson, 2001; Rountree et al., 2001; Goll and Bestor, 2005). The *de novo* DNA methylation of transposons in the germline is dependent on DNMT3L, an isoform of DNMT3a and DNMT3b that lacks methylation activity (Kato et al., 2007). DNA methylation is known to be associated with inactive chromatin states, and in most cases, with the repression of gene expression (Hendrich and Tweedie, 2003; Klose and Bird, 2006; Weber and Schubeler, 2007). Proper regulation of DNA methylation is critically important for normal development, cell proliferation, and the maintenance of genomic stability within a given organism (Ehrlich, 2002; Robertson, 2002; Jaenisch and Bird, 2003). The global loss of DNA methylation has been linked to the activation of transposable elements, elevated chromosome breakage, aneuploidy, increased mutation rates, and therefore to the phenomenon of genomic instability (Robertson, 2002; Weber and Schubeler, 2007; Weidman et

al., 2007). In addition, altered global DNA methylation patterns are a well known characteristic of cancer cells, and global loss of cytosine methylation was the first epigenetic abnormality discovered in cancer cells (Feinberg and Vogelstein, 1983; Flatau et al., 1983; Gama-Sosa et al., 1983; Feinberg, 2004). The DNA methylation profile of cancer cells is frequently characterized by global genome hypomethylation, as well as concurrent hypermethylation of selected CpG islands within gene promoters (e.g. tumor suppressor) (Jaenisch and Bird, 2003; Baylin, 2005; Baylin and Ohm, 2006; Weidman et al., 2007).

Consequently, it is not surprising that direct IR exposure has been reported to affect DNA methylation patterns. Acute exposures to low LET radiation such as X-rays and/or γ-rays have been noted to result in global genomic DNA hypomethylation (Kalinich et al., 1989). More recently, IR exposure has been found to lead to profound dose-dependent, as well as sex and tissue-specific global hypomethylation (Raiche et al., 2004; Pogribny et al., 2004, 2005; Koturbash et al., 2005; Loree et al., 2006). This loss of methylation was also associated with radiation-induced alterations in the expression of DNA methyltransferases, especially *de novo* methyltansferases DNMT3a, andDNMT3b (Raiche et al., 2004; Pogribny et al., 2005). Most importantly, the radiation-induced global genomic DNA hypomethylation patterns appear to be linked to genomic instability in exposed animals (Pogribny et al., 2004; Raiche et al., 2004; Pogribny et al., 2005; Loree et al., 2006).

DNA methylation also plays a role in radiation-induced bystander effects. Kaup and colleagues lead the way in showing the importance of DNA

methylation in the maintenance of radiation-induced bystander effects (Kaup et al., 2006). They demonstrated that dysregulation of DNA methylation patterns occurs in non-irradiated cells and can persists for 20 passages when they are treated with medium from irradiated cells (Kaup et al., 2006). These bystander cells, marked with aberrant methylation patterns, also exhibited numerous endpoints characteristic of genome instability (Kaup et al., 2006). The same pattern of genomic instability and significant loss of nuclear DNA methylation was also observed in 3D human tissue models (Sedelnikova et al., 2007).

As mentioned previously, much insight into the role of such epigenetic changes in bystander, as well as trangenerational effects *in vivo,* has come from the pioneering work of the Kovalchuk and Engelward laboratories. By demonstrating that radiation exposure limited to either half of the body leads to elevated levels of DNA strand breaks, and altered levels of key proteins involved in establishing and maintaining methylation marks, in lead shielded tissue at least 0.7 cm from irradiated tissue they produced the first data to clearly demonstrate that epigenetically regulated bystander effects occur *in vivo* (Koturbash et al., 2006a). Using localized cranial X-irradiation on a rat model, Koturbash et al. (2007) aslo demonstrated that localized IR exposure can induce profound global DNA hypomethylation in distant bystander tissue (spleen) that was observed 24 hours after exposure (Koturbash et al., 2007). Importantly, these changes were still observed seven months after exposure. This is relevant in terms of carcinogenesis due to the fact that the epigenetic manifestations of bystander effects persisted over a long period of time, roughly equivalent to ten years in

humans. Again, the profound and persistent reduction of methylation in the bystander spleen was paralleled by altered (decreased) levels of key proteins involved in the establishment and maintenance of methylation patterns (i.e. DNMT3a, DNMT1, and methyl-binding protein MeCP2). This was believed to contribute to the observed reactivation of the LINE1 retrotransposon in the bystander spleen (Koturbash et al., 2007). The observed hypomethylation was also manifested in the bystander germline of cranially exposed mice (Tamminga et al., 2008)

Consequently, the involvement of the same type of epigenetic effectors (global DNA methylation, and associated proteins), in transgenerational effects induced from the paternal whole body, as well as localized exposure to IR, have also been studied (Koturbash et al., 2006; Tamminga et al., 2008). Paternal whole body and cranially localized IR-exposures were shown to result in a significant global loss of DNA methylation in the thymus, bone marrow, and the spleen of F1 offspring (Koturbash et al., 2006; Tamminga et al., 2008). Whole body exposure also resulted in specific hypomethylation of LINE1 and SINE B2 in the germline of exposed males, which was further observed in the thymus of unexposed offspring (Filkowski et al., 2010). Correspondingly, the thymus from the progeny of paternal whole body IR exposures, and bone marrow from the offspring of cranial exposed fathers, where the most pronounced decreases in DNA methylation was observed, also exhibited significant decreases in the expression of DNMT1, DNMT3a, DNMT3b, and methyl binding protein MeCP2 (Koturbash et al., 2006; Tamminga et al., 2008). The global loss of DNA methylation and

altered levels of methyltransferases and methyl binding proteins can lead to the activation of transposable elements, contributing to genomic instability (Xu et al., 1999; Yu et al., 2001; Jirtle and Skinner, 2007). Accordingly, it can also be suggested that the global loss of DNA methylation observed in the progeny of irradiated fathers may influence retrotransposons and satellite DNA, thus underlying transgenerational genome instability. Such a hypothesis also corroborates, and may help elucidate, the increased mutation rates in satellite DNA and ESTR loci observed in the progeny of exposed parents (Barber and Dubrova, 2006). Even though these epigenetic alterations are well characterized consequences of radiation exposure, the underlying molecular mechanism that drive these alterations, especially site specific changes in DNA methyaltion patterns, remain elusive. Such molecular mechanisms may very well be chief contributors to IR-induced epigenetic alterations associated with germline genomic instability, and therefore, would be strongly implicated in facilitating epigenetic inheritance of transgenerational IR effects.

1.4.2 Histone Modifications

Indeed, changes in DNA methylation do not occur as isolated events, as they are closely connected to other components of chromatin structure, such as histone modifications (Jaenisch and Bird, 2003; Weidman et al., 2007). The main histone modifications include acetylation, methylation, phosphorylation, and ubiquitination (Jenuwein and Allis, 2001). There is a vast complexity of epigenetic control that can be exhibited from such modifications, as each of these modifications all have differing transcriptional consequences compounded by

further control depending on which residue is modified, and to what extent (e.g. mono-, di-, tri-methylated) (Cheung and Lau, 2005; Saha et al., 2006; He et al., 2007; Weidman et al., 2007). Recent studies have indicated that IR-induced global loss of DNA methylation may correlate with changes in histone methylation, specifically with the loss of histone H4 lysine trimethylation (Pogribny et al., 2005).

One of the best studied histone modifications, especially regarding IR exposure, is the phosphorylation of histone H2AX at serine 139 (γH2AX). γH2AX is possibly one of the earliest cellular responses to DSB, and therefore, to IR exposure. The formation of γH2AX is crucial for the repair of DSB, and for the maintenance of genome stability (Rogakou et al., 1998; Pilch et al., 2003; Sedelnikova et al., 2003). The involvement of H2AX posphorylation in bystander, as well as transgenerational IR-effects, has also been suggested. Elevated levels of γH2AX have been reported in somatic and notably germline bystander tissues *in vivo,* and this elevation has subsequently been observed in the offspring of exposed fathers (Barber et al., 2006; Koturbash et al., 2006a, 2006b, 2007; Tamminga et al., 2008).

1.4.3 Small RNA Mediated Events

Epigenetic control can also be regulated by small RNA mediated events (Bernstein and Allis, 2005). Here, I will discuss two types of small regulatory RNAs that are of particular interest: microRNAs (miRNA) and Piwi-interacting RNAs (piRNAs). MicroRNAs are abundant, small (~21-25 nt) single stranded non-coding RNAs that regulate gene expression primarily at the post-

transcriptional level (e.g. post transcriptional gene silencing, PTGS). Initially, miRNAs are endogenously transcribed as part of a primary transcript (pri-miRNA) that is able to form one or more hairpin structures (miRNA stem loops) from complementary sequences within the transcript. MiRNA genes can be transcribed independently, or clustered with others and transcribed as a polycistron (Chen and Meister, 2005). There are also a large number of intragenic miRNAs transcribed from within introns or exons of protein coding and non-coding genes (Rodriguez et al., 2004). These primary transcripts are then processed in the nucleus into stem-loop-structured miRNA precursors (pre-miRNA) approximately 70 nt long, by the RNase III enzyme Drosha. They are then exported to the cytoplasm where Dicer (RNase III enzyme) generates characteristic 21-25 nt long dsRNA that separate into two strands, one of which is incorporated into a member of the Argonaute protein family (AGO2), a central component the microRNA ribonucleoprotein complex (miRNP), commonly known as the RNA-induced silencing complex (RISC) (reviewed in Zeng, 2006). To control the translation of specific mRNAs, the miRNA guided RISC complex binds to the 3'UTR of target mRNAs with a similar sequence structure, thus serving as translational repressors that regulate protein synthesis by targeting specific mRNAs (Hutvagner and Zamore, 2002). Currently, it is believed that miRNAs exhibiting a high degree of complimentarity to their target mRNAs are able to repress translation through mRNA cleavage. However, most miRNAs have imperfections between the complementary sequences, and therefore, repress translation without cleavage (Yekta at al., 2004; Doench and Sharp, 2004).

Although the precise nature of such regulation remains unclear, it is suggested that the main mechanisms include alteration of pol(A) tail length and binding of regulatory proteins to the UTRs of target mRNAs (Grivna et al., 2006). One or many miRNAs can coordinate the expression of single/multiple genes, resulting in a complex mechanism for posttranscriptional gene regulation. Consequently, miRNAs can play key roles in numerous biological contexts, including cellular differentiation, proliferation, apoptosis, and even a predisposition to cancer (Shivdasani, 2006; Chang and Mendell, 2007; Fabbri et al., 2007). Altered levels of miRNAs have been reported in a variety of cancers (Volinia et al., 2006; Wiemer, 2007).

Not unexpectedly, miRNAs are also involved in IR-induced responses *in vivo*. IR exposure to one half of a mouse's body triggered a significant up-regulation of *miR-194* in distant bystander liver tissue, which was suggested to initiate and maintain the observed down regulation of DNMT3a and MeCP2 in the same bystander tissue (Koturbash et al., 2007). The expression patterns of miRNAs have also been profiled in directly exposed males, as well as their unexposed offspring, demonstrating the possibility that they may also play a role in trangenerational epigenetic inheritance of genomic instability (Filkowski et al., 2010).

Recently, an additional novel small RNA pathway has begun to be characterized, providing evidence for yet another small RNA mediated epigenetic effector. Known as the Piwi/piRNA pathway, it has several unique features that make it quite suitable as a mediator of epigenetic memory in germ cells. Here, I

will introduce key features of the piRNA pathway, followed by a further discussion later, in the context of spermatogenesis in the rodent germline.

Initially characterized in *Drosophila* (Aravin et al., 2003), the central component of the pathway is a large class of short, single stranded non-coding RNAs (~26-31 nt) and their Piwi protein partners, a subclass of the Argonuate protein family. Both Piwi interactin RNA (piRNAs) and Piwi proteins have expression patterns that are largely restricted to germ cells in nearly all multicellular animals studied (Aravin and Hannon, 2008). Piwi proteins are required for the production of their piRNA partners, and are essential for various stages of spermatogenesis, as well as germ stem cell self-renewal and transposon silencing (reviewed in Thomson and Lin, 2009; Aravin and Hannon, 2008). The best studied function of the piRNA pathway is to maintain genomic integrity by the suppression of transposable elements (TE), via transcriptional gene silencing (TGS) (Aravin and Hannon, 2008). TGS occurs through piRNA pathway mediated *de novo* methylation of the regulatory regions of retrotransposons in embryonic germ cells, which is believed to be subsequently maintained in germ and somatic cells throughout the life of the organism (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). While mutations in the DNMT family members impacted cytosine methylation, the piRNA pathway remained largely unaffected (Arivin et al., 2008). In contrast, a loss of the piRNA pathway prevents recognition and silencing of TE by the DNMT3L, supporting a model in which the piRNA pathway acts upstream of DNMT3L, and consequently DNMT3a and DNMT3b, to establish patterns of DNA methylation on TEs (Arivin et al., 2008).

PTGS also contributes during this process, as piRNA guided Piwi proteins also mediate cleavage of active transposon mRNA, from which primary piRNAs are believed to be derived in a process known as the "ping-pong" amplification cycle (Aravin et al., 2007a, b). However, it is important to note that the majority of mouse and rat piRNAs are not enriched for sequences from transposons and repeats. In mice and rats, repeats are underrepresented, since only ~17% of all piRNAs map to repetitive elements while a random distribution should yield close to 40%, which is the proportion of repetitive sequences in the genome (Vagin et al., 2006; Hartig et al., 2007). In mammals, piRNAs tend to cluster within certain regions of the genome, and a large number of piRNAs are derived from intergenic regions, but are also distributed among exonic, intronic, and as mentioned, repeat sequences (Grivna et al., 2006). A distinguishing feature of these clusters of uniquely mapping piRNAs is their pronounced strand bias, thereby leading to the proposal that the biogenesis of piRNAs involves a long, single stranded precursor (Seto et al., 2007). Since piRNA sequences correspond to a variety of genomic regions, the piRNA pathway may be involved in a more complex system, regulating the expression a plethora of genes other than repetitive elements.

Indeed, several recent studies suggest that the piRNA pathway is not limited to the repression of transposable and repetitive elements, and has additional diverse and complex roles in regulating gene expression at all known levels of epigenetic control. Piwi proteins and piRNAs together have been associated with mRNA, and mRNA cap binding proteins in polysomes and ribonucleoproteins (RNP), which play central roles in translational control;

however, the molecular mechanisms that achieve this translational regulation and the resulting outcome remains unclear (Grivna et al., 2006; Unhavaithaya et al., 2009; Thomson and Lin, 2009). Biochemically purified endogenous rat piRNA complex has been shown to exhibit RNA cleavage activity, presumably facilitated by the rat Piwi protein, Riwi (Lau et al., 2006). On the other hand, mouse Piwi proteins may actually be responsible for the stability of a subset of mRNAs, and positively regulating translation (Deng and Lin, 2002; Unhavaithaya et al., 2009). In addition, Grivna et al. (2006) showed a Piwi protein in mice (Miwi) is not only required for piRNA production, but also for a particular subset of miRNAs. Thus, the piRNA pathway may be involved in miRNA-mediated translational control. One common feature of *Piwi* gene mutations in mice is an increase in DNA damage marked by γH2AX foci, suggesting a possible link to DNA-damage repair/checkpoints (Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). It has been proposed that such dsDNA breaks are a result of over active transposons; however, this relationship is not fully understood, as dsDNA breaks could also be the cause of transposon activity, and not necessarily a result of it (Klattenhoff et al., 2007). Consistent with a possible role of mammalian Piwi-type proteins in DNA repair processes is the presence of RecQ1 in rat Piwi protein complexes (Lau et al., 2006). RecQ is a family of helicase enzymes that have highly conserved roles in dsDNA break repair through recombination (Hunter, 2008). The ability of the piRNA pathway to mediate epigenetic control of gene expression on the level of histone modifications has also been described. Human cells were transiently transfected with a human Piwi (Piwi-like4/Hiwi2) gene

containing a vector construct, which induced histone H3K9 methylation at the p16Ink41 locus, resulting in significant down regulation of p16 gene expression (Sugimoto et al., 2007). A more recent study has provided quite intriguing evidence for the production and function of a particular subset of abundant piRNAs, which are depleted in TE content and do not engage in the ping-pong cycle (Robine et al., 2009). They reported a substantial population of piRNAs derived from untranslated regions (UTR) of protein-coding genes. These genic piRNAs preferentially arise from 3'UTRs, and are produced by a piRNA biogenesis pathway that does not require ping-pong components, and are conserved across *Drosophila,* mice, and *Xenopus* (Robine et al., 2009). This breakthrough finding, as well as the previously discussed studies, provides overwhelming evidence for an additional and much larger breadth of piRNA pathway mediated gene regulation, in addition to TGS of TEs, which still remains unexplained.

The piRNA/Piwi pathway has several features that make it suitable as a mediator of epigenetic memory in germ cells. Mainly characterized by its ability to exert TGS by driving methylation of TE, it clearly has the ability impact genome stability in future generations. Moreover, even though this novel small RNA pathway has been shown to play a role in many of the epigenetic alterations that have been observed in response to IR, no experiments have been conducted to examine the possible role and response of this pathway to IR exposure. Because this pathway is mainly restricted to the male germline in mammals, it provides a novel mechanism to facilitate paternal epigenetic inheritance of IR-induced

genomic instability. This could also provide some insight into the observed loss of LINE1 and global DNA methylation, not only in the germline of exposed males, but more importantly, in the next generation (Koturbash et al., 2006; Tamminga et al., 2008; Filkowski et al., 2010). Understanding if and how the piRNA pathway responds to IR exposure could also potentially corroborate and help elucidate the increased mutation rates observed in satellite DNA and ESTR loci in the somatic and germline tissue from the progeny of exposed parents (Barber and Dubrova, 2006).

The mouse genome encodes three Piwi proteins, all of which play essential and non-redundant roles in virtually all stages of spermatogenesis (Deng and Lin, 2002; Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007; Kuramochi-Miyagawa et al., 2008). Therefore, I will introduce the relevant stages and cellular associations of the rodent germline in order to further discuss, in context, the known roles of the piRNA pathway in spermatogenesis.

1.5 CELLULAR ASSOCIATIONS OF THE TESTES

The model organisms of the studies presented herein are mice and rats; therefore, the focus of this section will be on the biology of the rodent germline. Starting from a self-renewing stem cell pool, male germ cells continually develop from puberty to old age/death. The complete process of male germ cell development is called spermatogenesis, and takes place within the testes (reviewed in Holstein et al., 2003). A testis can be divide into several hundred (~370) lobules that consist of the seminiferous tubules and intertubular tissue

(Fig. 1.1). The intertubular tissue contains groups of endocrine Leydig cells, as well as additional cellular elements. The seminiferous tubules are coiled loops that are connected at both ends to the rete testis (Fig. 1.1). The rete testis is a connecting network of delicate tubules located in the hilum of the testicle (mediastinum testis) that carries spermatozoa from the seminiferous tubules to the vasa efferentia (Fig. 1.1). Fluid containing immature spermatozoa is secreted by the seminiferous tubules and collected in the rete testis to be delivered to the excurrent ductal system of the epididymis where the spermatozoa mature into functional sperm (reviewed in Holstein et al., 2003).

The seminiferous tubules of the testes contain germ cells at various stages of development. The main stages of cell types, in sequential order of development, are known as spermatogonia, primary and secondary spermatocytes, and spermatids (Fig. 1.1). As the spermatogonia divide and mature into various cell types, they move progressively from the basal layer, through the adluminal compartment, to the lumen of the seminiferous tubule (Fig. 1.1). As a germ cell progresses from the basal layer to the lumen of the tubule in what is known as a spermatogenic cycle, it passes through three major stages of development, which are referred to as spermatogoniogenesis, meiosis (of spermatocytes), and spermiogenesis (maturation of spermatids into spermatozoa).

There are two types of spermatogonia, namely A-type and B-type. Type A spermatogonia belong to a self-renewing stem cell population, which divide continuously in successive mitosis to give rise to one A-type and one B-type spermatogonium. B-type spematogonia are committed to undergo further germ

cell development, in which an additional mitotic division gives rise to two primary spermatocytes. This marks the end of spermatogoniogenesis and the beginning of meiosis. Cells in meiosis are called spermatocytes. As the process of meiosis comprises two divisions, cells before the first division are called primary spermatocytes (2n), and cells after the first meiotic division are referred to as secondary spermatocytes (1n). These secondary spermatocytes then undergo the second division of meiosis, giving rise to four haploid round spermatids. These immature spermatids differentiate into spermatozoa in a process called spermiogenesis. Spermiogenesis ends when these cells are released from the adluminal compartment of the germinal epithelium to the lumen, at which point the free cells are called spermatozoa. Importantly, these aforementioned germ cell divisions are usually incomplete. After germ cells divide, their daughter cells remain interconnected by cytoplasmic bridges so that a clone, derived from one stem cell, forms a syncytium of cells (Greenbaum et al., 2007). Syncytial connections are maintained through spermatogonial and spermatocytic stages, and are dissolved only in advanced phases of spermatid development. This allows for rapid communication between cells, and this is believed to be the basis for the synchronous development of germ cells (Hamer et al., 2003).

The aforementioned germ cells of the seminiferous epithilium are located within invaginations of somatic Sertoli cells. These Sertoli cells are connected by specialized zones of tight junctions that separate the germinal epithelium in basal and adluminal compartments (Fig. 1.1, D). These specialized zones, or so-called "tight junctions," form the blood-testis barrier (Parreira et al., 2002). Once

maturing germ cells pass this blood-testes barrier, they are protected from exogenous substances, as well as the host's immune system (Itoh et al., 2005). Sertoli cells also function as "nurse cells" that regulate the flow of nutrients and growth factors required by germ cells (Russel and Griswold, 1993). Furthermore, Sertoli cells are also involved in the production of endocrine and paracrine substances that regulate spermatogenesis and the movement of germ cells within the seminiferous epithelium (reviewed in Mruk and Cheng, 2004; Peterson and Soder, 2006).

Normally, a new cycle of spermatogenesis begins before the preceding cycle has finished. Depending on the length of spermatogenesis and the frequency of new cycles (Hess et al., 1990), a cross section of the testis should reveal several hundred seminiferous tubules, each having a particular cellular association. These particular cellular associations have been categorized into a number of stages that make up a spermatogenic cycle, with 12 and 14 specific stages being identified in mice and rats, respectively (Fig 1.2) (reviewed in Hermo et al., 2010). The length of time that each cell remains in a particular stage is variable, and as a result, the frequency of time spent in each stage also varies (Hess et al., 1990). However, the seminiferous tubules are organized in such a way that these stages occur in a consecutive order. The sequential order and repetition of each stage along the tubule produces what is known as a "spermatogenesis wave" (reviewed in Hermo et al., 2010). The best way to envision this "wave" is that it is to space as the cycle is to time. The stages occur in descending order from the rete testis until approximately half way along the length of the seminiferous tubule where a

reversal is found, resulting in a new "wave," in which the stages will occur in ascending order up to the point of entry into the rete tesis (Hess, 1999). The presence of this wave is well documented in rodent species; however, its existence in humans is still controversial (Schultze, 1982; Johnson, 1994).

1.5.1 Piwi, PiRNAs and Spermatogenesis

Three murine Piwi-like proteins, Miwi (Piwil1), Mili (Piwil2), and Miwi2 (Piwil4), are essential and required for different stages of spermatogenesis. Moreover, they bind to distinct classes of their piRNA partners which are expressed during spermatogenic cycles, with particular sequence content distinguishing piRNA populations from embryonic and pre-meiotic germ cells from those that appear during meiosis throughout spermatogenesis (reviewed in Aravin and Hannon, 2008).

The expression of Miwi begins shortly after birth (14 dpp) and continues until old age/death starting in the pachytene stage of meiosis (spermatocytes) and into the round spermatid stage of germ cells during spermatogenesis (Fig. 1.3) (Deng and Lin, 2002). *Miwi*-null spermatocytes will arrest post-meiotically at the round spermatid stage (Fig. 1.2) (Deng and Lin, 2002). Although the basis for this developmental defect is unknown, Miwi has been posited to act in translational control, and loss of this control is thought to be a contributing factor (Grivna et al., 2006). Furthermore, the expression of Miwi strongly coincides with spermiogenesis, when chromatin is packed in such a manner that transcription does not occur at a significant level (Yu et al., 2003), at which point cells rely on stored mRNAs and posttranscriptional control of gene expression (Pentilla et al.,

1995; Yang et al., 2005). During meiosis, Miwi and Mili have overlapping expression patterns, during which time they both interact with an extremely abundant class of small piRNAs, known as pachytene piRNAs, the function of which remains elusive (Fig. 1.3) (Aravin et al., 2006; Girard et al., 2006). This class of pachytene piRNAs, derived mainly from non-repetitive genomic regions, is, for the most part, lost in *Miwi* mutants, which is also thought to be partially responsible for the post meiotic arrest of spermatogenesis in these animals (Aravin et al., 2006; Girard et al., 2006).

Of the three murine Piwi proteins, Mili is the most broadly expressed. Mili is detected in primordial germ cells (PGS) at 12.5 dpc, and persists during spermatogenesis up until the round spermatid stage (Fig. 1.3) (Aravin et al., 2008). Mili not only has overlapping temporal expression with both Miwi and Miwi2, but also associates with all developmental stage-dependent classes of piRNA (i.e. prenatal/prepachytene, and pachytene piRNAs) (Aravin et al., 2007, 2008; Kuramochi-Miyagawa at al., 2008).

The expression pattern of the third murine Piwi protein, Miwi2, is the most restricted, seen only perinatally in germ cells (gonocytes) from 15.5 dpc until a few days after birth (Fig.1.3) (Aravin et al., 2008). *Mili* and *Miwi2* mutants show quite similar phenotypes with the arrest of germ cell development due to apoptosis at the early pachytene stage of meiosis (Fig. 1.3) (Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007). Both mutants also exhibit enhanced retrotransposon expression in the male germline due to defective *de novo* DNA methylation of the derepressed TEs (Kuramochi-Miyagawa et al., 2008). The time

of overlapping expression of Mili and Miwi2 also coincides with the critical window of time during which male gametic *de novo* methylation patterns are established (Lees Murdock et al., 2003; Kato et al., 2007). It is now accepted that Mili and Miwi2 play distinct but complementary roles in establishing *de novo* methylation patterns that silence TEs in developing male germ cells. This was originally discovered because of their interactions with a discrete population of piRNAs (prepachytene/prenatal) that are expressed at this time (Aravin et al., 2008). These piRNAs are primarily derived from repetitive genomic regions, and show features of a "ping-pong" amplification cycle that drives the sequence specific methylation of TEs, while selectively consuming active TE transcripts to drive the generation of new piRNAs (Fig. 1.4) (Aravin et al., 2008, 2009). Before describing the ping-pong amplification cycle, it is necessary to define primary and secondary piRNAs. In general, piRNAs are designated as primary, not necessarily because of their order of production, but because they have a strong preference for a 5' uridine (1U). Pachytene piRNAs are exclusively primary; however, the subset of prepachytene piRNAs that are involved in the ping-pong cycle generate secondary piRNAs characterized by an adenine 10 nt from the 5' end (10A) (Fig. 1.4) (Aravin et al., 2008).

In the mammalian ping-pong cycle, it is believed that sense transcripts, likely mRNAs of active transposons, represent the major substrate for primary processing (process unknown) of piRNAs that then associate with Mili (Fig. 1.4) (Aravin et al., 2008). These primary sense piRNAs then guide Mili toward recognizing and cleaving antisense transcripts (possibly transcribed from genomic

piRNA gene clusters) that contain transposon sequences (Fig. 1.4). This produces a secondary antisense piRNA that complexes with Miwi2. Miwi2 and its secondary antisense piRNA partner can then either continue in this ping-pong cycle by recognizing complementary RNA transcripts (e.g. transposon mRNA), essentially regenerating a primary sense piRNA that would associate with Mili, or it can guide sequence specific DNA methylation of TE in the nucleus (Fig 1.4) (Aravin et al., 2008). Genetic and molecular characterizations of the interactions between methyltransferases and the piRNA pathway are consistent with piRNA/Piwi complexes directing DNMT3L, and indirectly active methyltransferases (DNMT3a, DNMT3b), to target loci based upon the sequence of their bound, small RNA guides (Aravin et al., 2008, 2009).

In summary, several broad conclusions can be drawn from the existing literature:

1) IR is an important DNA damaging agent that can cause a variety of cellular and biological responses leading to genomic instability, which can further manifest as transgenerational genome instability in unexposed offspring of preconception exposed parents.
2) The effects of radiation exposure, including genome instability (which is linked to carcinogenesis), can manifest in directly irradiated cells, as well as distant unexposed naïve cells, giving rise to the phenomenon known as the "bystander effect."

3) Bystander and transgenerational effects are thought have an epigenetic nature.
4) The germline specific piRNA/Piwi pathway provides a novel molecular mechanism to facilitate the paternal epigenetic inheritance of IR-induced genomic instability.

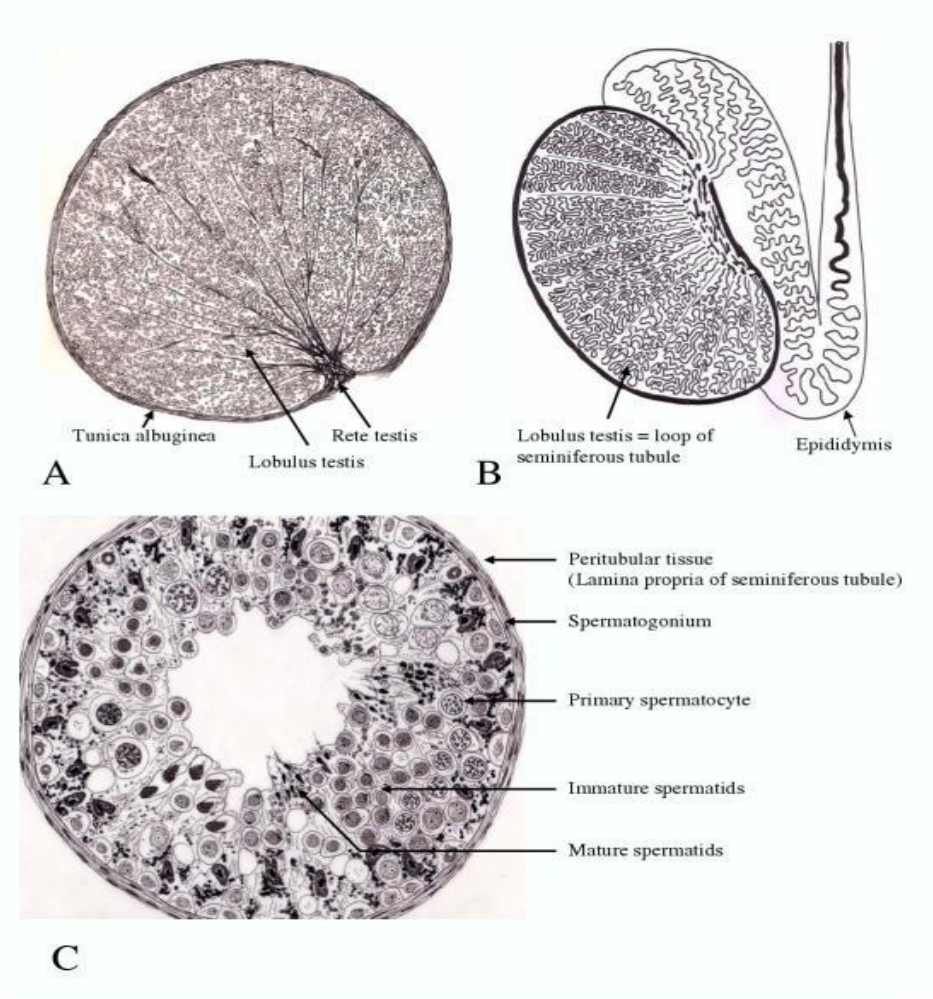
1.6 HYPOTHESES

The current study was designed to explore the possibility that Piwi and piRNA pathway protein levels are altered in response to whole body and localized IR exposure in mice and rats, thereby providing a possible novel molecular mechanism that could facilitate the paternal epigenetic inheritance of IR-induced genome instability.

We hypothesized that a novel small RNA pathway necessary for epigenetic regulation of genome stability in the male germline may play a role in the epigenetic inheritance of radiation induced genomic instability. We predict that acute whole body exposure to X-ray irradiation will lead to a time and dose dependent response in the piRNA pathway. We predict that this would manifest as significantly altered expression of key proteins involved in the small RNA pathway. In addition we predicted that localized exposure could result in bystander effects, manifested as similar significant altered expression of piRNA pathway proteins in the shielded male germline.

Several experiments were designed to test the proposed hypotheses. The experiments are further described as chapters of this thesis. Chapter 2 was

designed to examine if Piwi and piRNA proteins are altered in response to whole body X-irradiation in a time and dose dependent manner. Chapter 3 was designed to examine if localized X-ray exposure casues distant bystander-induced alterations to the piRNA pathway in the shielded male rodent germline (mice and rats)


Tunica albuginea
Rete testis
Lobulus testis
A
Lobulus testis = loop of seminiferous tubule
Epididymis
B
Peritubular tissue
(Lamina propria of seminiferous tubule)
Spermatogonium
Primary spermatocyte
Immature spermatids
Mature spermatids
C

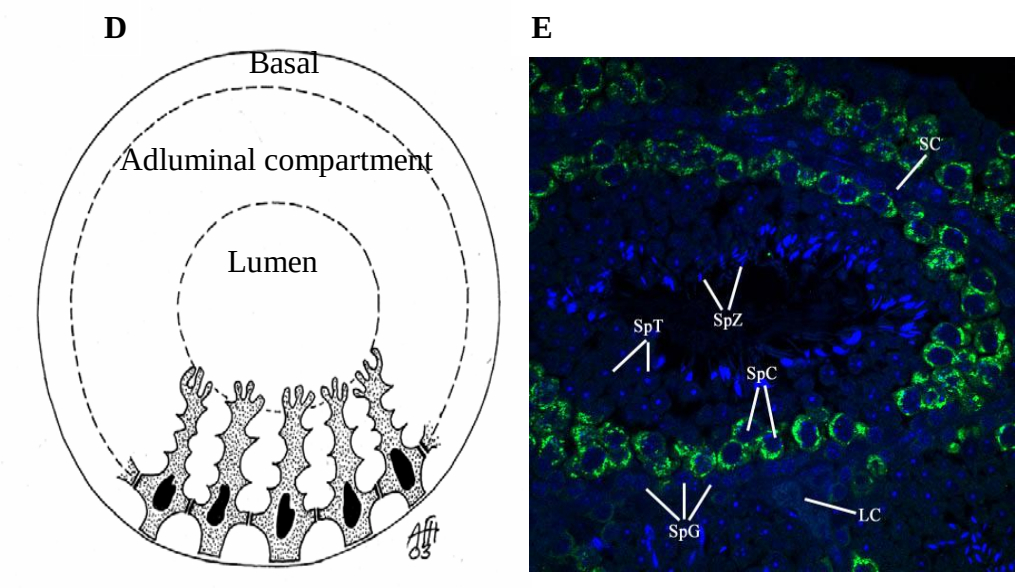

D
Basal
Adluminal compartment
Lumen
E
SC
SpZ
SpT
SpC
SpG
LC

Figure 1.1. Anatomy and cellular associations of the testes. (A) Drawing of a rodent testis cross-section. **(B)** Semi-schematic drawing showing arrangement of seminiferous tubules in rodent testis and of the excurrent ductular system of the epididymis. **(C)** Drawing of a cross section of a seminiferous tubule of a rodent testis. **(D)** Drawing of a cross section of a rodent seminiferous tubule showing Sertoli cells dividing the germinal epithelium into basal and adluminal compartments. **(E)** Seminiferous tubule from cross section of paraffin embedded mouse testis with Mili (green) and nuclear (DAPI) stain (blue). Image taken with a laser scanning confocal microscope (x60). Seminiferous tubule labeled with relevant cell types associated with spermatogenesis: SpG-spermatogonia, SpC spermatocyte, SpT-spermatid, SpZ-spermatozoa, SC-Sertoli Cell, LC-Leydig cell. Adapted with permission from Holstein et al. (2003)

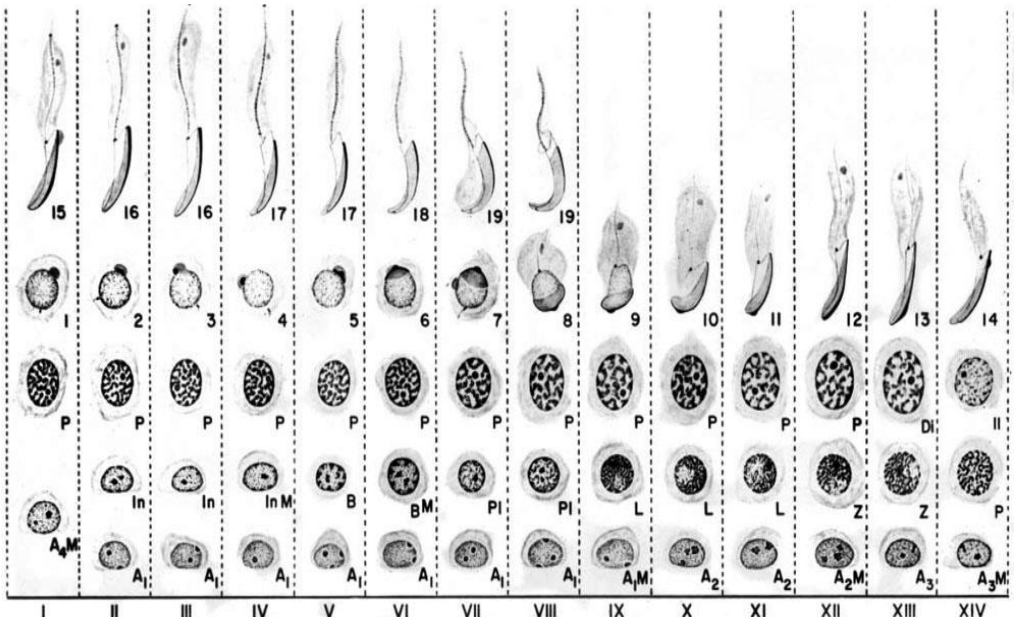


Figure 1.2. Schematic diagram of the 14 stages in the rat seminiferous epithelium. The stages are indicated by roman numerals I–XIV. The vertical columns illustrate the different cell types present in each. The cells illustrated are types A1–4 and intermediate (In), and type B spermatogonia; preleptotene, leptotene, zygotene, pachytene, and diplotene spermatocytes; and spermatids at steps 1–19 of spermiogenesis. M next to spermatogonia indicates that mitosis occurs for these cells, while II at stage XIV indicates secondary spermatocytes undergoing meiotic divisions. Adapted with permission from: Hermo et al. (2010).

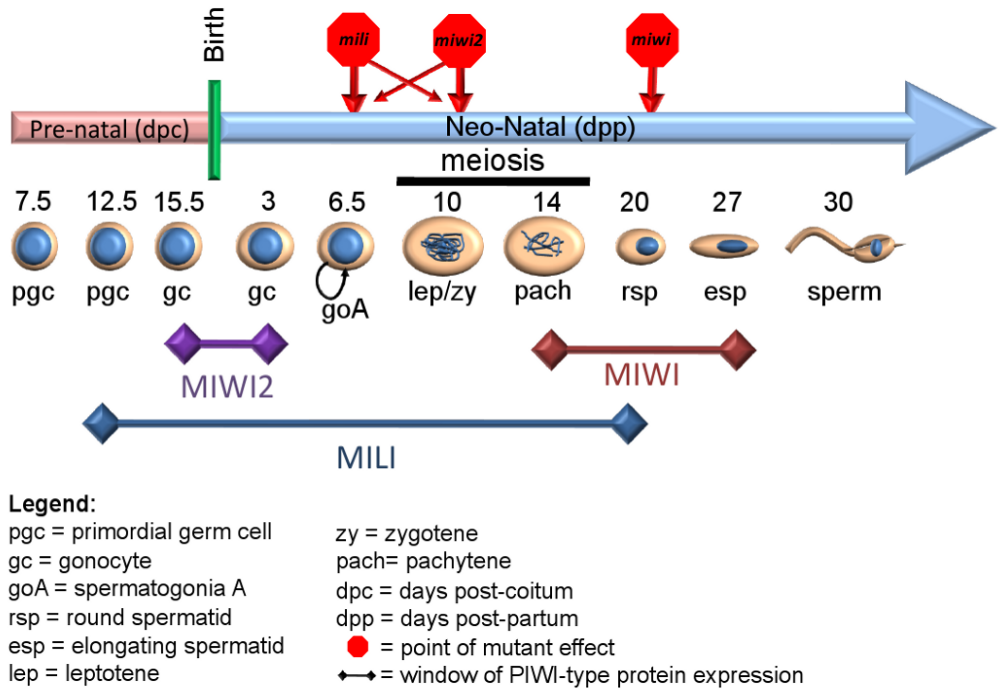


Figure 1.3. Expression of Piwi-like proteins during murine spermatogenesis. A schematic drawing of mouse spermatogenesis on a time coordinate, with MILI, MIWI, and MIWI2 expression periods indicated. Adapted with permission from: Thomson and Lin (2009).

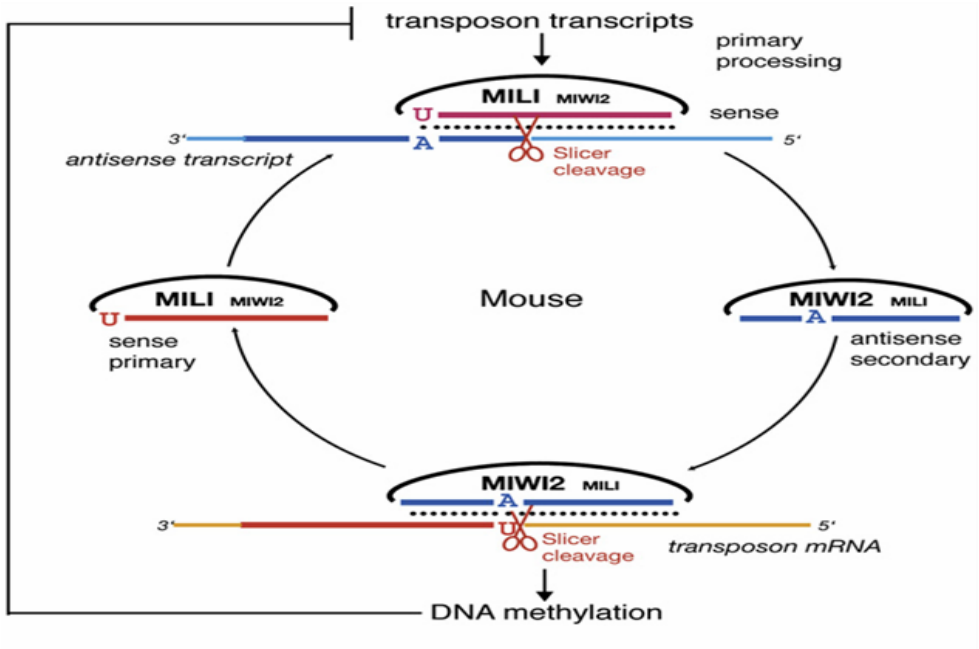


Figure 1.4. The Ping-Pong model for piRNA amplification in mice. Adapted with permission from: Aravin et al. (2008)

CHAPTER 2: ACUTE X-RAY EXPOSURE INDUCES ALTERED EXPRESSION OF PiRNA PATHWAY PROTEINS ESSENTIAL FOR EPIGENETIC MAINTENANCE OF GERMLINE GENOME STABILITY

2.1 ABSTRACT

The number of people that receive acute/chronic exposure to ionizing radiation (IR) via occupational, diagnostic, or treatment related modalities is progressively rising. It is now accepted that the negative consequences of radiation exposure are not isolated to exposed cells or individuals. Exposure to IR can induce genome instability in the germline, and is further associated with transgenerational genomic instability in the offspring of exposed males. The exact molecular mechanisms of transgenerational genome instability have yet to be elucidated, although there is support for it being an epigenetically-induced phenomenon.

The main focus of this study is on the protein component of a novel, small RNA pathway (piRNA pathway). Of primary interest, the Piwi proteins play an integral role in the piRNA pathway, and are essential for epigenetic regulation of gametogeneses and maintenance of germline genome integrity in male mice.

Utilizing western immunoblotting and immunohistochemical analyses, we have demonstrated that the expression of murine piRNA pathway proteins is significantly altered in a time and dose dependent manner in response to whole body X-ray exposure. IR-induced aberrant expression of piRNA pathway proteins may play a key role in producing epigenetic alterations in the male germline that may impact future generations.

2.2 INTRODUCTION

While cancer radiation therapy has led to increased patient survival rates, the risk of secondary treatment related complications is a growing concern. Based on current statistics, approximately 12% of newly diagnosed cancers will occur in patients younger than 50 years of age, of which an estimated 37% will occur in males (Canadian Cancer Society, 2010). Moreover, it is now widely accepted that pre-conception whole-body exposure to ionizing radiation (IR) poses a significant secondary threat to the progeny of irradiated parents, largely attributed to transgenerational genomic instability (Morgan, 2003b, c). Consequently, a major quality of life issue faced by cancer survivors is not only the increased risk of developing secondary treatment related cancer, but also the ability to produce healthy offspring.

IR is well known for being a potent damaging agent that can affect a variety of regulatory processes in exposed cells. Most notably, IR is known to be capable of inducing DNA damage, such as single and double strand breaks, as well as nucleotide base damage, such as cross linking or dimer formation (Little, 2000). IR also has the ability to affect a variety of processes within exposed cells, such as 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; Jeggo and Lobrich, 2006; Valerie et al., 2007; Rodemann and Blaese, 2007). The accumulation of DNA damage caused by IR in conjunction with disrupted cellular

regulation processes can lead to genomic instability (Little, 2000; Barcellos-Hoff, 2005; Sowa et al., 2006)

Genomic instability is characterized by an increased rate of acquisition of alterations within the genome. It manifests as an induction of chromosomal aberrations, aneupliody, micronuclei, gene mutations and amplification, and microsatellite/ESTR instability (Huang, et al., 2003; Morgan, 2003a, b, c; Suzuki et al., 2003). IR-induced genomic instability can be observed in high frequencies in cells generations after the initial exposure (Morgan, 2003a; Limoli et al., 1999, 2000). The loss of genomic stability is believed to be a hallmark of many cancers, as well as an important prerequisite for cancer formation (Goldberg, 2003; Little, 2003; Loeb et al., 2003).

In males, germ cells continually and rapidly divide during spermatogenesis, and thus, are important targets for the effects of IR. IR-induced genomic instability has been well documented in the male germline, and is thought to lead to transgenerational genomic instability in the offspring of preconception exposed males (Dubrova, 2003a, b; Aitken and De Iuliis, 2007). Consequently, there can be a transmission of increased cancer risk to the untreated progeny of parents exposed to radiation; thus termed transgenerational carcinogenesis (Nomura, 2003; Cheng et al., 2004).

Notwithstanding, the exact molecular mechanism of IR-induced genomic instability and transgenerational effects are not well understood, yet accumulating evidence points to the epigenetic nature of this phenomenon (reviewed in Kovalchuk and Baulch, 2008; Jirtle and Skinner, 2007; Dubrova 2003).

Epigenetic alterations are mitotically stable and meiotically heritable changes in gene expression without mutation (i.e. no change in DNA sequence), which include DNA methylation, histone modifications and small RNA mediated events (Jaenisch and Bird, 2003). In particular, a large number of studies have demonstrated the importance of DNA methylation changes in the molecular etiology of IR-induced transgenerational genome instability (Kovalchuk and Baulch, 2008). DNA methylation is an important epigenetic mechanism that regulates gene expression and chromatin structure, and is critically important for normal development, cell proliferation, and the maintenance of genomic stability in a given organism (Ehrlich, 2002; Robertson, 2002; Jaenisch and Bird, 2003; Klose and Bird, 2006; Weber and Schubeler, 2007). Accordingly, altered global DNA methylation patterns are a well known characteristic of cancer cells, frequently characterized by a loss of global genome methylation concurrent with methylation of selected promoter regions, such as tumor suppressors or apoptotic genes (Jaenisch and Bird, 2003; Baylin, 2005; Baylin and Ohm, 2006; Weidman et al., 2007).

Recently a novel, small RNA pathway that is the only epigenetic mechanism known to be able to direct sequence specific methylation of the mammalian genome has been discovered. This newly discovered class of small RNA appears to be specific to animals and interacts with an animal specific class of Argonaute proteins known as the Piwi family (Aravin et al., 2007a). These appropriately named Piwi-interacting RNAs (piRNAs) approximately 26-31nt long have been identified in virtually all animals studied, including mammals

where their expression patterns are largely restricted to the male germline (reviewed in Aravin and Hannon, 2008). The mouse genome encodes three *Drosophila* Piwi protein homologs, Miwi, Mili and Miwi2, all three of which are expressed in high levels in testes at different, but not completely exclusive, times during spermatogenesis (reviewed in Klattenhoff and Theurkauf, 2008). Piwi proteins are required for piRNA production, and all three of the murine Piwi proteins bind piRNAs, and a single null mutation in any of these genes will result in complete male sterility (Deng and Lin, 2002: Kuramochi-Miyagawa et al., 2004; Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006; Carmell et al., 2007). Mili has overlapping expression with Miwi2 and Miwi, and it is first expressed in primordial germ cells (PGS) and subsequently in testis during spermatogenesis up until the round spermatid stage (Fig. 1.3) (Aravin et al., 2008). The expression of Miwi begins ~14 days after birth, as it is expressed during the pachytene stage of meiosis (spermatocytes), and into the elongating spermatid stage during spermatogenesis (Fig, 1.3) (Deng and Lin, 2002). The expression pattern of the third murine Piwi protein Miwi2 is the most restricted, seen only perinatally in germ cells (gonocytes) from 15.5dpc until a few days after birth (Aravin et al., 2008). The developmental stage of arrest during spermatogenesis in *Miwi and, Mili* mutants predominantly coincides with their expression patterns (Carmell et al., 2007; Thomson and Lin 2009; Unhavaithaya et al., 2009).

There are also several non Piwi family proteins that have been recently discovered to play important biological function in the piRNA pathway. For

example, a study has shown that all Piwi proteins in *Drosophila*, mice, and *Xenopus* contain symmetrical dimethyl arginines (sDMAs) (Kirono et al., 2009). The factor that mediates this post-translational modification is a protein complex termed the methylosome that is known to contain an arginine methyltransferase Prmt5 and its co-factor Mep50 (Friesen et al., 2002). Proteomic analyses of murine Piwi proteins have led to the belief that the biological functions of Piwi proteins and the piRNA pathway are highly regulated by Tudor proteins via associations with the Pmt5/Mep50 induced sDMA modifications on Piwi proteins (Kirino et al., 2009; Vagin et al., 2009; Siomi et al., 2010). In addition, Maelstrom (Mael), a murine protein homolog of the *Drosophila* gene *maelstrom* is a protein of unknown biochemical function; however, structural examination has led to the suggestion that the C-terminal contains a domain having the potential to exhibit nuclease activity or an RNA-binding ability that may be implicated in piRNA biogenesis (Zhang et al., 2008). Studies have demonstrated the involvement of Mael in the piRNA pathway supporting a key regulatory relationship with Mili (Soper et al., 2008; Aravin et al., 2009).

The most well characterized function of Piwi proteins in the piRNA pathway is to drive transcriptional gene silencing (TGS) of selfish genetic elements. Mili and Miwi2 facilitate TGS through piRNA sequence driven recognition of the regulatory regions of active transposable elements that need to be targeted for *de novo* methylation in embryonic germ cells (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). Mili and Miwi are then thought to maintain these methylation patterns in the germline throughout life (Aravin et al., 2008;

Aravin and Hannon, 2008). Although the piRNA/Piwi pathway's role in TGS of TEs has been well established, the full extent to which this pathway is able to epigenetically regulate gene expression remains mostly elusive. In mice and rats, piRNA associated with repeats are underrepresented, as approximately 40% of their genome consists of TE, while only ~17% of all piRNAs map to repetitive elements (Vagin et al., 2006; Hartig et al., 2007). In mammals, large numbers of piRNAs are derived from intergenic regions, but are also distributed among exonic and intronic sequences (Grivna et al., 2006). Recently, it has been shown that piRNAs are also generated from the 3'UTRs of a broad set of cellular transcripts (Robine et al., 2000). In addition, numerous recent studies have implicated this pathway in histone modifications, translational regulation, and micro RNA mediate events (Grivna et al., 2006; Lau et al., 2006; Sugimoto et al., 2007; Unhavaithaya et al., 2009).

The piRNA pathway of the male germline has strikingly unique features that make it suitable as a mediator of epigenetic memory in germ cells. In addition to being directly involved in the maintenance of genomic instability, by facilitating DNA methylation of TE, the piRNA pathway has been implicated in the other epigenetic alterations that affect a variety of cellular regulation processes. Many of these alterations, such as histone modifications, microRNAome dysregulation, and most notably, DNA methylation, have been implicated in IR-induced genome instability (reviewed in Kovalchuk and Baulch, 2008). Therefore, it seems that the piRNA pathway may be a major contributor to the phenomenon known as epigenomic instability, used to describe the possible

epigenetic mechanisms underlying certain transgenerational effects (Baulche and Raabe, 2005). However, no studies have ever been undertaken in order to examine whether the piRNA/Piwi pathway plays a role in such responses to radiation exposure. We hypothesized that the piRNA pathway may play a role in the epigenetic alterations involved in the production/inheritance of IR-induced genomic instability. In the current study, we used a well recognized murine model to establish a plausible link between *in vivo* exposure to IR and epigenetic alterations that could be caused by a possible response from the piRNA pathway.

2.3 MATERIALS AND METHODS

2.3.1 Animal Model and Exposure

Mice (mature, 56 day-old males, 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 on Animal Care. The procedures were approved by the University of Lethbridge Animal Welfare Committee. Animals were housed in a virus free facility at the University of Lethbridge in a temperature-controlled room with a 12 h light/dark cycle, housed 3 per cage, and given food and water *ad libitum*.

The first cohort consisted of 41 animals, of which 21 received 2.5 Gy (3 Gy/min) of X-rays (90 kV, 5 mA) to the entire body, and 20 control animals were sham treated. In our previous studies, this dosing scheme led to significant deleterious effects and epigenetic alterations in the germline and progeny of exposed male mice (Filkowski et al., 2010; Koturbash et al., 2006b). For the

irradiation procedure, animals were placed in small (10x5x6 cm) vented plastic containers. These containers safely limit the movement of the animals and insure dose uniformity. For sham treatment, mice were placed in the plastic containers and into the irradiator machine, but the X-rays were not turned on. Four days (96 hrs) after exposure, mice were humanely sacrificed, and then their testis were sampled and processed for further molecular studies. One testis of each animal was immediately frozen in liquid nitrogen and stored at -80ºC, while the other one was fixed in 4% paraformaldehyde (J.T. Baker) for approximately 48 hrs at 4 ºC.

In order to examine a temporal response relationship, the experiment was independently reproduced with a second cohort of 30 animals (20 exposed and 10 controls); however, half of the exposed animals were sacrificed at an earlier time point after irradiation (6 hrs) in addition to the 96 hr time point. Their tissue was harvested and treated in the same way.

In order to examine if there was some type of dose response relationship, a third cohort of animals of the same sex and age were kept in the same manner and exposed to higher [5 Gy (3 Gy/min) (90 kV, 5 mA)] and lower (0.05 Gy) doses of whole body X-irradiation. All animals were humanely sacrificed 6 hours after exposure (6 Sham control, 6 high, and 6 low dose exposed animals). Testes were immediately frozen in liquid nitrogen and stored at -80ºC until further analyses.

2.3.2 Western Immunoblotting

Testis tissue samples destined for protein extraction were snap frozen in liquid nitrogen immediately after removal and stored at -80ºC until further

analysis. Whole testis were sonicated in 1% SDS, and subsequently heated to 95ºC for 5 min, and centrifuged at 10 000 rcf for 5 min. The supernatant (protein extract) was then removed and stored for further analysis after small (25 µl) aliquots were reserved for protein concentration determination using protein assay reagents from BioRad (Hercules, CA). Equal amounts of protein, either 20 µg or 15 µg were loaded per gel, and separated by SDS-polyacrylamide electrophoresis in slab gels of 8% polyacrylamide, and transferred to PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ). Three Membranes were produced for each protein of interest. From the first cohort of animals 18 samples from each group (control and 96 h) were randomly chosen. From the second cohort of animals 9 samples from each group (control, 6 h, and 96 h) were randomly chosen. From the third cohort of animals a sample from every animal in each group was used (control, 5 Gy, 0.05 Gy) with each sample being used in duplicate. The membranes were incubated with antibodies against Miwi (1:1000 rabbit anti-Miwi, Cell Signaling Technology Inc., Boston MA), Mili (1:1000 rabbit anti-Mili, Cell Signaling Technology Inc.), Maelstrom (1:000 rabbit anti-Mael, Abcam Cambridge, MA), Mep50 (1:2000 rabbit anti-Mep50, Cell Signaling Technology Inc.), Prmt5 (1:1000 rabbit anti-Prmt5, Cell Signaling Technology Inc.), actin (loading control) (1:5000; Goat anti-β-actin, Abcam), and α-Tubulin (loadin control) (1:10 000; rabbit ani-α-tubulin, Abcam). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000 goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology Inc.) and ECL plus immunoblotting detection system (GE

Healthcare Biosciences). Chemoluminescence was detected by means of Biomax MR films (Eastman Kodak, New Haven, CT). 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. Potein signals were quantified using NIH ImageJ 1.63 Software and normalized to the actin, tubulin, or the Mr 50 000 protein level, which produced consistent results. Protein levels in the exposed cohorts were related to the controls and presented as percent (100%) change from control.

2.3.3 Immunofluorescence (IF)

The testis tissues destined for IF and IHC were immediately fixed in 4% PFA, pH 7.4, for approximately 48 hrs at 4ºC, rinsed with sterile 1X PBS, pH 7.4, and then stored in 70% ethanol until paraffin embedding. Paraffin embedding and sectioning was conducted at Pantomics Inc. Richmond, CA. The tissues were assembled into tissue microarrays (TMAs) with sections 5 µm thick with a diameter of 4.5 mm by Pantomics Inc. Immunofluorescent staining was conducted using the antibodies against Miwi (1:400, rabbit anti-Miwi, Cell Signaling Technology Inc), Mili (1:300 rabbit anti-Mili, Cell Signaling Technology Inc.), and Maelstrom (1:500 rabbit anti-Mael, Abcam), in accordance with the manufacturers' recommendations. In brief, upon deparaffinization, slides were subjected to heat induced epitope retrieval in a citrate buffer solution, pH 7.4 (Dako Canada, Inc. Burlington, ON), washed 3X 5 minutes in 1X PBS, pH 7.4, then subjected to serum blocking (5% BSA, 5% goat serum in 1X PBS, pH 7.4) for 1.5 hrs to prevent non-specific binding of immunoglobulin. TMAs were then

incubated with a primary antibody diluted in a blocking serum overnight at 4ºC in a humidified chamber. The following day, they were washed 3 X 5 min in 1X PBS, pH 7.4, with 0.1% Tween, followed by a two-hour incubation with a secondary antibody diluted in a blocking serum (1:700, Alexa Fluor Goat anti-rabbit 488 nm, Invitrogen), washed 3X 5 min in 1X PBS, pH 7.4, with 0.1% Tween, dehydrated, and counterstained with Prolong Gold antifade mounting media with 4', 6-diamidino-2-phenyl-iodole, dihyrochloride (DAPI) (Invitrogen Canada Inc. Burlington, ON). For imaging, a laser scanning confocal microscope was used (x60).

2.3.4 Immunohistochemistry (IHC)

Parafin embedding, sectioning, and TMA assembly for IHC analysis were conducted at Pantomics Inc. IHC staining was conducted using the antibody against Miwi (1:300, rabbit anti-Miwi, Cell Signaling Technology, Inc), in accordance with the manufacturers' recommendations. In brief, upon deparaffinization, slides were subjected to heat induced epitope retrieval in a citrate buffer solution, pH 7.4 (Dako Canada, Inc. Burlington, ON), washed 3X 5 minutes in 1X PBS, pH 7.4, subjected to peroxidase blocking for 15 min with 3% hydrogen peroxide in 1XPBS, pH 7.4, washed 3X 5 minutes in 1X PBS, pH 7.4, followed by serum blocking (5% BSA, 5% goat serum in 1X PBS, pH 7.4) for 1.5 hrs to prevent non-specific binding of the immunoglobulin. TMAs were then incubated with a primary antibody diluted in a blocking serum overnight at 4ºC in a humidified chamber, then washed 3X 5 min in 1X PBS, pH 7.4, with 0.1% Tween, followed by incubation with a secondary biotinylated antibody diluted in

a blocking serum for at least 1 hr, subjected to HRP-Streptavidin detection, and counterstained with hematoxylin. Cells positive for Miwi presented as brown, while cells not positive for Miwi stained blue. For imaging, a compound light microscope (x100) was used.

2.3.5 Statistical Analysis of Western Immunoblots

After each sample singnal was quantified and normalized to the loading conrol, each sample value was standardized in order to combine the data from the three membranes that were produced for each protein of interest. Values were standardized by dividing each data point per membrane by the same value (i.e. the mean of the control values on that membrane). Each value was then multiplied by 100 thereby representing percent change from control. A Student's *t* test for independent variance was used to determine significance ($p \leq 0.05$). Statistical analysis and plotting of the results were performed using MS Excel for Windows XP software. The results are presented as mean percent values ± standard error of the mean (SEM).

2.4 RESULTS AND DISCUSSION

2.4.1 Radiation-Induced ChangesIin The Levels of Key PiRNA Pathway Proteins

Analysis of the western immuno blot data revealed altered levels of murine Piwi proteins (i.e. Miwi and Mili) after full body exposure to 2.5 Gy of X-rays (Fig. 2.1). This result was also confirmed by similar observable changes in protein levels detected by IF and IHC (Fig. 2.1, D, and E). Mili protein levels

were significantly altered at both time points after exposure; however, Miwi displayed a time dependent alteration to protein levels as the change in protein level was not significant until 96 hrs after treatment. By 96 hrs post exposure, all of the major proteins of the piRNA pathway analyzed demonstrated a significantly ($p \leq 0.05$) altered amount of protein compared to those of the sham treated animals, while only two proteins were significantly altered 6 hrs post exposure (Fig 2.1). This observation is consistent with a time dependant response of the piRNA pathway after exposure to IR. It should be noted that the sample size for the 6 hr timepoint group was smaller than the 96hr group, which may have contributed to this result.

2.4.2 Mili and Miwi

Most intriguing was the pattern of altered protein levels demonstrated by the two murine Piwi proteins Mili and Miwi, as Miwi levels were significantly increased 4 days after exposure, while Mili was significantly decreased 6 hrs after treatment (Fig 2.1, C). These two proteins are both involved in and necessary for the piRNA pathway, and are sometimes expressed at the same times during spermatogenesis; however, they also have exclusive expression patterns during certain stages of spermatogenesis (Fig. 1.2) (Deng and Lin, 2002; Aravin et al., 2006; Girard et al., 2006; Aravin et al., 2008). Mili and Miwi have been posited to different, but not necessarily mutually exclusive, roles in the epigenetic regulation of gene expression. Specifically, Mili has been shown to additionally bind to a certain population of piRNAs that appear before the pachytene stage of meiosis, which are correspondingly known as pre-pachytene piRNAs (Aravin et al., 2006).

The defining feature of these piRNAs is that a large majority of them map to transposon and retrotransposon repeats (Aravin et al., 2007b). Indeed, in *Mili* mutant mice long interspersed element-1 (LINE-1), and intercisternal A particles (IAP) are derepressed, which is associated with a loss of methylation marks on these repetitive elements (Aravin et al., 2007b; Carmell, 2007; Kuramochi-Miyagawa et al., 2008). It is, therefore, understood that one major role of Mili and pre-pachytene piRNAs is to guide sequence specific *de novo* methylation of certain TEs, which is also believed to be subsequently maintained by Mili (Aravin et al., 2008). The reduction of Mili protein signal in IF stained testis, observed after irradiation at both time points, was most visible in spermatogonia and early spermatocytes (Fig. 2.1, D). Importantly, this also occurs at a stage where pre-pachytene (repeat-associated) piRNAs are primarily associated with Mili, until the onset of meiosis when Miwi is expressed in primary spermatocytes (Fig 1.3) (Aravin et al., 2008, 2009). This may represent a developmental window during spermatogenesis where Mili is required for maintainance of TGS, particularly of TEs.

Previous studies examining transgenerational genome instability examined the mutation rates in repeat elements, known as expanded simple tandem repeats (ESTR) loci, which showed increased mutation rates in the directly exposed germline, as well as in the unexposed progeny of exposed mice (Barber et al., 2002; Yauk et al., 2002; Dubrova et al., 2003). Importantly, studies have shown that irradiation can also cause profound hypomethylation of LINE-1 and SINE B2 retrotransposons in the male rodent germline (Filkowski et al.,

2010). These epigenetic alterations were further observed in somatic tissues of unexposed progeny. Recently, it has also been found that over expression of the human Mili homolog (Hili) in Hela cancer cells causes a significant decrease in the expression of LINE-1 mRNA (Lu et al., 2010). Therefore, it is plausible that the observed significant reduction of Mili in the germline of exposed male mice could result in loss of maintenance of methylation patterns on repetitive elements that could further manifest in the unexposed offspring and contribute to elevated mutation rates and genomic instability. Further analyses into what effect an IR-induced reduction of Mili has on the type/populations of bound piRNA sequences could either substantiate or provide further insight into such a speculation.

As previously mentioned, the effect of IR on Miwi expression was opposite of that observed for Mili (Fig. 2.1). This result also seems to correspond to and illustrate the proposed specialized role of Miwi in the piRNA pathway. Several lines of evidence have demonstrated that Miwi has the potential to play a key role in translation regulation (Grivna et al., 2006; Lau et al., 2006; Unhavaithaya et al., 2009). Miwi proteins and their piRNAs partners together have been associated with mRNA, and mRNA cap binding protein eIF4E, in polysomes and ribonucleoprotein fractions (RNP), which play fundamental roles in translational control (Grivna et al., 2006; Unhavaithaya et al., 2009). Biochemically purified endogenous rat piRNA complexes are also able to exhibit RNA cleavage activity, presumably directly facilitated by Riwi the rat homolog of Miwi (Lau et al., 2006). Additionally, Miwi is found in ribonucleic protein fractions along with a testis-expressed kinesin, KIF17b, which is believed to

function in nuclear to cytoplasmic (i.e. to the chromatoid body) shuttling of mRNA and RNA binding proteins in regulating translation during spermiogenesis (Kotaja et al., 2006). Miwi is also suspected to play an additional, more indirect, role in translational regulation, as it is required for a particular subset of testis specific microRNAs in addition to its associated piRNAs. Thus, Miwi may also be involved in microRNA pathway-mediated translational control (Grivna et al., 2006). It has been found that hypomethylation and genome instability in the germline of exposed fathers and their progeny is associated with altered miRNA expression, and therefore, this altered miRNA expression could be connected to IR-induced alteration of Miwi levels (Filkowski et al., 2010).

There is certainly a large and increasing body of evidence suggesting that Miwi and the piRNA pathway have some role in translational regulation, which if true, could provide insight into the up regulation of Miwi that was observed in the exposed mice. If Mili has a primary involvement in TGS, and is reduced in response to IR, whether this is a deleterious or protective response, it may cause a downstream effect in the expression of Miwi. Mili is expressed before Miwi in a subset of spermatogonia until the round spermatid stage, when only Miwi is expressed (Fig 1.2) (Kuramochi-Miyagawa et al., 2004; Unhavaithaya et al., 2009), which is also when transcription no longer occurs in these cells at significant levels (Yu et al., 2003). At this stage of development, these cells rely on stored mRNAs and posttranscriptional control of gene expression (Pentilla et al., 1995; Yang et al., 2005). Importantly, the qualitative analysis of our IF and IHC data revealed that Miwi expression appeared to be largely increased in round

spermatid stage cells (Fig 2.0., D, and E). There was a noticeable increase in Miwi levels in what appeared to be secondary spermatocytes as well. The increased expression of Miwi in spermatids might have a larger significance because this corresponds to time when Miwi is acting alone, as Mili is no longer expressed. The increase in Miwi protein levels could be related to an increased need for translational repression, as a result of decreased Mili expression, and therefore, possibly altered TGS. As further research is needed to verify such a relationship, isolation of the populations of Miwi and Mili bound piRNAs after such IR exposure, and elucidation of their functional targets could provide such evidence.

The translational control of transposon activity by the piRNA pathway was also highlighted in a study where an insertion mutation in a piRNA cluster coding region (Nct1/2) caused substantial decrease of a select population of piRNAs of which many contained sequences complementary to the 3'UTR of a LINE-1 retrotransposon (Xu et al., 2008). In contrast to *Mili* and *Miwi* mutants, in which all piRNA production is lost, this *Nct1/2* mutant displayed normal spermatogegnesis and fertility rates (Xu et al., 2008). One of the most striking contrasts was that there was no major increase in the expression of LINE-1 mRNA; however, the LINE1-encoded protein (ORF1) was markedly increased (up to 15-fold) (Xu et al., 2008). The small increase in LINE-1 mRNA and large increase in ORF1 suggests posttranscriptional gene regulation. It should be noted, however, that there was no change in Miwi levels and whether the knocked down piRNAs interacted with Miwi, Mili, or both, was not looked into. However,

immunohistochemistry of testes sections did show that ORF1 was up-regulated in elongating spermatids when only Miwi is expressed (Deng and Lin, 2002; Xu et al., 2008).

2.4.3 Maelstrom

In order to gain further insight into the response of the piRNA pathway to radiation exposure we also looked at the level of Maelstrom (Mael), a murine protein homolog of the *Drosophila* gene *maelstrom* (Clegg at al., 1997). Mael is a protein of unknown biochemical function, but is has been found to contain an N-terminal domain with weak similarity to a DNA binding motif known as a HMG domain (Bianchi and Agresti, 2005). Further structural examination has led to the suggestion that the C-terminal contains a domain having the potential to exhibit nuclease activity or an RNA-binding ability that may be implicated in piRNA biogenesis (Zhang et al., 2008). Indeed, there is a direct involvement of Mael in the rodent piRNA pathway (Soper et al., 2008; Aravin et al., 2009). The studies that have demonstrated the involvement of Mael in the piRNA pathway also provide evidence in support of a key regulatory relationship with Mili. This may also partially explain the significant down regulation of Mael 96 hrs after treatment which correspondingly was also observed in Mili protein levels (Fig. 2.1).

Importantly, *Mael*-mutant mice show almost identical phenotypic traits as those in *Mili*-mutants (Soper et al., 2008). This includes complete arrest of spermatogenesis due to apoptosis during meiotic prophase I (pachytene stage), DNA damage, reduced DNA methylation, and derepression of LINE1

retrotransposons (Kuramochi-Miyagawa et al., 2004; Carmell et al., 2007, Soper et al., 2008). In a recent study, *Mili*-mutant mice lost almost all Mael protein signals in IF stained testes sections and no Mael granules or nuclear localization could be detected (Aravin et al., 2009). We were not able to clearly identify the loss of perinuclear localization of Mael; however, there was an observable loss of Mael signals from the chromatoid body of spermatids (Fig 2.1 D). It should be noted, however, that there was also loss of Mael protein signal throughout the seminiferous epithelium from spermatogonia until spermatozoa (Fig 2.1 D). The only cells expressing Mael that demonstrated little visible loss of protein signals after exposure were somatic Sertoli cells (Fig 2.0 D). This may be due to a specialized role of Mael in the germ cell restricted piRNA pathway response to IR.

Interestingly, in one of the first *Mael* knockout mouse studies, it was shown that there was loss of LINE1 methylation, which corresponded to the transcriptional derepression of LINE1 retrotransposons (Soper et al., 2008). This analysis was performed on adult mouse testes, whereas a more recent *Mael* knockout study was performed on fetal testes (i.e. gonocytes), and they found that the loss of Mael did not derail the acquisition of *de novo* methylation of LINE1 elements (Aravin et al., 2009). These data suggest that Mili and Mael cooperate in certain functions of the piRNA pathway, with Meal expression or localization being reliant on Mili. Specifically, these data imply that Mael is not necessarily required for *de novo* methylation, but does appear to be required for maintenance of this methylation. Therefore, Mili and Mael may also cooperate in some way,

during a possible piRNA pathway response to radiation exposure, possibly in the maintenance of *de novo* methylation patterns.

2.4.4 Prmt5 and Mep50

All three mouse Piwi proteins have been demonstrated to complex with the Prmt5/Mep50 methylosome (Vagin et al., 2009). SDMAs are known to be able to modify the stability of a protein, as well as its ability to perform biological functions (Bedford and Clarke, 2009; Kirono et al., 2009). These arginine methyl marks are recognized by a family of Tudor domain proteins, whose members have also shown specificity for interaction with all three murine Piwi proteins (Vagin et al., 2009). Tudor family members interact with particular proteins via the methylated arginines of their target proteins, thus giving them the ability to regulate their function (Cote and Richard, 2005). In fact, genetic studies have linked Tudor family members to gametogenesis even before the discovery of piRNAs and characterization of Piwi proteins (Boswell and Mahowals, 1895; Chuma et al., 2006). Although studies describing mouse *Prmt5*-mutants have not yet been published, studies on *Drosophila Prmt5*-knockouts and proteomic analysis of murine Piwi proteins have led to the belief that the biological functions of Piwi proteins and the piRNA pathway are highly regulated by Tudor proteins via associations with the Pmt5/Mep50 induced sDMA modifications on Piwi proteins (Kirino et al., 2009; Vagin et al., 2009; Siomi et al., 2010). Therefore, we decided to analyze the protein levels of Prmt5 and its Mep50 cofactor in order to further verify another level of piRNA pathway response to radiation. We observed a similar significant down regulation of protein levels 96

hrs after exposure for Prmt5 and Mep50; however, a similar alteration to protein levels was not the case 6 hrs after irradiation as only Mep50 was significantly decreased (Fig 2.1). A similar pattern of altered expression in both of these proteins was expected to occur, solely based on the reasoning that together, they make up the main components of the methylosome complex, and therefore, should exhibit similar altered protein levels (Friesen et al., 2002). Importantly, the Mep50 cofactor is needed for the interaction of Prmt5 with mouse Piwi proteins, and is thought to specifically bridge the interactions between the mouse Piwi proteins and Prmt5 (Vagin et al., 2009). Therefore reduction of Mep50 alone may specifically alter the methylosomes' ability to incur sDMA modifications on Piwi proteins. Owing to some discrepancies in the methylation status observed in mouse Piwi proteins, it has also been suggested that Piwi protein methylation is regulated dynamically through spermatogenesis (Siomi et al., 2010). This dynamic regulation of post-translational modifications may also apply to the piRNA pathway's response to radiation. The altered regulation of Prmt5 and Mep50 protein levels observed in response to radiation exposure provides further support for the piRNA pathways involvement in germline responses to radiation, and possibly, epigenetic alterations that can effect future generations.

2.4.5 Effect of High and Low Level Radiation Exposure On PiRNA Pathway Protein Levels

After observing a significant response in the regulation of piRNA pathway protein levels after 2.5 Gy of whole body X-ray exposures, we then decided to examine if there was a different or dose dependent reaction in response to high

and low doses of X-ray exposure. Because cellular responses to low levels of radiation (i.e. 0.05 Gy) may be difficult to detect at later time points, and because high doses may result in a major loss of certain germ cell types we analyzed the same protein levels 6 hours after 5 Gy and 0.05 Gy X-ray exposures. We observed an overall trend in protein level changes that was similar to the overall trend previously observed in animals exposed to 2.5 Gy at the same time point (Fig. 2.1 and 2.2). Even though a similar trend of Miwi being up regulated with Mili, Mael, Mep50, and Prmt5 being down regulated was observed, we also observed key differences in the responses induced by the high and low exposure levels. In particular, the low dose of 0.05 Gy actually induced a larger change in all protein levels than that of the high 5 Gy dose (Fig. 2.2). The protein level change induced by 0.05 Gy exposure at 6 hrs was also greater than that of the 2.5 Gy exposure group at 6hrs in all proteins, with the exception of Mael (Fig. 2.1, and 2.2). This seems to suggest that the piRNA pathway is a sensitive responder to radiation exposure even at low-level radiation, and that these low-level doses may actually induce the largest responses. It should be noted, however, that the sample size for the 0.05 Gy group was n=12 with each animal being represented twice (Fig 2.2) . Whereas the 2.5 Gy (6 hr) group had a smaller sample size of n=9 with each animal being represented once (Fig. 2.1). Indeed, the biological effects of low-level radiation differ from those of high-level radiation, including a non-linear dose to damage relationship (i.e. increase dose ≠ increased damage), as well as its stimulatory effect on cellular metabolisms and defense systems (Cia, 1999; Feinendegen, 2005; Liu, et al., 2006), and therefore, the same may apply for the

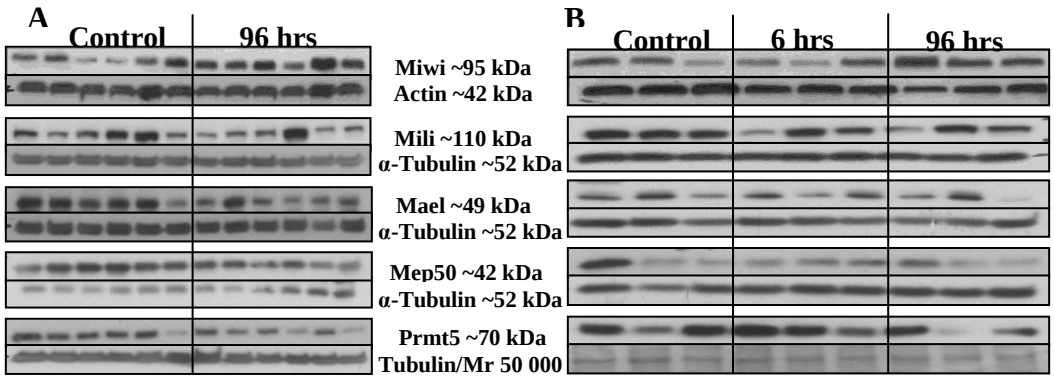
piRNA pathway. One other possible explanation for the high-level radiation dose group displaying less altered protein levels, especially Miwi, may be the increased induction of apoptotic pathways at higher doses of radiation. At higher doses of radiation, there can be a significant increase in the loss of primary spermatocytes due to apoptosis (Cordelli et al., 2002). This is most likely attributed to stage-dependent variations in radiosensitivity. The most radiosensitive germ cells are differentiating type A spermatogonia, and mitotic phase type B spermatogonia. Therefore, at higher doses, there will be an increase in the induction of apoptosis in these cell types, resulting in less primary spermatocytes (West and Lahdetie, 2001; Cordelli et al., 2002). Importantly, apoptosis that regulates germ cell density during normal spermatogenesis occurs in large populations of interconnected spermatogonia, because apoptotic signals are transmitted through intercellular bridges that connect the spermatogonia (Huckins, 1978). However, radiation induced apoptosis occurs through an alternative pathway, as spermatogonia individually undergo apoptosis without affecting other interconnected spermatogonia (Hamer et al., 2003). Because Miwi expression begins in primary spermatocytes, an increase in radiation, and therefore, in the induction of apoptosis, could result in less overall Miwi protein, and because non-apoptotic affected spermatocytes should still exhibit IR-induced increases in Miwi protein levels, the total Miwi protein level may therefore more closely resemble that of the control levels (Fig 2.2). Although an increase in the induction of apoptotic pathways may differentially affect the levels of piRNA pathway proteins, it does not explain the trend of altered protein levels we observed for the

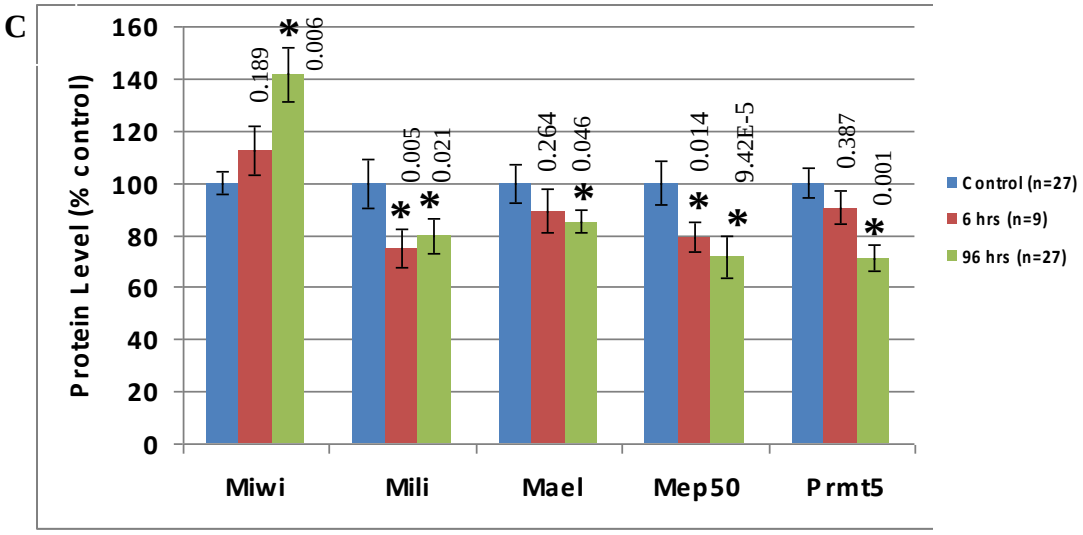
high and low dose exposure groups. We looked at protein levels 6 hrs after exposure, and therefore, the full effects of apoptosis on cell populations should not be overly significant, as studies examining radiation induced death of mouse germ cells typically use 12 hrs post exposure as a starting point (Liu et al., 2006). However, this does not rule out the possibility that the induction of apoptotic pathways had some effect on piRNA protein levels or vice versa. For instance, it has been shown that the over expression of human Piwil2 (Hili) can inhibit apoptosis through activation of the Stat3/Bcl-X_L pathway (Lee at al., 2005). Although future studies are needed in order to address this issue, we have provided compelling evidence for a radiation-induced dose dependent response of the piRNA pathway.

2.5 CONCLUSIONS

This was the first ever study to show that there is a piRNA pathway response to genotoxic stress exposure. PiRNA pathway protein levels were significantly altered in a time and dose dependent manner in response to whole body X-irradiation. Whole body exposure to IR is known to cause germline epigenetic alterations that can lead to genome and epigenome instability in unexposed progeny (Kovalchuk and Baulch, 2008). The piRNA pathway is a novel epigenetic mechanism that regulates various cellular processes during spermatogenesis, including the maintenance of genome stability. We have demonstrated that this may very well include germline responses to genotoxic stress. Whether the piRNA pathway response to radiation exposure is deleterious and/or protective remains to be seen; however, the piRNA pathway does provide

a novel epigenetic mechanism poised to be involved in transgenerational radiation effects, such as genome and epigenomic instability. Further studies are clearly needed to understand the molecular, biological, and evolutionary consequences of piRNA pathway protein level changes induced by radiation exposure and the impact this may have on male germline genome integrity.


A
Control
96 hrs
B
Control
6 hrs
96 hrs
Miwi ~95 kDa
Actin ~42 kDa
Mili ~110 kDa
α-Tubulin ~52 kDa
Mael ~49 kDa
α-Tubulin ~52 kDa
Mep50 ~42 kDa
α-Tubulin ~52 kDa
Prmt5 ~70 kDa
Tubulin/Mr 50 000


C
Protein Level (% control)
160
140
120
100
80
60
40
20
0
0.189
0.006
0.005
0.021
0.264
0.046
0.014
9.42E-5
0.387
0.001
Miwi
Mili
Mael
Mep50
Prmt5
Control (n=27)
6 hrs (n=9)
96 hrs (n=27)

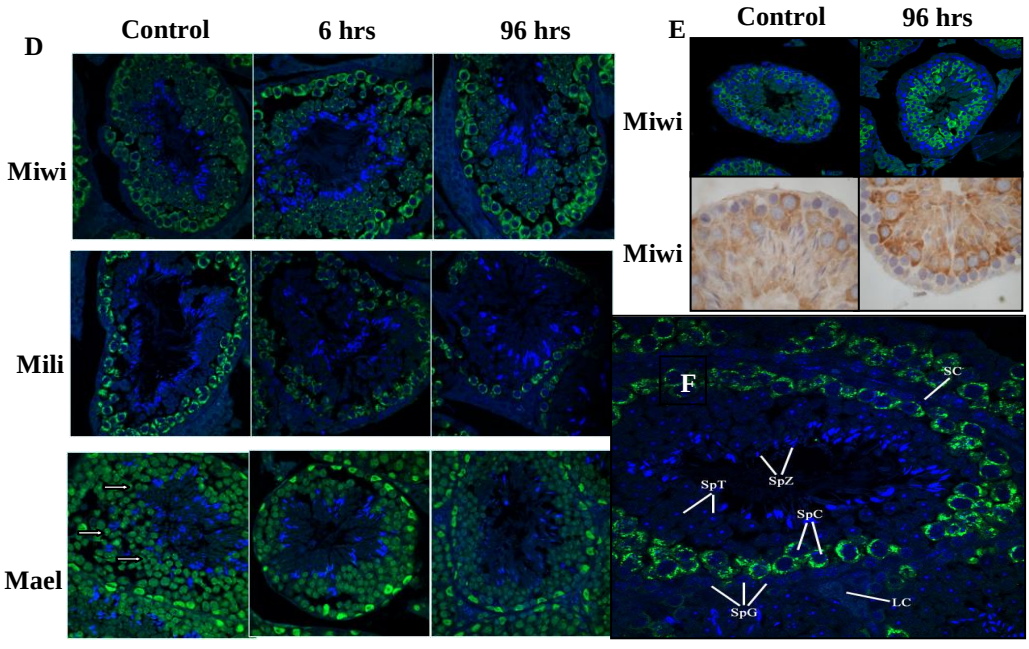

D
Control
6 hrs
96 hrs
Miwi
Mili
Mael
E
Control
96 hrs
Miwi
Miwi
F
SC
SpZ
SpT
SpC
SpG
LC

Figure 2.1. Acute whole body exposure to 2.5 Gy of X-irradiation leads to altered regulation of piRNA pathway protein levels. (A, B) Western immunoblot of whole testis lysate using antibodies against Miwi, Mili, Mael, Mep50 and Prmt5 proteins and actin, α-tubulin (loading controls), and coomassie stain (loading control) . **(C)** Analysis of western immunoblot. Protein levels relative to controls (100%) are shown, ±SE, *$p \leq 0.05$, data labels represent p-value. **(D)** Immuno-fluorescent analysis of protein levels in sections of paraffin embedded mouse testes using antibodies against Miwi, Mili and Mael (green), with nuclear (DAPI) stain (blue), block arrows on Mael picture (control) showing chromatoid body, images taken with a laser scanning confocal microscope (x60). **(E)** Immuno-fluorescent and immuno-histochemical analysis of Miwi protein levels in paraffin embedded mouse testes using antibodies toward Miwi (green/brown, upper and lower panel respectively) and counterstained with DAPI nuclear stain (blue, upper panel) and Hemotoxylin (blue, lower panel). IF images taken with a laser scanning confocal microscope (x60).IHC images taken with compound light microscope (x100). **(F)** Seminiferous tubule from cross section of paraffin embedded mouse testis with Mili (green) and nuclear (DAPI) stain (blue). Image taken with a laser scanning confocal microscope (x60). Seminiferous tubule labeled with relevant cell types associated with spermatogenesis: SpG-spermatogonia, SpC spermatocyte, SpT-spermatid, SpZ-spermatozoa, SC-Sertoli Cell, LC-Leydig cell.

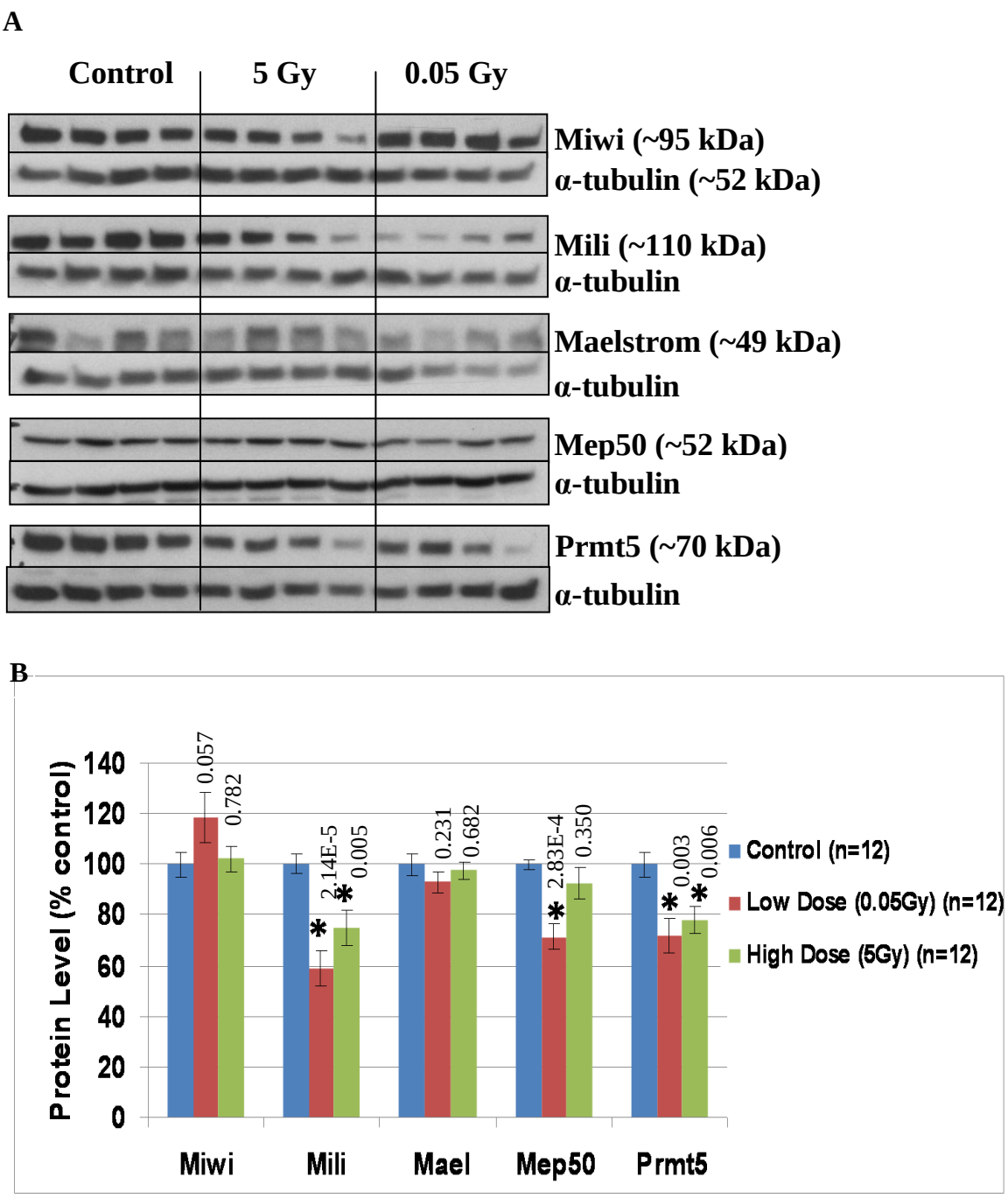


Figure 2.2. Altered regulation of piRNA pathway protein levels after high and low-level X-ray exposure. (A) Western blot of whole testis lysate using antibodies against Miwi, Mili, Mael, Mep50, Prmt5, and α-Tubulin proteins (loading control). **(B)** Analysis of western blot. Protein levels relative to controls (100%) are shown, ±SE, *p≤0.05, data labels represent p-value.

CHAPTER 3: LOCALIZED X-RAY EXPOSURE CAUSES DISTANT BYSTANDER-INDUCED ALTERATIONS TO THE PiRNA PATHWAY IN THE SHIELDED MALE RODENT GERMLINE

3.1 ABSTRACT

It is now generally accepted that paternal whole body irradiation causes transgenerational genome and epigenome instability in the offspring. The majority of human exposures to radiation, such as therapeutic and diagnostic irradiations, are localized and focused. Even though 'bystander' effects are accepted as a ubiquitous consequence of radiation exposure, which can result in the genome instability in distant unexposed cells, bystander effects on the germline remain under investigated. Localized radiation exposure has only recently been demonstrated to cause distant bystander effects in the male germline, leading to epigenetic alterations linked to genome instability in the offspring.

A novel small RNA pathway (piRNA) that specializes in epigenetic regulation of male germ cell genome integrity during spermatogenesis has also recently been discovered. The involvement of the piRNA pathway in germline responses to ionizing radiation has never been studied. Here we report that X-ray exposure localized to the cranium or liver induces a significant alteration in the levels of key regulatory piRNA pathway proteins, in the shielded male germline. Significant radiation-induced alteration to key piRNA pathway protein levels was observed in both mice and rats suggesting that altered piRNA pathway protein levels may represent a conserved male germline response to radiation exposure.

3.2 INTRODUCTION

Currently, it is well accepted that pre-conception whole body radiation exposure poses a significant threat to the progeny of irradiated fathers by inducing DNA damage in sperm cells (Aitken and De Iuliis, 2007). This damage has not been fully characterized, though in addition to direct DNA damage such as strand breaks it is known to include epigenetic alterations such as DNA methylation (Aitken and De Iuliis, 2007). Such damage to sperm cells may affect fertilization and embryo development and cause a number of harmful phenotypic and genotypic effects in the offspring (Aitken and De Iuliis, 2007). Phenotypic effects include decreased fertility rates and a variety of teratogenic effects, whereas genotypic effects consist of increased mutation rates and elevated frequencies of chromosome aberrations, micronuclei formation, altered gene expression, and many other hallmarks of radiation-induced genomic instability (Dubrova, 2003a; Morgan, 2003b; Nomura, 2003; Barber and Dubrova, 2006; Morgan and Sowa, 2007). Therefore, exposure to ionizing radiation (IR) leads to genomic instability in exposed cells including the germline, and further to transgenerational genomic instability in the unexposed offspring from fathers exposed to radiation prior to conception. This could predispose the offspring to an increased risk of genetic diseases, infertility, and cancer (Mohr et al., 1999; Dubrova, 2003b; Barber and Dubrova, 2006).

The majority of studies on germline and transgenerational radiation effects have analyzed the consequences of whole body parental exposure; however, such exposure in human populations is relatively rare. In contrast, localized exposure

of body parts or organs occurs much more frequently in humans during radiation therapy and diagnostics. One third of people alive today are likely to develop cancer, and more than half of these people will receive some form of radiation treatment (American Cancer Society, 2003). Of all the radiotherapy procedures, brain irradiation is among the most widespread, as brain tumors are the second most common cancers in young adults of reproductive age (Huff, 2007; Lyons and Vora, 2007). The recent advances in radiation therapy have increased the successful treatment of cancer, resulting in increased patient survival rates. Yet a possible quality-of- life issue that these survivors face is not only an increased risk of developing a secondary treatment-related cancer but also the production of healthy offspring. The potential for localized radiation exposure to affect the germline and thus induce genomic instability and deleterious changes in unexposed progeny needs to be addressed.

Delayed and non-targeted effects of IR have become especially important in recent years, as it is now known that exposed cells can communicate radiation-induced damage to distant unexposed cells, resulting in genomic instability (Morgan, 2003a, b, c; Morgan and Sowa, 2007). This phenomenon is widely known as a "bystander" effect and has been well documented *in vitro* using cell and tissue culture models (Persaud et al., 2005; Sedelinkova et al., 2007). Consequently, bystander effects are now accepted as a ubiquitous consequence of radiation exposure (Mothersill and Seymour, 2004; Morgan and Sowa, 2007). Several studies have demonstrated that bystander effects also occur *in vivo*, and that localized body or cranial irradiation can yield profound epigenetic changes in

distant protected tissues and organs, including the rodent germline (Koturbash et al., 2006a, 2007, 2008; Tamminga et al., 2008; Filkowski et al., 2010). These epigenetic changes primarily encompass profound alteration of DNA methylation and methylation machinery, microRNAome, and histone modifications, which are believed to be an underlying force driving genomic instability (reviewed in Kovalchuk and Baulch, 2008). Epigenetic alterations can induce mitotically stable and meiotically heritable changes in gene expression and are believed to play a key role in the molecular etiology of IR-induced transgenerational genomic instability (Kovalchuk and Baulch, 2008). Indeed, it has been found that epigenetic changes in the irradiated male germline can further result in profound epigenetic dysregulation in unexposed offspring, including hypomethylation of retrotransposable elements (Tamminga et al., 2008; Filkowski et al., 2010).

Small RNAs such as miRNAs play crucial roles in the epigenetic regulation of many aspects of cell growth and differentiation and have even been implicated in predisposition to cancer (Chang and Mendell, 2007; Fabbri et al., 2007). The role of miRNAs also includes cellular responses to radiation; in particular, aberrant miRNA expression has been associated with germline responses to direct IR and bystander effects, which are further altered in unexposed offspring and therefore associated with transgenerational effects (Tamminga et al., 2008; Filkowski et al., 2010). The altered miRNA expression is also indirectly implicated in altered DNA methylation levels as regulatory miRNAs targeting methylation-specific proteins are altered in response to IR (Tamminga et al., 2008b; Filkowski et al., 2010).

Recently, a novel small RNA pathway in addition to the miRNA pathway has been discovered, which has added another level of small RNA–mediated epigenetic regulation. Compared to miRNAs, this newly discovered pathway has several known features that make it suitable as a chief mediator of epigenetic memory in germ cells (reviewed in Aravin and Hannon, 2008). This novel pathway involves a new class of small RNA that specifically interacts with an animal-specific clade of Argonaute proteins, the Piwi family (Aravin et al., 2007). These Piwi-interacting RNAs (piRNAs) have been described in most animals studied, including mammals, where their expression pattern is largely restricted to the male germline (Aravin and Hannon, 2008). Although our understanding of Piwi proteins and piRNA biology has grown substantially during the last several years, major gaps still exist regarding the full biological roles of this enigmatic piRNA pathway. Importantly, the piRNA pathway has clear roles in maintaining germline genome stability, as it enforces silencing of transposable elements by directing site-specific methylation during male germ cell development (Aravin et al., 2007a, b; Aravin et al., 2008; Kuramochi-Miyagawa, 2008). As such, the piRNA pathways represent perhaps the only currently known sequence-specific mechanism for deposition of DNA methylation in mammals. Interestingly, the piRNA pathway is known to be specifically involved in the methylation of retrotransposable elements such as LINE-1 and SINE B2, which have been found to be hypomethylated in the germline of X-ray exposed parents as well as their offspring (Aravin et al., 2007a, b; Aravin et al., 2008; Kuramochi-Miyagawa, 2008; Filkowski et al., 2010). It is clear that the piRNA pathway has key roles in

such maintenance of genome stability as well as additional biological roles that are essential for epigenetic regulation of germ cell development (Aravin and Hannon, 2008). However, the possibility that the piRNA pathway may play a key role in epigenetic responses of the male germline associated with radiation exposure has never been addressed.

We therefore decided to investigate the possible role of the piRNA pathway in germline responses associated with IR-induced bystander effects. We recently found that key piRNA pathway proteins are altered in response to direct IR exposure (unpublished). Because bystander effects are considered ubiquitous consequences of radiation exposure, which also produce epigenetic alterations in the shielded germline, we decided to examine changes in piRNA pathway protein levels after localized exposure to X-rays. This would therefore, in part, define the alteration of piRNA pathway protein levels as a conserved male germline response to IR. IR-induced alteration to protein levels intimately involved in the piRNA pathway may be involved in producing epigenetic alterations in the male germline associated with IR exposure and transgenerational genome instability.

3.3 MATERIALS AND METHODS

3.3.1 Animal Model: Irradiation Scheme and Tissue Sampling

Five-month-old male Long–Evans rats (36 animals) and 56-day-old male C57BL/6 mice (36 animals) were randomly assigned to the different treatment groups. Mice treatment groups consisted of cranial-exposed, body-exposed, and sham-treated animals (12 animals per group), while rats had an additional group of liver-exposed animals (10 animals per exposure group, and 6 sham-treated animals). Certain organs such as the spleen and liver are known to be targets of and behave differently to radiation exposure, including bystander effects (Brooks, 2004; Koturbash et al., 2006a, 2007, 2008). Therefore we also subjected a cohort of rats to localized liver X-irradiation to examine if different organs have distinct roles in producing germline bystander responses in the piRNA pathway. Handling and care of animals were performed in accordance with the recommendations of the Canadian Council on Animal Care. The procedures were approved by the University of Lethbridge Animal Welfare Committee. Animals were housed in a virus-free facility at the University of Lethbridge in a temperature-controlled room with a 12 h light/dark cycle and were given food and water *ad libitum.*

The body exposed cohort of animals received 2.5 Gy (3 Gy/min) of X-rays (90 kV, 5 mA) to the entire body. The cranial-exposed cohort received 2.5 Gy (3 Gy/min) of X-rays (90 kV, 5 mA) to the entire (mice) or hippocampal area (rats) of the skull. The cohort of liver-exposed rats received 2.5 Gy (3 Gy/min) of X-rays (90 kV, 5 mA) to a small (~1 cm^2) section of the abdomen where the liver is located. During localized exposures, the rest of the animals' bodies were

protected by a ~3 mm-thick lead shield, the same type used for the protection of humans during diagnostic radiology and as previously published (Koturbash et al., 2006a, b, 2007). Control animals were sham treated by placing them in the irradiator machine completely shielded by lead. The protection of the shielded "bystander" tissue and sham-treated animals was complete, as verified by careful dosimetry using the RAD-CHECK^{TM} monitor (Nuclear Associates div. of Victoreen Inc., Carle Place, NY). For whole body exposures, animals were housed singly in plastic vented containers. For cranial exposures in mice, plastic containers that left only the head of the animals unshielded were used. Rats had to be sedated for localized exposures using ketamine/xylazine. All animals were humanely sacrificed at two different time points. Half of the animals in each treatment group were sacrificed 96 hrs after exposure and the other half 14 days after exposure. Testes of rats were snap frozen in liquid nitrogen and stored at -80ºC. For mice, one testis was snap frozen and stored at -80ºC, while the other one was fixed in 4% paraformaldehyde (J.T. Baker) for approximately 48 h at 4ºC.

3.3.2 Western Immunoblotting

Whole testes were sonicated in 1% SDS and subsequently heated to 95ºC for 5 min, then centrifuged at 10 000 RCF for 5 min. The supernatant (protein extract) was removed and stored for further analysis after small (25 µl) aliquots were reserved for protein concentration determination using protein assay reagents from BioRad (Hercules, CA). Equal amounts of proteins of either 20 µg or 15 µg per gel were separated by SDS-polyacrylamide electrophoresis in slab

gels of 8% polyacrylamide and transferred to PVDF membranes (GE Healthcare Biosciences, Piscataway, NJ). Three membranes were produced for each protein of interest at both time points for mice. For rats four membranes were produced for each protein of interest at both time points. For mice each sample (animal) was represented twice. For rats each control sample (animal) was represented four times, while exposed samples were represented at least twice (two randomly chosen samples are represented three times). The membranes were incubated with antibodies against Miwi (1:1000 rabbit anti-Miwi, Cell Signaling Technology Inc., Boston, MA), Mili (1:1000 anti-Mili, Cell Signaling Technology Inc.), Maelstrom (1:1000 rabbit anti-Mael, Abcam, Cambridge, MA), Mep50 (1:2000 rabbit anti-Mep50, Cell Signaling Technology Inc.), Prmt5 (1:1000 rabbit anti-Prmt5, Cell Signaling Technology Inc.), and actin (loading control) (1:5000 goat anti-β-actin, Abcam). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (1:5000 goat anti-rabbit IgG-HRP, Santa Cruz Biotechnology Inc.) and ECL plus immunoblotting detection system (GE Healthcare Biosciences). Chemoluminescence was detected by means of Biomax MR films (Eastman Kodak, New Haven, CT). 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. Protein signals were quantified using NIH ImageJ 1.63 Software and normalized to the actin or the Mr 50 000 protein level, which gave consistent results. Protein levels in the exposed cohorts were related to the controls and presented as percent change from control.

3.3.3 Immunofluorescence

Testis tissues destined for IF staining were immediately fixed in 4% PFA, pH 7.4, for approximately 48 h at 4ºC; rinsed with sterile 1X PBS, pH 7.4; then stored in 70% ethanol until paraffin embedding. Paraffin embedding and sectioning was conducted at Pantomics Inc. (Richmond, CA). The tissues were assembled into tissue microarrays (TMAs) with sections 5 µm thick with a diameter of 4.5 mm at Pantomics Inc. Immunofluorescent staining was conducted using the antibodies against Miwi (1:400 rabbit anti-Miwi, Cell Signaling Technology Inc.), Mili (1:300 rabbit anti-Mili, Cell Signaling Technology Inc.), and Maelstrom (1:500 rabbit anti-Mael, Abcam) in accordance with the manufacturers' recommendations. In brief, upon deparaffinization, slides were subjected to heat-induced epitope retrieval in citrate buffer solution pH 7.4 (Dako Canada Inc., Burlington, ON), washed 3X 5 min in 1X PBS pH 7.4, then subjected to serum blocking (5% BSA, 5% goat serum in 1X PBS, pH 7.4) for 1.5 h to prevent non-specific binding of immunoglobulin. TMAs were then incubated with primary antibody diluted in blocking serum overnight at 4ºC in a humidified chamber. The following day, they were washed 3X 5 min in 1X PBS pH 7.4 with 0.1% Tween followed by 2-h incubation with a secondary antibody diluted in blocking serum (1:700, Alexa Fluor Goat anti-rabbit 488nm, Invitrogen), washed 3X 5 min in 1X PBS pH 7.4 with 0.1% Tween, dehydrated, and counterstained with ProLong® Gold antifade mounting media with 4', 6-diamidino-2-phenyl-iodole, dihyrochloride (DAPI) (Invitrogen Canada Inc., Burlington, ON). A laser-scanning confocal microscope at 60X was used for imaging.

3.3.4 Statistical Analysis of Western Immunoblots

After each sample singnal was quantified and normalized to the loading conrol, each sample value was standardized in order to combine the data from all of the membranes that were produced for each protein of interest at each time point. Values were standardized by dividing each data point per membrane by the same value (i.e. the mean of the control values on that membrane). Each value was then multiplied by 100 thereby representing percent change from control. A Student's *t* test for independent variance was used to determine significance ($p \leq 0.05$). Statistical analysis and plotting of the results were performed using MS Excel for Windows XP software. The results are presented as mean percent values ± standard error of the mean (SEM).

3.4 RESULTS AND DISCUSSION

3.4.1 Induction of Bystander Effect In The Testis of Rats and Mice Subjected To Localized X-Irradiation

We utilized an animal model whereby sexually mature male Long–Evans rats and C57BL/6 mice were subjected to a localized 2.5 Gy X-ray exposure to the skull, while the rest of the body was protected by a medical-grade shield, to simulate a brain irradiation scenario. We utilized two rodent models to examine wether IR exposure induces altered piRNA pathway proteins across species. The lead shielding used for this study was similar to those used for patients exposed to radiation in the clinic. The same shielding was also used in a published study on bystander effects in the rat germline, which also simulated the brain irradiation scenario (Tamminga et al., 2008). Even though the shielding of an animal body

may be complete, X-rays can be reflected as they pass through tissue, thus forming a small "scatter" dose in the protected tissue (Koturbash et al., 2006a). The study by Tamminga et al. (2008) that used an X-ray dose of 20 Gy demonstrated that the epigenetic germline effects they observed were not induced by scatter irradiation. It can therefore be assumed that the germline effects we are interested in represent true bystander effects/responses that are not due to a scatter dose exposure.

.3.4.2 Bystander-Induced Alteration of PiRNA Pathway Protein Levels In Mice

Adult mice and rats express two homologous Piwi proteins, Miwi/Riwi and Mili/Rili, respectively (Girard et al., 2006). Mili and Miwi proteins are required for the expression of their small RNA partners, which include a class of pachytene piRNAs that are primarily void of repetitive sequences and whose function remains elusive (Aravin et al., 2006; Grivna et al., 2006; Aravin and Hannon, 2008). However, this class of piRNA is needed for spermatogenesis, particularly spermiogenesis (Deng and Lin, 2002; Aravin and Hannon, 2008), and has been implicated in regulating translation as some of these piRNAs putatively target 3'UTRs of a broad set of cellular mRNA transcripts (Robine et al., 2009). Studies have further suggested that Miwi may play a more significant role in translational regulation (Grivna et al., 2006; Lau et al., 2006; Thomson and Lin, 2009). Mili, on the other hand, additionally binds to another class of piRNAs rich in repetitive sequences, whose appearance precedes the expression of Miwi and pachytene piRNAs (Aravin et al., 2006). As the repetitive nature of these pre-

pachytene piRNAs suggests, they are involved in the silencing of repetitive elements, particularly transposons (Aravin et al., 2007b). One major role of Mili and pre-pachytene piRNAs is to guide and maintain sequence-specific *de novo* methylation of certain transposable elements TEs (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). Of particular interest, it has been found that Mili is required for the repression and methylation of LINE1 elements (Aravin et al., 2007b; Carmell, 2007; Kuramochi-Miyagawa et al., 2008). This is the same retrotransposable element that is hypomethylated in the male germline of exposed mice and their offspring (Filkowski et al., 2010).

We observed a significant suppression of Mili protein after whole body and localized cranial exposure in mice at both time points studied (Fig. 3.1). The amount of reduction in Mili protein levels 96 h after whole body X-ray exposure was almost identical to that of the localized cranial-exposed group, reminiscent of a true bystander response (Fig. 3.1). Visual analysis of IF stained testis sections also confirmed a reduction in Mili protein levels between the whole body and localized groups 96 h after exposure (Fig 3.2). Even though the reduced Mili signal in IF stained testis sections was not overly prominent, it appeared as though most of the signal reduction occurred in spermatogonia and early spermatocytes (Fig. 3.2). This is consistent with our previous results (unpublished) and our suggestion that the down regulation of the Mili level is primarily occurring at a developmental stage when Mili is known to associate with pre-pachytene piRNAs. We therefore hypothesize that this reduction may result in altered transcriptional gene silencing TGS of TEs, particularly LINE1, thereby

contributing to germline genomic instability, and epigenetic alterations that can further manifest in future generations as hypomethylation of LINE1 elements and predisposition to genome instability. A recent study has corroborated a portion of this theory by showing that over expression of Hili, the human equivalent of Mili, in a Hela cancer cell line results in a significant reduction of LINE1 mRNA expression (Lu et al., 2010). Therefore, it is plausible that decreased expression of Mili after IR exposure could result in increased LINE1 mRNA expression. In the future, it would be pertinent to analyze the effect of altered Mili expression on the population of bound piRNAs. Subsequent identification of the possible functional targets of these altered piRNAs, followed by characterization of target site methylation status in exposed animals and their offspring, could substantiate this hypothesis.

In addition to Mili, we also observed a statistically significant bystander-induced reduction of Mael, as well as methylosome proteins Mep50 and Prmt5 at both time points (Fig. 3.1). Originally characterized in *Drosophila*, it is now clear that Mael is a key regulatory protein needed for proper functioning of the piRNA pathway (Soper et al., 2008; Aravin et al., 2009). Mainly through the examination of *Mael*-mutant mice, it is now known that Mael interacts with Piwi proteins in order to regulate the elaborate cytoplasmic compartmentalization needed for the proper functioning of the piRNA pathway and maintenance of genome stability (Soper et al., 2008; Aravin et al., 2009). Mael is also believed to be involved in the biogenesis of certain piRNA species, as studies have suggested that Mael has nuclease activity and RNA-binding abilities that may be implicated in piRNA

biogenesis (Zhang et al., 2008). Moreover, it has also been found that Mael is required for the production of certain pre-pachytene (fetal) piRNAs, DNA methylation, and efficient down regulation of TE. Specifically, it has been shown that LINE1 is derepressed due to loss of methylation in adult *Mael*-mutant mice (Soper et al., 2008). However, it was recently found that loss of Mael in fetal mice does not derail the acquisition of *de novo* methylation of these transposable elements (Aravin et al., 2009). This seems to suggest that Mael may not be essential for establishing methylation of these elements, but instead it may be needed for subsequent maintenance of this *de novo* methylation. Therefore, the loss of Mael and Mili that we observed may contribute to the loss of methylation of LINE1 in the germline of exposed animals (Filkowski et al., 2010). Indeed, the reduction of Mael could be directly connected to the loss Mili. IF stained testis of Mili-mutant mice have exhibited a significant reduction of a Mael protein signal, along with a complete loss of signal in certain germ cell–specific granules where Mael normally accumulates (Aravin et al., 2009). However, analysis of the IF stained testes in our study revealed that the loss of the Mael signal appeared to be uniform across the seminiferous epithelium from spermatogonia to spermatid stage cells for both direct and localized exposure groups (Fig. 3.2). Mael is also probably involved with Miwi in the piRNA pathway as well, as Mael coimmunoprecipitates with Miwi (Costa et al., 2006). However, little is known about this relationship, as *Mael*-mutant mice show almost the same phenotypic traits as *Mili*-mutant mice (Soper et al., 2008). This includes complete arrest of spermatogenesis due to apoptosis during meiotic prophase I (pachytene stage).

This corresponds to the time in germ cell development when Miwi expression begins (Fig. 1.3); therefore, it is not possible to examine the effect of losing Mael on Miwi at this stage of the piRNA pathway.

There was also a corresponding significant reduction of methylosome proteins Prmt5 and Mep50 at both 96 h and 14 days after whole body and localized X-ray exposure (Fig 3.1). Again, this is consistent with a piRNA pathway response to IR-induced germline bystander effects. Mep50 and Prmt5 are the main protein components of a complex known as the methylosome (Friesen et al., 2002). We therefore expected to see fairly similar patterns of altered expression between these two proteins at both time points, which was indeed observed (Fig. 3.1). This methylosome complex is responsible for mediating a post-translational modification of proteins by facilitating symmetrical dimethylation of arginines (sDMAs) (Friesen at al., 2002). All three mouse Piwi proteins are known to complex with the Prmt5/Mep50 methylosome as well as have sDMA modifications (Kirino et al., 2009; Vagin et al., 2009). These arginine methyl marks are recognized by a family of Tudor domain proteins, whose members have also shown specificity for interaction with all three murine Piwi proteins (Vagin et al., 2009). Tudor family member proteins recognize and interact with other proteins via their sDMA modifications, thus giving them the ability to regulate their function (Cote and Richard, 2005). Studies on *Drosophila Prmt5*-knockouts and proteomic analysis of murine Piwi proteins have led to the belief that the biological functions of Piwi proteins and the piRNA pathway are highly regulated by Tudor proteins via associations with the Pmt5/Mep50-induced

sDMA modifications (Kirino et al., 2009; Vagin et al., 2009; Siomi et al., 2010). Studies on *Drosophila Prmt5*-knockouts have also found that sDMA modifications can also directly affect the stability of Piwi proteins (Kirino et al., 2009). Moreover, there has been some recent experimental evidence that suggests Piwi protein methylation is regulated dynamically through spermatogenesis (Siomi et al., 2010). This dynamic regulation of post-translation modifications may also apply to the piRNA pathway's response to radiation. We therefore suggest that the altered expression of methylosome proteins in response to IR exposure and bystander effects may underlie a regulatory response of the piRNA pathway.

There was also evidence of a response or alteration to the piRNA pathway after localized IR exposure demonstrated by the dynamic alteration of Miwi protein levels observed between the two time points (Fig 3.1). A significant up regulation of Miwi protein levels 96 h after whole body exposure was evident (Fig. 3.1). An increase in Miwi protein levels in cranial-exposed animals was observed at the same time point but was not significant. Analysis of the IF stained testes section also revealed an increase in Miwi 96 h after exposure, which was more pronounced in the animals that received whole body irradiation (Fig. 3.2). In contrast to our Western blot analysis, the change in the Miwi signal appeared to be larger than that of Mili and Mael (Fig. 3.1). The loss of the Miwi protein signal appeared to primarily occur in spermatids during spermiogenesis. This may reflect an important role for Miwi in translational regulation, which has been reported previously (Grivna et al., 2006). Interestingly, and in contrast to the other

piRNA pathway proteins, after 14 days the up regulation of Miwi was reversed, and there was actually a reduction in the level of protein. Although the bystander response was similar to that of the whole body exposure group, the decrease in Miwi protein levels was not significant (Fig. 3.1). It should be noted, however, that this change was very close to being statistically significant, as the bystander and whole body exposure groups had p-values of 0.051 and 0.086, respectively. Without further analysis, it is difficult to infer the causes and outcome of this dynamic alteration of Miwi levels. However, it does suggest that the piRNA pathway is affected by or responding to radiation exposure including bystander effects, and that different Piwi proteins might play distinct but not necessarily mutually exclusive roles in this response.

3.4.3 Bystander-Induced Alteration of PiRNA Pathway Protein Levels In Rats

To investigate if the piRNA pathway plays a conserved role in responses to radiation, we also looked into the effects of localized and whole body X-ray exposure on piRNA pathway protein levels in a rat model.

We have previously found an overall pattern exhibited in the alteration of murine piRNA pathway proteins after exposure to IR. Generally, we have found that within a few days of exposure there is up regulation of Miwi and down regulation of Mili, Mael, and the methylosome proteins. We also found that 14 days after exposure Miwi is no longer up regulated but instead actually appears to begin to be suppressed along with the other piRNA pathway proteins. Interestingly, we also observed a very similar trend in the response of rat piRNA

pathway proteins after localized and whole body exposure (Fig 3.3). The amount of increase in rat Miwi levels 96 h after exposure and subsequent down regulation 14 days later was statistically significant (Fig. 3.3, C and D). As mentioned, this was not the case for our mice Western blot data, as this change was not significant except for the whole body exposure group 96 h after exposure (Fig 3.1). The significant increase in rat Miwi 96 h after exposure in all groups does seem to further support the marked increase of the Miwi signal we observed in our IF stained sections of mouse testes 96 h after localized cranial and whole body X-ray exposure (Fig 3.2). As mentioned, we also included an additional cohort of rats that received localized X-irradiation of the liver in order to examine the possibility that different organs produce different bystander effects. For the most part, we observed similar alteration to protein levels after localized cranial and liver exposures. Nevertheless, we did find some fairly consistent trends in some of the differences observed between the responses induced by liver and cranial exposures. Mainly, it appeared as though the liver-exposed group induced smaller alterations to piRNA pathway protein levels, which was most evident in the animals that were examined 14 days after exposure (Fig 3.3). This provides some indirect evidence to support the theory that certain organs such as the spleen and liver behave differently to radiation, which could also include differences in the bystander effects that they produce (Brooks, 2004; Koturbash et al., 2006a, 2007, 2008).

Overall, these data demonstrate that IR exposure including bystander effects induce altered regulation of piRNA pathway protein levels in both rat and

mice germlines. These data also support our hypothesis that the piRNA pathway may represent a conserved epigenetic regulatory mechanism that is either affected by or responding to radiation exposure, including IR-induced germline bystander effects.

3.5 CONCLUSIONS

This is the first study to show that the highly conserved germline piRNA pathway is profoundly altered in response to radiation exposure. Specifically, we found that levels of key regulatory proteins intimately involved in the piRNA pathway are significantly altered in a time-dependent manner after whole body and localized X-irradiations in both rats and mice. Our study provides only partial preliminary data supporting the role of the piRNA pathway in germline responses to radiation exposure. Further studies are greatly needed to understand the molecular, biological, and evolutionary consequences of piRNA pathway protein responses to radiation exposure and its impact on germline genome integrity. The piRNA pathway has many features that make it suitable as a mediator of epigenetic memory in germ cells. Gaining a further understanding of the outcomes associated with piRNA pathway protein level changes in response to IR exposures may very well provide important information necessary to elucidate the molecular mechanisms underlying IR-induced epigenetic dysregulation in the male germline and therefore transgenerational genomic instability and carcinogenesis.

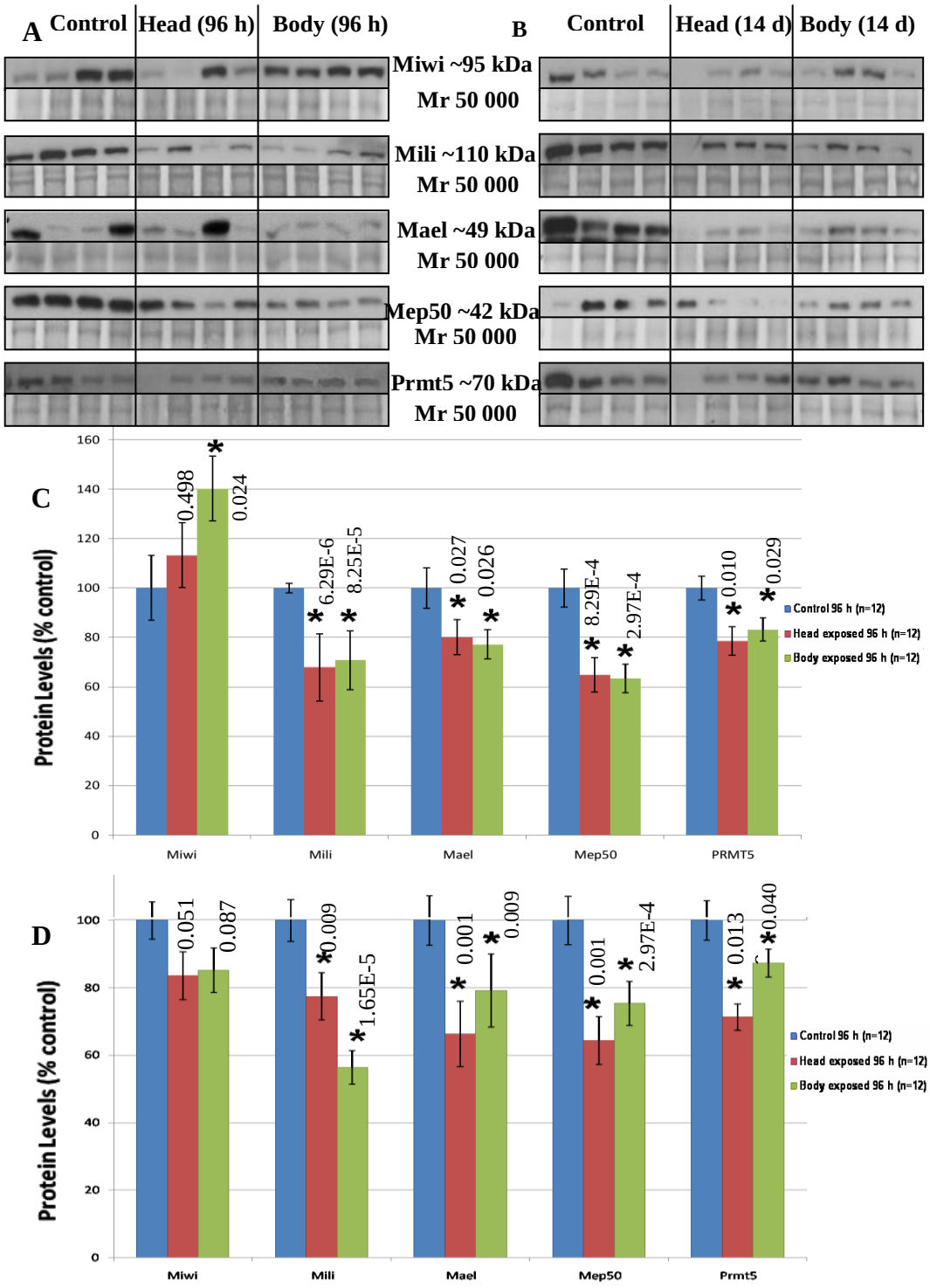


Figure 3.1. Bystander induced deregulation of piRNA pathway protein levels in mice. (A, B) Western blot of whole testis lysate from animals sacrificed 96hrs, and 14 days after exposure using antibodies against Miwi, Mili, Mael, Mep50, Prmt5, and coomassie stain (loading control). **(C, D)** Analysis of western blots from animals sacrificed 96 hrs and 14 days after exposure respectively. Protein levels relative to controls (100%) are shown, ±SE, *p≤0.05.

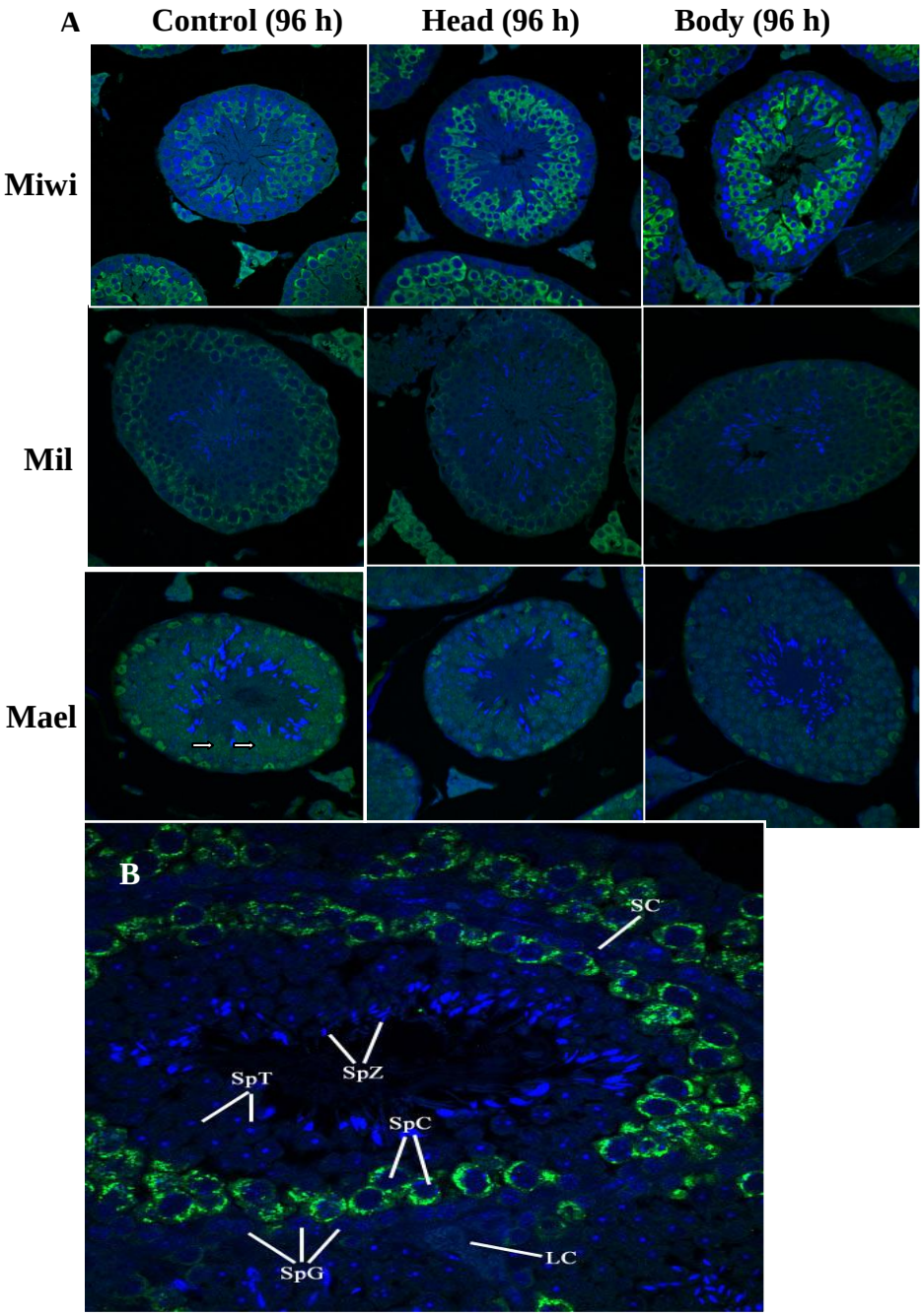


Figure 3.2. Immuno-fluorescent analysis of piRNA pathway protein levels. **(A)** Seminiferous tubules from sections of paraffin embedded mouse testes using antibodies against Miwi, Mili and Mael (green), with nuclear (DAPI) stain (blue), block arrows on Mael (control) showing chromatoid body, images taken with a laser scanning confocal microscope (x60). **(B)** Seminiferous tubule from cross section of paraffin embedded mouse testis with Mili (green) and nuclear (DAPI) stain (blue). Image taken with a laser scanning confocal microscope (x60). Seminiferous tubule labeled with relevant cell types associated with spermatogenesis: SpG-spermatogonia, SpC spermatocyte, SpT-spermatid, SpZ-spermatozoa, SC-Sertoli Cell, LC-Leydig cell

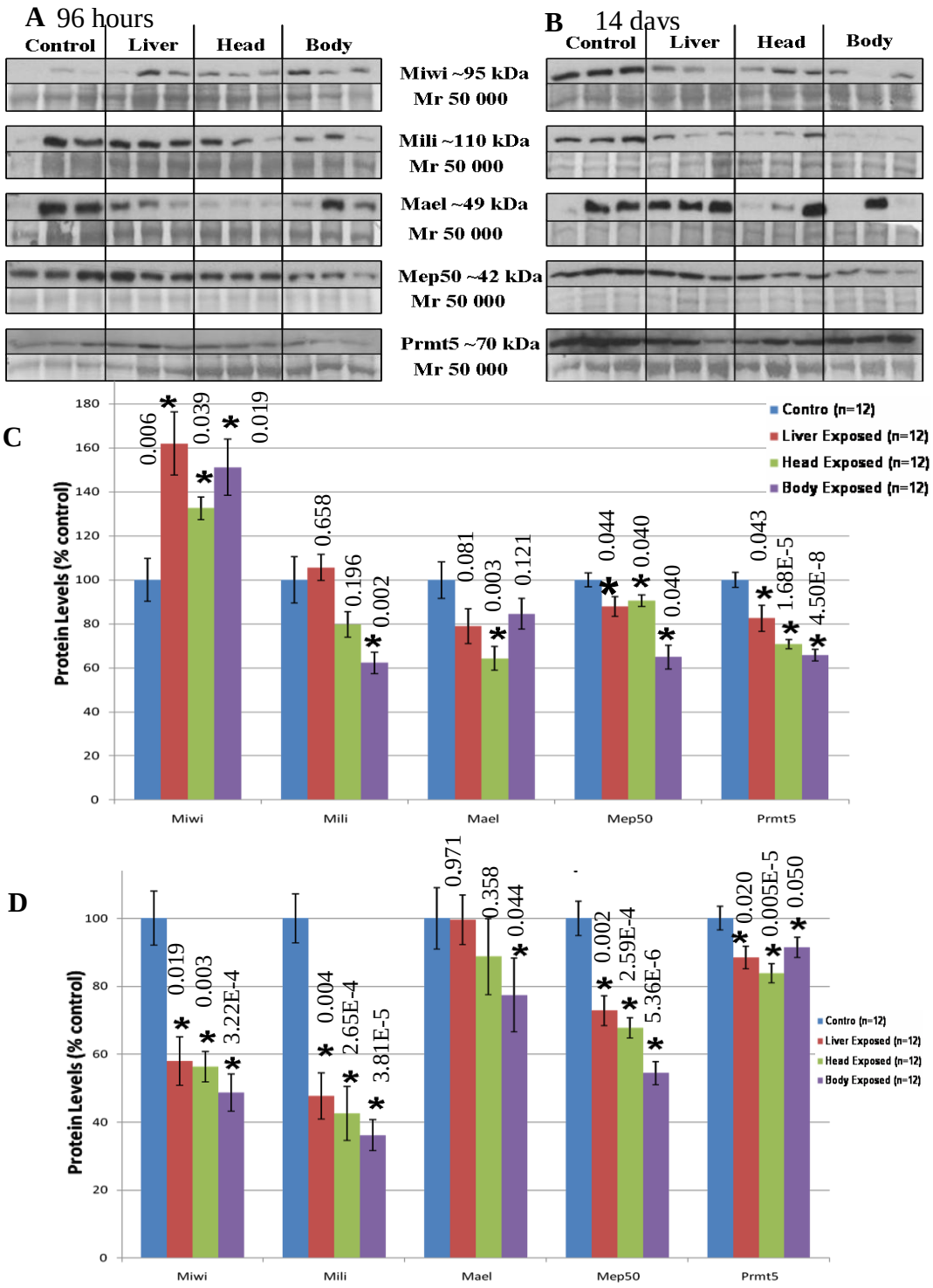


Figure 3.3. Bystander induced deregulation of piRNA pathway protein levels in rats. (A, B) Western blot of whole testis lysate from animals sacrificed 96hrs, and 14 days after exposure using antibodies against Miwi, Mili, Mael, Mep50, Prmt5, with coomassie stain (loading control). **(C, D)** Analysis of western blots from animals sacrificed 96 hours and 14 days after exposure respectively. Protein levels relative to controls (100%) are shown, ±SE, *p≤0.05, data labels represent p-values.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

Owing to a marked increase in accessibility and the introduction of innovative techniques that utilize ionizing radiation (IR), the number of people that receive chronic and/or acute exposure to radiation via occupational, diagnostic, or treatment related modalities is progressively rising. In particular, the use of radiation therapy in modern cancer treatment regimes has significantly lowered cancer mortality rates in men of all ages (Canadian Cancer Society, 2008). Even though cancer is primarily associated with the aged, it frequently occurs in people of reproductive age with current incidence rates showing that approximately 12% of newly diagnosed cancers occur in people under the age of fifty (Canadian Cancer Society, 2008). The potential for adverse biological effects associated with radiation exposure have consequently gained a lot of interest in recent years. The problem of potential deleterious effects of radiation exposure on subsequent progeny of exposed parents has become an issue of utmost importance.

The primary negative biological effects of IR have historically been accepted as direct damage to DNA. It is now known that this damage, in conjunction with the disruption of a variety of cellular regulation processes, can lead to the phenomenon of genomic instability that is linked to carcinogenesis (Little, 2000; Barcellos-Hoff, 2005; Sowa et al., 2006). IR induced genome instability can occur in the descendents of directly exposed cells for many generations, as well as in naïve un-irradiated bystander cells (Morgan, 2003a, b).

The testes are one of the most radiosensitive organs (Feinendegen, 2005). Even if it is directed to distant body parts, IR exposure can lead to genomic instability in the germline and further to transgenerational genome instability in the unexposed offspring of parents exposed before conception (Dubrova, 2003b; Morgan, 2003a, b, c; Tamminga et al., 2008). Although it is clear that IR-induced bystander and transgenerational effects are linked to genome instability, the exact molecular mechanisms that lead to their development are only beginning to be understood. Accumulating evidence suggests that epigenetic alterations are key factors underling the molecular etiology of the above mentioned phenomena (Kovalchuk and Baulch, 2008).

DNA methylation is one of the main epigenetic mechanisms that safeguard genome stability in cells and regulates gene expression and chromatin structure. The germline specific piRNA pathway has an established role in maintaining genome stability as it enforces the silencing of transposable elements by directing site specific methylation during male germ cell development (Aravin et al., 2007a, b, 2008; Kuramochi-Miyagawa, 2008). As such, the piRNA pathways represent perhaps the only currently known sequence-specific mechanism for deposition of DNA methylation in mammals.

The aim of this study was to investigate wether key piRNA pathway protein levels are effected by, or responding to, IR exposure in a time and dose dependent manner across two rodent species. The results presented here demonstrated that piRNA pathway protein levels in mice and rats are significantly altered in a time and dose dependend manner after full body and localized

exposures to X-rays. Alteration to protein levels that are intimately involved in the germline specific piRNA pathway may be involved in molecular and epigenetic consequences associated with direct and indirect radiation exposure upon the male germline. These findings provide preliminary evidence in support of the piRNA pathway representing a novel molecular mechanism that may facilitate the epigenetic inheritance of IR-induced genome instability.

The three major findings of this thesis are:

1) Full body X-irradiation significantly alters the regulation of Piwi and key piRNA pathway protein levels in the exposed rodent (rat, mice) germline. The alteration of piRNA pathway protein levels after IR exposure is dose and time dependent.
2) Localized cranial or liver X-ray exposure induces a significant altered regulation of Piwi and piRNA pathway protein levels in the lead shielded germline of rats and mice.
3) The Piwi and piRNA pathway proteins are part of a conserved epigenetic mechanism necessary for germline genome stability, and these proteins are either responding to or altered by exposure to IR. IR-induced alterations to Piwi and piRNA pathway proteins that are necessary for the proper functioning of the germline specific piRNA pathway may underly a response of this novel molecular mechanism (piRNA pathway) that might play a key role in facilitating epigenetic inheritance of radiation effects, including genomic instability.

4.1 FUTURE DIRECTIONS

The findings presented in this thesis have only provided primary evidence for the involvement of the piRNA pathway in the germline responses to radiation exposure, which may further lead to germline and transgenerational epigenetic consequences associated with IR exposure. What remains to be discovered are the possible epigenetic consequences associated with altered piRNA pathway protein levels in germline responses to IR exposure. Further studies are greatly needed in order to understand the possible deleterious and/or protective biological outcomes associated with piRNA pathway protein responses to IR, and how these responses may cause alterations to the germline epigenome which can affect future generations. Similarly, further studies are required to understand mechanisms associated with germline bystander effects and the nature of bystander signaling. The following are suggestions for future studies:

1) As mentioned, the current study only classified alterations to the protein component of the piRNA pathway after exposure to IR. Further studies will be required to examine the effect of altered piRNA pathway protein levels on the small RNA component of the pathway. Immunoprecipitation of Piwi proteins after IR exposure and the subsequent analysis and sequencing of their piRNA partners will guide us in understanding the effects of altered piRNA pathway protein levels on the epigenome. With the rate of advancement in sequencing techniques and bioinformatics we will soon be able to identify the functional targets of these piRNAs, which will guide us in

understanding the biological consequences of piRNA pathway protein responses to IR that may be linked to heritable effects associated with IR exposure (i.e. transgenerational genomic instability and carcinogenesis)

2) Perhaps the most important problem will be deducing the combined contribution of piRNA pathway responses with DNA damage, hypomethylation, and gene dysregulation to transgenerational genomic instability. Dissecting the molecular events that follow fertilization by radiation-affected sperm would contribute significantly to a 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 influences genome destabilization in unexposed offspring.
3) The exact nature of the bystander signal still needs to be delineated. Most importantly, mechanisms that facilitate the communication of IR exposure to distant unexposed cells need to be identified. One conceivable explanation is that the signal originates from exposed cells in the blood, and thus the bystander signal is transmitted to distant tissue/organs via the blood. During localized cranial/liver irradiation, a certain amount of blood cells are present in the field of exposure. 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. Although candidate molecules are numerous, current literature

suggests key players include reactive oxygen species (ROS) such as superoxide and hydrogen peroxide (Morgan, 2003a, b, c; Lyng et al., 2000, 2002; Azzam et al., 2003b), short RNAs (Koturbash et al., 2007; Kovalchuk and Baulch, 2008), Ca^{2+} ions (Lyng et al., 2006), and cytokines (Facoetti et al., 2006; Iyer and Lennert, 2000). It needs to be further established what and how inflammatory responses mounted by the host's immune system contribute to bystander signaling and responses.

4) Normal regulation of spermatogenesis largely depends on the proper functioning of the hypothalamic-pituitary-gonad (HPG) axis. The HPG axis primarily involves complex interactions between endocrine cells of the testes, such as Leydig and Sertoli cells, and the pituitary gland. Future studies will need to analyze the impact of radiation damage on the HPG axis, particularly the pituitary. Radiation damage to the pituitary may affect the secretion of hormones, which may also contribute to bystander signaling and responses seen from the male germline during spermatogenesis.

REFERENCES

Aitken RJ, De Iuliis GN. Origins and consequences of DNA damage in male germ cells. Reprod Biomed Online 2007; 14:727-733

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

Aravin AA, Lagos-Quintana M, Yalcin A, Zavolan M, Marks D, Snyder B, Gaasterland T, Meyer J, Tuschl T. The small RNA profile during *Drosophila melanogaster* development. Dev Cell. 2003; 5(2): 337–350.

Aravin A, Gaidatzis D, Pfeffer S, Lagos-Quintana M, Landgraf P, Iovino N, Morris P, Brownstein MJ, Kuramochi-Miyagawa S, Nakano T, Chien M, Russo JJ, Ju J, Sheridan R, Sander C, Zavolan M, Tuschl. A novel class of small RNAs bind to MILI protein in mouse testes. Nature. 2006; 442:203-7.

Aravin AA, Hannon GJ, Brennecke J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. Science. 2007a; 318: 761-764

Aravin AA, Sachidanandam R, Girard A, Fejes-Toth K, Hannon GJ. Developmentally regulated piRNA clusters implicate Mili in transposon control. Science. 2007b; 316:744-747

Aravin AA, Hannon GJ. Small RNA silencing pathways in germ and stem cells. Cold Spring Harb Symp Quant Biol. 2008; 73:283-290

Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, Bestor T, Hannon GJ. A piRNA pathway primed by individual transposons is linked to *de novo* DNA methylation in mice. Mol Cell. 2008; 31(6):785-99.

Azzam EI, de Toledo SM, Little JB. Expression of CONNEXIN43 is highly sensitive to ionizing radiation and other environmental stresses. Cancer Res. 2003a; 63:7128-35

Azzam EI, de Toledo SM, Little JB. Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. Oncogene. 2003b; 22:7050-7

Balonov MI. The Chernobyl Forum: major findings and recommendations. J Environ Radioact. 2007; 96(1-3):6-12

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 USA. 2002; 99:6877-82

Barber RC, and 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; 15:138-148

Baylin SB. DNA methylation and gene silencing in cancer. Nat Clin Pract Oncol. 2005;
2:S4-11

Baylin SB, Ohm JE. Epigenetic gene silencing in cancer - a mechanism for early oncogenic pathway addiction? Nat Rev Cancer. 2006; 6:107-16

Bedford Mt, Calrke SC. Protein arginine methylation in mammals: who, what, and why. Mol Cell. 2009; 33:1-13

Belyakov OV, Mitchell SA, Parikh D, Randers-Pehrson G, Marino SA, Amundson SA, Geard CR, Brenner DJ. Biological effects in unirradiated human tissue induced by radiation damage up to 1 mm away. Proc Natl Acad Sci USA. 2005; 102:14203-8

Bernstein E, Allis CD. RNA meets chromatin. Genes Dev. 2005; 19:1635-55

Bianchi ME, Agresti A. HMG proteins: dynamic players in gene regulation and differentiation. Curr Opin Genet Dev. 2005; 15:496-506

Bishayee A, Hill HZ, Stein D, Rao DV, Howell RW. Free radical-initiated and gap junction-mediated bystander effect due to nonuniform distribution of incorporated radioactivity in a three-dimensional tissue culture model. Radiat Res. 2001; 155:335-44

Bogdanova TI, Zurnadzhy LY, Greenebaum E, McConnell RJ, Robbins J, Epstein OV, Olijnyk VA, Hatch M, Zablotska LB, Tronko MD. A cohort study of thyroid cancer and other thyroid diseases after the Chornobyl accident: pathology analysis of thyroid cancer cases in Ukraine detected during the first screening (1998-2000). Cancer. 2006; 107:2559-66

Boswell RE, and Mahowald AP. *Tudor,* a gene required for assembly of the germ plasm in *Drosophila melangaster.* Cell. 1985; 43:2484-89

Brenner DJ, Hall EJ. Risk of cancer from diagnostic X-rays. Lancet. 2004; 363:2192

Brooks AL, Retherford JC, McClellan RO. Effect of 239PuO2 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

Burrel 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 137Cs gamma-rays. Mutat Res. 1997; 381:59-66

Cai L. Research of the adaptive response induced by low-dose-radiation: where have we been and were should we go? Hum Exp Toxicol. 1999; 18:419-425

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

Canadian Cancer Society/National Cancer Institute of Canada. Canadian Cancer Statistics 2010, Toronto, Canada: 2010. p. 1-28

Carmell MA, Girard A, van de Kant HJ, Bourc'his D, Bestor TH, de Rooij DG, Hannon GJ. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev Cell. 2007; 12:503–514

Carmichael A, Sami AS, Dixon JM. Breast cancer risk among the survivors of atomic bomb and patients exposed to therapeutic ionizing radiation. EJSO. 2003; 29:475-9

Cattanach BM, Papworth D, Patrick G, Goodhead DT, Hacker T, Cobb L, Whitehill E. Investigation of lung tumor induction in BALB/cJ mice following paternal X-irradiation. Int J Radiat Biol. 1995; 67:607-615

Cattanach BM, Papworth D, Patrick G, Goodhead DT, Hacker T, Cobb L, Whitehill E. Investigation of lung tumor induction in C3H/HeH mice, with and without tumor promotion with urethane, following paternal X-irradiation. Mutat Res. 1998; 403:1-12

Chang TC, Mendell JT. The roles of microRNAs in vertebrate physiology and human disease. Annu Rev Genomics Hum Genet. 2007; 8:215-239

Chen PY, Meister G. Micro-RNA-guided posttranscriptional gene regulation. Biol Chem. 2005; 386:1205-1218

Cheng KC, Loeb LA. Genomic instability and tumor progression: mechanistic considerations. Adv Cancer Res. 1993; 60:121-156

Cheng RY, Hockman T, Crawford E, Anderson LM, Shiao YH. Epigenetic and gene expression changes related to transgenerational carcinogenesis. Mol Carcinog. 2004; 40:1-11

Cheung P, Lau P. Epigenetic regulation by histone methylation and histone variants. Mol Endocrinol. 2005; 19:563-73

Chuma S, Hosokawa M, Kitamura K, Kasai S, Fujioka M, Hiyoshi M, Takamune K, Noce T, Nakatsuji N. Tdrd1/Mtr-1, a tudor-related gene, is essential for male germ-cell differentiation and nuage/germinal granule formation in mice. Proc Natl Acad Sci U S A. 2006; 43:15894-9

Cordelli E, Fresegna AM, Leter G, Eleuteri P, Spano M, Villani P. Evaluation of DNA damage in different stages of mouse spermatogenesis after testicular X irradiation. Radiat Res. 2003; 160:443-451

Costa, Y, Speed RM, Gautier P, Semple CA, Maratou K, Turner JM, Cooke HJ. Mouse maelstrom: link between meiotic silencing of unsynapsed chromatin and microRNA pathway? Hum mol Genet. 2006; 15:2324-2334

Cote J, Richard S. Tudor domains bind symmetrical dimethylated arginines. J Biol Chem. 2005; 280:28476-83

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

Deng W, Lin H. Miwi, a murine homolog of piwi, encodes a cytoplasmic protein essential for spermatogenesis. Dev Cell 2002; 2:819–830

Dickinson HO, and Parker L. Leukemia and non-Hodgkin's lymphoma in children of male Sellafield radiation workers. Int J Cancer. 2002; 99:437-444

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 mutation induced by ionizing radiation. Nat Genet. 1993; 5:92-94

Dubrova YE, Nestrova VN, Krouchinsky NG, Ostapenko VA, Neumann R, Neil DL, Jeffrys AJ. Human minisatellite mutation rate after Chernobyl accident. Nature. 1996; 380; 683-686

Dubrova YE, Grant G, Chumak AA, Tezhka VA, Karakasian AN. Elevated minisatellite mutation rate in the post-Chernobyl families from the Ukraine. Am J Hum Genet. 2002a; 71:801-809

Dubrova YE, Bersimbaev RI, Djansugurova LB, Tankimanova MK, Mamyrbaeva ZZ, Moustonen R, Londholm C, Hultin M, Salomaa S. Nuclear weapons tests and human germ line mutation rate. Science. 2002b; 295:1037

Dubrova Y. Long-term genetic effects of radiation exposure. Mutat Res. 2003a; 544:433–439.

Dubrova Y. Radiation-induced transgenerational instability. Oncogene. 2003b; 22:7087-93

Ehrlich M. DNA methylation in cancer: too much, but also too little. Oncogene. 2002; 1:5400-13

Emerit I, Levy A, Cernjavski L, Arutyunyan R, Oganesyan N, Pogosian A, Mejlumian H,
Sarkisian T, Gulkandanian 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, Oganesian 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

Erven K, Van Limbergen E. Regional lymph node irradiation in breast cancer. Future Oncol. 2007; 3:343-52

Fabbri M, Ivan M, Cimmino A, Negrini M, Calin GA. Regulatory mechanisms of microRNAs involvement in cancer. Expert Opin Biol Ther. 2007; 7:1009-19

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

Fei P, and El-Deiry WS. P53 and radiation responses. Oncogene. 2003; 22:5774-83

Feinberg AP, Vogelstein B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature. 1983; 301:89-92

Feinberg AP. The epigentics of cancer etiology. Semin Cancer Biol. 2004; 6:427-32.

Feinendegen LE. Evidence for beneficial low level radiation effects and radiation hormesis. Br J Radiol. 2005; 78:3-7

Filkowski JN, Ilnytskyy Y, Tamminga J, Koturbash I, Golubov A, Bagnyukova T, Pogribny IP, Kovalchuk O. Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression. Carcinogenesis. 2010; 6:1110-5

Flatau E, Bogenmann E, Jones PA. Variable 5-methylsytosine levels in human tumor cell lines and fresh pediatric tumor explants. Cancer Res. 1983; 43:4901-05

Folkard M, Vojnovic B, Prise KM, Bowey AG, Locke RJ, Schettino G, Michael BD. A Charged particle microbeam: I. Development of an experimental system for targeting cells individually with counted particles. Int J Radiat Biol. 1997; 72:375-378

Folley JH, Borges W, Yamasaki T. Incidence of leukemia in survivors of the atomic bomb in Hiroshima and Nagasaki. Am J Med. 1952; 13:311-21

Frankenberg-Scwager M. Induction, repairand biological relevance of radiation-induced DNA lesions in eukaryotic cells. Radiat Environ Biophys. 1990; 29-273-92

Freeman SM, Aboud CN, Whartenby KA, Packman CH, Koeplin DS, Moolten FL,
Abraham GN. The “bystander effect”: Tumor regression when a fraction of the tumor mass is genetically modified. Cancer Res. 1993; 53:5274-83

Friesen WJ, Wyce A, Paushkin S, Abel L, Rappsilber J, Mann M, Dreyfuss G. A novel WD repeat component of the methylosome binds SM proteins. J Biol Chem. 2002; 277:8243-47

Gama-Sosa MA, Slagel VA, Trewn RW, Oxenhandler R, Kuo KC, Gehrke CW, Ehrlich M. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res. 1983; 11:6883-94

Gardner MJ, Snee MP, Hall AJ, Powell CA, Downes S, and Terrell JD. Results of case-control study of leukemia and lymphoma among young people near Sellafield nuclear plant in West Cumbria. Br Med J. 1990; 300:423-429

Girard A, Sachidanandam R, Hannon GJ, Carmell MA. A germline-specific class of small RNA binds mammalian Piwi proteins. Nature. 2006; 199-202

Girard A, and Hannon GJ. Conserved themes in small-RNA-mediated transposon control. Trends Cell Biol. 2008; 18:136-148

Gluzman D, Imamura N, Sklyarenko L, Nadgornaya V, Zavelevich M, Machilo V.
Malignant diseases of hematopoietic and lymphpoid tissues in Chernobyl clean-up workers. Hematol J. 2005; 5:565-71

Goh K, Sumner H. Breaks in normal human chromosomes: Are they induced by the transferable substance in the plasma of persons exposed to total-body irradiation? Radiat Res. 1968; 35:171-81

Goll MG, Bestor TH. Eukaryotic cytosine methyltransferases. Ann. Rev. Biochem. 2005; 74:481-514

Goldberg Z, Lehnert BE. Radiation-induced effects in unirradiated cells: A review and implications in cancer. Int J Oncol. 2002; 21:337-49

Goldberg Z. Clinical implications of radiation-induced genomic instability. Oncogene. 2003; 22:7011-7017

Goodhead DT. Initial events in the cellular effects of ionizing radiation: Clusters damage in DNA. Int J Radiat Biol. 1994; 65:7-17

Gramegna A. Un cas d'acromegalie traitè pour la radiotherapie. Rev. Neurol. 1909; 17**:**15-17

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

Grivna ST, Pyhtila B, Lin H. MIWI associates with translational machinery and PIWI-interacting RNAs (piRNAs) in regulating spermatogenesis.Proc Natl Acad Sci USA. 2006; 103(36):13415-20

Hall EJ. Lessons we have learned from our children: cancer risks from diagnostic radiology. Pediatr Radiol. 2002; 32:700-6

Hall EJ. The bystander effect. Health Phys. 2003; 85:31-5

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

Hartig J, Tomari Y, Forstemann K. Pi-RNAs-the ancient hunters of genome invaders. Genes and Dev. 2007; 21:1707-1713

Hermo L, Pelletier RM, Cyr DG, Smith CE. Surfing the wave, cycle, life history, and genes/proteins expressed by testicular germ cells. Part 1: Background to spermatogenesis, spermatogonia, and spermatocytes. Microsc Res Tech. 2010; 4:241-78

He S, Dunn KL, Espino PS, Drobic B, Li L, Yu J, Sun JM, Chen HY, Pritchard S, Davie JR. Chromatin organization and nuclear microenvironments in cancer cells. J Cell Biochem. 2008; 104(6):2004-15

Hendrich B, Tweedie S. The methyl-CpG binding domain and the evolving role of DNA methylation in animals. Trends Genet. 2003; 19:269-77

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; 43:317-24

Hess RA. Spermatogenesis, Overview. Encyclopedia of Reproduction. 1999; 4:539-45

Hildreth NG, Shore RE, Dvoretsky PM. The risk of breast cancer after irradiation of the thymus in infancy. N Engl J Med. 1989; 321:1281-4

Hollowell JG Jr, Littlefield LG. Chromosome damage induced by plasma of x-rayed patients: An indirect effect of x-ray. Proc Soc Exp Biol Med. 1968; 129:240-4

Holstein AF, Schulze W, Davidoff M. Understanding spermatogenesis is a prerequisite for treatment. Reprod Biol Endocrinol. 2003:1-107

Hoyes KP, Lord BI, McCann C, Hendry JH, Morris ID. Transgenerational effects of preconception paternal contamination with (55)Fe. Radiat Res. 2001; 56:488-494

Huang L, Snyder AR, Morgan WF. Radiation-induced genomic instability and its implications for radiation carcinogenesis. Oncogene. 2003;22:5848-54

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/emrg/topic334.htm

Hunter N. The RecQ DNA helicases: Jacks-of-all-trades or master-tradesmen? Cell Res 2008; 18:328–330.

Hutvagner G, Zamore PD. A miRNA in a multiple-turnover RNAi enzyme complex. Science. 2002; 297:2056-60

Iliakis G, Wang Y, Guan J, Wang H. DNA damage checkpoint control in cells exposed to ionizing radiation. Oncogene. 2003; 22:5834-47

Infante-Rivard C, Mathonnet G, Sinnett D. Risk of childhood leukemia associated with diagnostic irradiation and polymorphisms in DNA repair genes. Environ Health Perspect. 2000; 108:495-8

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 and Lehnert BE. 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 PA, Lobrich M. Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability. DNA Repair. 2006; 5:1192-8

Jenuwein T, Allis CD. Translating the histone code. Science. 2001; 293:1074-80

Jirtle RL, Skinner MK. Environmental epigenomics and disease susceptibility. Nat Rev Genet. 2007; 8:253-62

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; 15:435-441

Kalinich JF, Catravas GN, Snyder SL. The effect of gamma radiation on DNA methylation. Radiat. Res. 1989; 117:185-97

Kaplan MI, Limoli CL, Morgan WF. Perpetuating radiation-induced chromosomal instability. Radiat Oncol Investig. 1997; 5:124-128

Kato Y, Kaneda M, Hata K, Kumaki K, Hisano M, Kohara Y, Okano M, Li E, Nozaki M, Sasaki H. Role of the Dnmt3 family in de novo methylation of imprinted and repetitive sequences during male germ cell development in the mouse. Hum Mol Genet. 2007; 16(19):2272-80.

Kaup S, Grandjean V, Mukherjee R, Kapoor A, Keyes E, Seymour CB, Mothersill CE,
Schofield PN. Radiation-induced genomic instability is associated with DNA methylation changes in cultured human keratinocytes. Mutation Res. 2006; 597:87-97

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

Kirino Y, Kim N, de Planell-Saguer M, Khandros E, Chiorean S, Klein PS, Rigoutsos I, Jongens TA, Mourelatos Z.Arginine methylation of Piwi proteins catalysed by dPRMT5 is required for Ago3 and Aub stability.Nat Cell Biol. 2009; 11:652-8

Klattenhoff C, Bratu DP, McGinnis-Schultz N, Koppetsch BS, Cook HA, Theurkauf WE. *Drosophila* rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. Dev Cell 2007; 12:45–55

Klattenhoff C, Theurkauf W. Biogenesis an germline functions of piRNAs. Dev. 2008; 135-3-9

Kleinerman RA. Cancer risks following diagnostic and therapeutic radiation exposure in children. Pediatr Radiol. 2006; 36:121-5

Klose RJ, Bird AP. Genomic DNA methylation: The mark and its mediators. Trends Biochem Sci. 2006; 31:89-97

Kossenko MM. Cancer mortality in the exposed population of the Techa River area. World Health Stat Q. 1996; 49:17-21

Kotaja N, Lin H, Parvinen M, Sassone-Corsi P. Interplay of PIWI/Argonaute protein MIWI and kinesin KIF17b in chromatoid bodies of male germ cells. J Cell Sci. 2006; 119:2819-25.

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, Baker M, Loree J, Kutanzi K, Hudson D, Progribny 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 R, Tryndyak V, Kovalchuk I,
Pogribny I, 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, 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. 2008; 70:554-562

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

Kuramochi-Miyagawa S, Kimura T, Ijiri TW, Isobe T, Asada N, Fujita Y, Ikawa M, Iwai N, Okabe M, Deng W, Lin H, Matsuda Y, Nakano T. Mili, a mammalian member of piwi family gene, is essential for spermatogenesis. Development 2004; 131:839–849

Kuramochi-Miyagawa S, Watanabe T, Gotoh K, Totoki Y, Toyoda A, Ikawa M, Asada N, Kojima K, Yamaguchi Y, Ijiri TW, Hata K, Li E, Matsuda Y, Kimura T, Okabe M, Sakaki Y, Sasaki H, NakanoT. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. Genes Dev 2008; 22(7):908–917

Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE. Characterization of the piRNA complex from rat testes. Science 2006; 313:363–367

Lee JH, Schutte D, Wulf G, Fuzesi L, Radzun HJ, Schweyer S, Engel W, Nayernia. Stem-cell protein Piwil2 is widely expressed in tumors and inhibits apoptosis through activation of the Stat3/Bcl-XL pathway. Hum Mol Gen. 2006; 15:201-211

Lees-Murdock DJ, De Felici M, Walsh CP. Methylation dynamics of repetetive DNA elements in the mouse germ cell lineage. Genomics. 2003; 82:230-237

Leone G, Mele L, Pulsoni A, Equitani F, Pagano L. The incidence of secondary leukaemias. *Haematologica*. 1999; 84:937-45

Likhtarov I, Kovgan L, Vavilov S, Chepurny M, Ron E, Lubin J., Bouville A, Tronko N,
Bogdanova T, Gulak L, Zablotska L, Howe G. Post-Chernobyl thyroid cancers in Ukraine. Report 2: risk analysis. Radiat Res. 2006; 166:375-86

Limoli CL, Corcoran JJ, Milligan JR, Ward JF, Morgan WF. Critical target and dose-rate responses for the induction of chromosomal instability by ionizing radiation. Radiat Res. 1999; 151:677-685

Limoli CL, Ponnaiya B, Corcoran JJ, Giedzinski E, Kaplan MI Hartman A, Morgan WF. Genomic instability induced by high and low LET ionizing radiation. Adv Space Res. 2000; 25:2107-2117

Little JB. Radiation-induced genomic instability. Int J Radiat Biol. 1998; 74:663-71

Little JB. Induction of genetic instability by ionizing radiation. C R Acad Sci III. 1999; 322:127-34

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

Little JB. Genomic instability and radiation. J Radiol Prot. 2003; 23:173-81

Little JB. Cellular radiation effects and the bystander response. Mutat Res. 2006; 597:113-8

Liu D, Momoi H, Li L, Ishikawa Y, Fukumoto M. Microsatellite instability in thorotrast-induced human intrahepatic cholangiocarcinoma. Int J Cancer. 2002; 102:366-71

Liu G, Gong P, Zhao H, Wang Z, Gong S, Cai L. Effect of low-level radiation on the death of male germ cells. Radiat Res. 2006; 165:379-389

Liu Z, Mothersill CE, McNeill FE, Lyng FM, Byun SH, Seymour CB, Prestwich WV. A dose threshold for a medium transfer bystander effect for a human skin cell line. Radiation Res. 2006; 166:19-23

Loeb LA, Loeb KR, Anderson JP. Multiple mutations and cancer. Proc Natl Acad Sci U.S.A. 2003; 776-781

Lord BI, Woolford LB, Wang L, McDonald D, Lorimore SA, Stones VA, Wright EG, Scott D. Induction of lymphohaemopoietic malignancy: Impact of preconception paternal irradiation. Int J Radiat Biol. 1998; 74:721-728

Loree J, Koturbash I, Kutanzi K, Baker M, Pogribny I, Kovalchuk O. Radiation-induced molecular changes in rat mammary tissue: Possible implications for radiation-induced carcinogenesis. Int J Radiat Biol., 82:805-15 2006;

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

Lu Y, Li C, Zhang K, Sun H, Tao D, Liu Y, Zhang S, Ma Y. Identification of piRNAs in Hela cells by massive parallel sequencing. BMB Rep. 2010; 43:635-41

Luning KG, Frolen H, Nilsson A. Genetic effects of 239Pu salt injections in male mice. Mutat Res. 1976; 539-542

Lyon MF, Philips RJS, Searle AG. The overall rates of dominant and recessive lethal and visible mutation induced by spermatogonial X-irradiation of male mice. Genet Res. 1964; 5:448-467

Lyons MK, Vora SA. Brain tumors: Current issues in diagnosis and management. Semin Neurol. 2007; 27:312-324

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. Brit J Cancer. 2000; 83:1223-30

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

Maguire P, Mothersill C, McClean B, Seymour C, Lyng FM. Modulation of radiation responses by pre-exposure to irradiated cell conditioned medium. Radiation Res. 2007; 167:485-92

McKinney PA, Fear NT, Stockton D. Parental occupation at periconception: finding from the United Kingdom childhood cancer y. Occup Environ Med. 2003; 60:901-909

Mohr SU, 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. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. Radiat Res. 2003a; 159:567-80195

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. 2003b; 159:581-96

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, Sowa MB. Effects of ionizing radiation in non-irradiated cells. Proc Natl Acad Sci USA. 2005; 102:14127-28

Morgan WF, Sowa MB. Non-targeted bystander effects induced by ionizing radiation. Mutat Res. 2007; 616:159-64

Morimura K, Romanenko A, Min W, Salim EI, Kinoshita A, Wanibuchi H, Vozianov A,
Fukushima S. Possible distinct molecular carcinogenic pathways for bladder cancer in Ukraine, before and after the Chernobyl disaster. Oncol Rep. 2004; 11:881-6

Morin DM, Lonstein JE, Stovall M, Hacker DG, Luckyanov N, Land CE. Breast cancer mortality after diagnostic radiography: Findings from the U.S. Scoliosis Cohort Study. Spine. 2000; 25:2052-63

Mothersill C, Seymour CB. Radiation-induced bystander effects – implications for cancer. Nat Rev Cancer. 2004; 4:158-64

Mothersill C, Smith RW, Agnihotri N, Seymour CB. Characterization of a radiation-induced stress response communicated in vivo between zebrafish. Environ Sci Technol. 2007; 41:3382-7

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

Murphy JB, Morton J. Action of serum from x-rayed animals on lymphoid cells in vitro. J Exp Med.1915; XX11:800

Nagar S, Smith LE, Morgan WF. Characterization of a novel epigenetic effect of ionizing radiation: the death-inducing effect. Cancer Res. 2003; 63:324-8

Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Res. 1992; 52:6394-6

Nomura T. Parental exposure to X-rays and chemicals induces heritable tumors and anomalies in mice. Nature.1982; 296:575-577

Nomura T. Leukemia in children whose parents have been exposed to radiation. Br Med J. 1993; 306:1412

Nomura T. Transgenerational carcinogenesis: Induction and transmission of genetic alterations and mechanisms of carcinogenesis. Mutat Res. 2003; 544425-32

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, Russel 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 sterna bone marrow following roentgen-ray therapy to the spleen in chronic granulocytic leukaemia. Cancer. 1954; 7:179-89

Paqutte B, Little JB. *In vivo* enhancement of genomic instability in minisatellite sequences of mouse C3/10Ht1/2 cells transformed in vitro by X-rays. Cancer Res. 1994; 54:3173-78

Pentilla TL, Yuan L, Mali P, Hoog C, Parvinen M. Haploid gene expression: Temporal onset and storage patterns of 13 novel transcripts during rat and mouse spemiogenesis. Biol Reprod. 1995; 53:499-510

Persaud R, Zhou H, Baker SE, Hei TK, Hall EJ. Assessment of low linear energy transfer radiation-induced bystander mutagenesis in a three-dimensional culture model. Cancer Res. 2006; 65:9876-82

Petersen C, Soder O. The sertoli cell--a hormonal target and ‘super’ nurse for germ cells that determines testicular size. Horm Res. 2006; 66:153-161

Pilch DR, Sedelnikova OA, Redon C, Celeste A, Nussenzweig A, Bonner WM. Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. Biochem Cell Biol. 2003; 81:123-9

Pils S, Muller WU, Streffer C. Lethal and teratogenic effects in two successive generations of the HLG mouse stain after radiation exposure of zygotes-association with genomic instability? Mutat Res. 1999; 429:85-92

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, Koturbash I, Tryndyak V, Hudson D, Stevenson SML, 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

Pollack A, Zagars GK, Smith LG, Lee JJ, von Eschenbach AC, Antolak JA, StarkschallG, Rosen I. Preliminary results of a randomized radiotherapy dose-escalation study comparing 70 Gy with 78 Gy for prostate cancer. J Clin Oncol. 2000; 18:3904-11

Potter de P, Levecq L, Godfraind C, Renard L. Primary orbital melanoma treated with iodine-125 plaque radiotherapy. Am J Ophtalmol. 2006; 142:864-6

Powell SN, and 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

Preston-Martin S, Thomas DC, Yu MC, Henderson BE. Diagnostic radiography as a risk factor for chronic myeloid and monocytic leukaemia (CML). Br J Cancer. 1989; 59:639-44

Prysyazhnyuk A, Gristchenko V, Fedorenko Z, Gulak L, Fuzik M, Slipenyuk K, Tirmarche M.Twenty years after the Chernobyl accident: solid cancer incidence in various groups of the Ukrainian population. Radiat Environ Biophys. 2007; 46:43-51

Pukkala E, Kesminiene A, Poliakov S, Ryzhov A, Drozdovitch V, Kovgan L, Kyyrönen P, Malakhova IV, Gulak L, Cardis E. Breast cancer in Belarus and Ukraine after the Chernobyl accident. Int J Cancer. 2006; 119:651-8

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

Rander-Pehrson G, Gerad CR, Johnson G, Elliston CD, Brenner DJ. The Columbia University single-ion microbeam. Radiat Res. 2001; 156:210-214

Robertson KD. DNA methylation, methyltransferases, and cancer. Oncogene. 2001; 20:3139-55

Robertson KD. DNA methylation and chromatin – unraveling the tangled web. Oncogene. 2002; 21:5361-79

Robine N, Lau NC, Balla S, Jin Z, Okamura K, Kuramochi-Miyagawa S, Blower MD, Lai EC. A broadly conserved pathway generates 3'UTR-directed primary piRNAs. Curr Biol. 2009; 19(24):2066-76

Rodeman 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

Roman E. Doyle P, Maconochie N, Davies G, Smith PG, Beral V. Cancer in children of nuclear industry employees: Report on children aged under 25 years from nuclear industry family study. BMJ. 1999; 318:1443-1450

Romanenko A, Morell-Quadreny L, Nepomnyaschy V, Vozianov A, Llombart-Bosch A. Pathology and proliferative activity of renal-cell carcinomas (RCCs) and renal oncocytomas in patients with different radiation exposure after the Chernobyl accident in Ukraine. Int J Cancer 2000; 87:880-3

Roof KS, Fidias P, Lynch TJ, Ancukiewicz M, Choi NC. Radiation dose escalation in limited-stage small-cell lung cancer. Int J Radiat Oncol Biol Phys. 2003; 57:709-16

Rountree MR, Bachman KE, Herman JG, Baylin SB. DNA methylation, chromatin inheritance, and cancer. Oncogene. 2001; 20:3156-65

Russell LD, Griswold MD. The Sertoli cell. Cache River Press, Clearwater FL. 1993

Saha A, Wittmeyer J, Cairns BR. Chromatin remodelling: the industrial revolution of DNA around histones. Nat Rev Mol Cell Biol. 2006; 7:437-47

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

Sedelnikova O, 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

Seto AG, Kingston RE, Lau NC. The coming age for Piwi proteins. Mol Cell. 2007; 26:603-609

Shao C, Furusawa, Aoki M, Ando K. Role of gap junctional intercellular communication in radiation-induced bystander effects in human fibroblasts. Radiat Res. 2003; 160:318 23

Shilnikova NS, Preston DL, Ron E, Gilbert ES, Vassilenko EK, Romanov SA, Kuznetsova IS, Sokolnikov ME, Okatenko PV, Kreslov VV, Koshurnikova NA. Cancer mortality risk among workers at the Mayak nuclear complex. Radiat Res. 2003; 158:787
98

Shiono PH, Chang CS, Mryianthopoulos NC. Preconception radiation, intrauterine diagnostic radiation and childhood neoplasia. J Natl Cancer Inst. 1980; 65:681-686

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

Shu XO, Potter JD, Linet MS, Severson RK, Han D, Kersey JH, Neglia JP, Trigg ME,
Robison LL. Diagnostic X-rays and ultrasound exposure and risk of childhood acute lymphoblastic leukemia by immunophenotype. Cancer Epidemiol Biomarkers Prev. 2002; 11:177-85

Shu XO, Gao YT, Brinton LA, Linet MS, Tu JT, Zheng W, and Fraumeni JF. A population-based case-control study of childhood leukemia in Shanghai. Cancer. 1988; 62:635-644

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

Siomi Mc, Mannen T, Siomi H. How does the royal family of Tudor rule the piwi-interacting RNA pathway. Genes and Development. 2010; 24:636-646

Soper S, Godfied WV, Hardiman TC, Goodheart M, Martin SL, de Boer P, Botvin A. mouse maelstrom, a component of the nuage is essential for spermatogenesis and transposon repression in meiosis. Develop Cell. 2008; 15:285-297.

Sowa M, Arthurs BJ, Morgan WF. Effects of ionizing radiation on cellular structures, induced instability and carcinogenesis. EXS. 2006; 96:293-301

Sugimoto K, Kage H, Aki N, Sano A, Kitagawa H, Nagase T,Yatomi Y, Ohishi N, Takai D. The induction of H3K9 methylation by PIWIL4 at the p16Ink4a locus. Biochem Biophys Res Com. 2007; 359: 497–502

Suzuki M, Zhou H, Hei TK, Tsuruoka C, Fujitaka K. 2003. Induction of a bystander chromosomal damage of He-ion microbeams in mammalian cells. Biol Sci Space. 2003; 17:251-252

Suzuki M, Tsuruoka C. Heavy charged particles produce a bystander effect via cell-cell junctions. Biol Sci Space. 2004; 18:241-6

Tamminga J, Koturbash I, Baker M, Kutanzi K, Kathiria P, Progribny 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. 2008a; 7(9):1238-45

Tamminga J, Kathiria P, Koturbash I, Kovalchuk O. DNA damage-induced upregulation of miR-709 in the germline downregulates BORIS to counteract aberrant DNA hypomethylation. Cell Cycle. 2008b; 73731-6

Thomson T, and Lin H. The biogenesis and function piwi proteins and piRNAs: Progress and prospect. Annu Rev Cell Dev Biol. 2009; 25: 355–376

Unhavaithaya Y, Hao Y, Beyret E, Yin H, Kuramochi-Miyagawa S, Nakano T, Lin H. MILI, a piRNA binding protein, is required for germline stem cell self-renewal and appears to positively regulate translation. J Biol Chem.2009; 284(10):6507-19

Vagin V, Signova A, Li C, Seitz H, Gvozdev V, Zamore P. A distinct small RNA pathway silences selfish genetic elements in the germline. Science. 2006; 313:320-324

Vagin VV, Wohlschlegel J, Qu J, Jonsson Z, Huang X, Chuma S, Girard A, Sachidanandam R, Hannon GJ, Aravin AA. Proteomic analysis of murine Piwi proteins reveals a role for arginine methylation in specifying interaction with Tudor family members. Genes Dev. 2009; 23:1749-62

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

Volinia S, Calin GA, Liu CG, Ambs S, Climmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M, Prueitt RL, Yanaihara N, Lanza G, Scarpa A, Vecchione A, Negrini M, Harris CC, Croce CM. A microRNA expression signature of

human solid tumors defines cancer gene targets. Proc Natl Acad Sci USA. 2006; 103:2257-2261

Wakabayashi T, Kato H, Ikeda T, Schull WJ. Studies of the mortality of A-bomb survivors, report 7. Part III. Incidence of cancer in 1959-1978, based on the tumor registry, Nagasaki. Radiat Res. 1983; 93:112-46

Ward JF. Radiation mutagenesis: The initial DNA lesions responsible. Radiat Res. 1995; 142:362-368

Watanabe S, Shimosato Y, Okita T, Ezaki H, Shigemitsu T. Leukemia and thyroid carcinoma found among A-bomb survivors in Hiroshima. Recent Results Cancer Res. 1972; 39:57-83

Watson GE, Lorimore SA, Clutton SM, Kadhim MA, Wright EG. Genetic factors influencing alpha-particle-induced chromosomal instability. Int J Radiat Bio1997; 71:497-503

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

Weidman JR, Dolinoy DC, Murphy SK, Jirtle RL. Cancer susceptibility: epigenetic manifestation of environmental exposures. Cancer J. 2007; 13:9-16

West A, Lahdetie J. X-irradiation-induced chanegs in the progression of type B spermatogonia and preloptotene spermatocytes. Mol Reprod Dev. 2001; 58:78-87

Wiemer EA. The role of microRNAs in cancer: no small matter. Eur J Cancer. 2007; 43:1529-44

Wiley LM, Baulch JE, Raabe OG, Straume T. Impaired cell proliferation in mice that persists across at least two generations after paternal irradiation. Radiat Res. 1997; 148:145-151

Williams ED. Chernobyl and thyroid cancer. J Surg Oncol. 2006; 94:670-7

Wright EG, Coates PJ. Untargeted effects of ionizing radiation: implications for radiation pathology, Mutation Res. 2006; 597:19-32

Wu LJ, Rander-Pehrson G, Xu A, Waldren CA, Geard CR, YU Z, Hei TK, Targeted cytoplasmic irradiation with alpha particles induces mutations in mammalian cells. Proc Natl Aca Sci. USA. 1999; 96:4959-4964

Xu GL, Bestor TH, Bourc'his D, Hsieh CL, Tommerup N, Bugge M, Hulten M, Qu X,

Russo JJ, Viegas-Pequignot E. Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene. Nature. 1999; 402:187-91

Xu M, You Y, Hunsicker P, Hori T, Small C, Griswold MD, Hecht NB. Mice deficient for small cluster of piwi-interacting RNAs implicate piwi-interacting RNAs in transposon control. Biol Reprod. 2008; 79:51-57

Yang H, Asaad N, Held KD. Medium-mediated intercellular communication is involved in bystander responses of X-ray-irradiated normal human fibroblasts. Oncogene. 2005; 24:2096-103

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 USA, 2005; 102:1513-1518

Yauk CL, Dubrova YL, Grant GR, Jeffreys AJ. A novel single molecular analysis of spontaneous and radiation induced mutation at a mouse tandem repeat locus. Mutat Res. 2002; 500:147-156

Yekta S, Shih IH, Bartel DP. MicroRNA-directed cleavage of HoxB8 mRNA. Science. 2004; 304:594-596

Yu F, Zingler N, Schumann G, Stratling WH. Methyl-CpG-binding protein 2 represses LINE-1 expression and retrotransposition but not alu transcription. Nucl. Ac. Res. 2001; 29:4493-501

Yu Z, Guo R, Ge Y, Ma J, Guan R, 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

Zeng Y. Principles of micro-RNA production and maturation. Oncogene. 2006; 25(46):6156-62.

Zhang D, Xiong H, Shan J, Xia X, Trudeau VL. Functional insight into Maelstrom in the germline piRNA pathway: a unique domain homologous to the DnaQ-H 3'–5' exonuclease, its lineage-specific expansion/loss and evolutionarily active site switch. Biology Direct. 2008; 3:48

Zhou H, Suzuki M, Geard CR, Hei TK. Effects of irradiated medium with or without cells on bystander cell responses. Mutat Res. 2002; 499:135-41