Non-targeted effects of ionizing radiation in vivo: epigenetic aspects

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ABSTRACT

The classical paradigm of radiation biology is based on the notion that ionizing particle has to traverse a nucleus of a living cell in order to damage genetic material either directly or via production of short living free radicals. After DNA damage is introduced it can be either safely repaired and the cell can continue divisions unaltered; or it can result in a failure to repair and cells death; or finally, upon misrepair, the cell would be carrying genetic alteration that could result in cancer or developmental abnormality. Therefore modern risk estimations are based on the notion that nucleus is the true target of radiation effects and those are essentially stochastic with linear dependence on the dose.

During the last two decades or so, a different idea was developed based on the observation that irradiated cells can communicate radiation induced stress signals to their unaffected neighbors and themselves become reprogrammed to maintained abnormal radiation-induced phenotype across multiple cellular divisions. Even more astonishingly this phenotype maybe transmitted by irradiated germ cells to unexposed progeny. Here we suggest that these non-targeted effects are maintained by epigenetic mechanisms and examine epigenetic underpinnings of bystander and transgenerational effects in vivo.

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LIST OF ABBREVIATIONS

[H3]dCTP – tritium labeled deoxycytidine triphosphate

ATP – adenosine triphosphate

CGI – CpG island

CHO – Chinese hamster ovary

dCTP - deoxycytidine triphosphate

DNA – deoxyribonucleic acid

DNMT – DNA methyl transferase

DSB – double strand break

ECL – enhanced chemiluminescence

HDAC – histone deacetylases

Hr – hour

IR – ionizing radiation

LET – low energy transfer

LINE – long interspersed nuclear element

LOWESS – local weighted scatterplot smoothing

LTR – long tandem repeat

LSH – lymphoid specific helicase

ncRNA – non-coding ribonucleic acid

NIH – national institute of health

nt - nucleotide

ORF – open reading frame

PCR – polymerase chain reaction

piRNA – piwi ribonucleic acid

PPI – paternal preconceptual irradiation

PVDF - polyvinylidene fluoride

qRT-PCR – quantitative real-time polymerase chain reaction

RIGI – radiation-induced genomic instability

RISC – RNA-induced silencing complex

RNA – ribonucleic acid

ROS – reactive oxygen species

RPM – read per million

SCE – sister chromatid exchanges

sncRNA – small non-coding ribonucleic acid

SOD – superoxide dismutase

TE – transposable element

TF – transcription factor

UTR – untranslated region

GENERAL INTRODUCTION

The central paradigm of classical radiation biology is based on a model that considers only the direct interaction of ionizing radiation (IR) with genetic material in the nucleus. Such an interaction leads to a mutational event. This model predicts that radiation-induced mutational events occur randomly within an IR-targeted nucleus within short period of time after exposure. It also predicts a linear dependence of the frequency of mutations on the dose of IR.

This central paradigm of radiation biology was challenged in 1992 by the discovery of non-targeted radiation-induced bystander effects of IR by Nagasawa and Little (1992). After α-particle irradiation of only 1% of cells in a cell population, 30% of cells exhibited increased levels of sister chromatid exchanges, suggesting the existence of communication between IR-damaged and intact naïve bystander cells. Their discovery brought about a new model of interaction between an exposed cellular system and IR.

This new theory suggests that IR can induce mutational events not only within targeted nuclei, but also within non-targeted intact nuclei when cellular cytoplasm is traversed. IR can also induce effects in neighboring unexposed naïve “bystander” cells and even in distant tissues and organs of an organism that has received a localized IR exposure. Moreover, a memory of the initial radiation injury is maintained in the progeny of irradiated cells in the form of an altered phenotype that favors genomic instability (Kadhim, Macdonald et al. 1992); repeated evidence suggests that a similar phenotype can be triggered in the embryo conceived from irradiated germ cells and maintained in the adult (Wiley, Baulch et al. 1997; Barber, Plumb et al. 2002; Streffer 2004). The phenomenon whereby cells are able to communicate radiation-induced stress signals to

unexposed cells is called the *bystander effect* and the phenomenon of the propagation of radiation-induced injury across cellular divisions is termed *radiation-induced genomic instability*.

Manifestations of non-targeted effects are well described, but their mechanisms are still not well understood. Strong evidence points to the involvement of altered cellular signaling processes maintained by heritable alterations in the chromatin and in the expression of non-coding RNAs (Koturbash, Baker et al. 2006; Koturbash, Boyko et al. 2007). This type of effect is termed "epigenetic" since the primary structure of the DNA is not altered. Here we review some of the experimental evidence of non-targeted effects in vitro and in vivo, and their possible connection to changes in epigenetic programming.

1.1. EXPERIMENTAL EVIDENCE OF NON-TARGETED EFFECTS OF IONIZING RADIATION

1.1.1. Bystander effect in vitro

The "bystander effect" refers to a relatively new radiobiological phenomenon that manifests as the ability of irradiated cells to transmit stress signals, causing the appearance of a wide spectrum of measurable radiation damage end-points in unexposed neighboring cells. Such end-points usually appear to be detrimental, and include chromosomal breakage, sister chromatid exchange (SCE), gene mutations, apoptosis, and malignant transformation (Morgan 2003). The induction of protective mechanisms in bystander cells, such as secretion of inhibitory factors (such as TGF-β2, inhibin-β, serine protease inhibitors) (Komarova, Diatchenko et al. 1998), enhanced cell differentiation (Belyakov, Folkard et al. 2002), and radio-adaptation (Iyer and Lehnert 2002).

A number of experimental protocols have been developed over the years to study the bystander effects of IR exposure in vitro, all of which can be classified into four categories: (1) experiments involving low fluency particle irradiation; (2) co-culturing of irradiated and naïve cells; (3) media transfer from irradiated to unirradiated cells; and (4) experiments involving microbeam exposure. Here we present a brief and by no means complete overview of the experimental evidence collected using these methods, and insights with regard to the possible molecular mechanisms of bystander effects and the nature of the signals transmitted by irradiated cells.

1.1.2. Low fluency broad-beam radiation experiments

Historically, the earliest studies of bystander effects involved low fluency α-particles. Nagasawa and Little (1992) observed close to a 30% increase in SCE in Chinese hamster ovary (CHO) cells at the very low dose of 0.31 mGy. As predicted, 1% of nuclei were directly traversed by ionizing particles at the given dose. The same experiment showed an unusual dose-response, as the frequency of induced SCE plateaued at 2.5 mGy, instead of increasing linearly as predicted by the target model of radiation exposure. Interestingly, this effect was specific for SCE, as a previous study detected a linear increase in chromosomal aberrations with a similar dose delivery (Nagasawa, Little et al. 1992). The authors came to the conclusion that α-particles induce an increase in the recombination process in unexposed nuclei, possibly mediated by autocrine signaling cascades induced by reactive oxygen species (ROS). The increase in SCE was observed only in the low-dose range consistent with occupational radiation exposures (Nagasawa, Little et al. 1992). These experimental findings were confirmed by Deshpande and colleagues (1996), who observed excessive induction of SCEs in normal human lung

fibroblasts in the low-dose range. Furthermore, short-lived SCE inducing factors were generated by α-particles in a cell-free medium containing serum; however, long-lived (≈24 hr) SCE inducing factors were produced only in media containing living cells. In both cases they were inhibited by ROS scavengers (Lehnert, Goodwin et al. 1997).

Mutations of the mutational marker Hprt (hypoxanthine-guanine phosphoribosyltransferase) loci were significantly induced at very low doses of α-particles in CHO cells, and the frequency of mutations was estimated to be increased five-fold over that expected from direct nuclear traversals (Nagasawa and Little 1999). Furthermore, the bystander effects (based on the frequency of Hprt mutations) were greatly enhanced in double-strand break (DSB) repair deficient CHO cells in comparison to the wild type, demonstrating that bystander-induced signaling may be linked to DSB repair (Nagasawa, Huo et al. 2003). The spectrum of mutations was also different, with mainly point mutations in wild type cells and deletions in DSB repair deficient cells (Nagasawa, Huo et al. 2003). DNA damage signaling pathways were activated in bystander lung fibroblasts as p53 protein accumulation was detected in 5.3% of cells, while only 0.45% of the nuclei were traversed by ionizing particles within the exposed population (Hickman, Jaramillo et al. 1994).

Bystander effects can be induced not only by α-particles but also by low-LET radiation such as X-rays, which caused bystander effects in human fibroblasts independent of the dose above 0.1 Gy (Yang, Asaad et al. 2005). The bystander response to X-rays manifested in the accumulation of γH2AX foci (indicators of DNA double-strand breaks), induction of the DNA damage sensor p21Waf1, and decreased clonogenic survival of bystander cells (Yang, Asaad et al. 2005). Treatment with ROS scavengers

decreased the accumulation of DSBs and stress inducible p21Waf1 (cycline dependent kinase inhibitor 1), but had no effect on clonogenic survival, indicating that the irradiated cells released factors other than ROS into the cellular environment (Yang, Asaad et al. 2005).

Experiments by Azzam and colleagues showed that DNA damage sensing and homologous recombination pathways were induced in bystander cells and that these effects could be suppressed by the interruption of intercellular gap junctions (Azzam, de Toledo et al. 1998). Furthermore, the same group showed that bystander induction of p21Waf1 and micronucleus formation were abrogated in cells deficient in gap-junction communication due to loss-of-function mutations in Connexin43 (Azzam, de Toledo et al. 1998). Recently, an elegant study addressed the question of the efficient distance of bystander signaling. The cells were grown on a plate overlaid with an ultra-thin solid-state nuclear detector, allowing visualization of directly traversed and bystander cells harboring DNA damage (Gaillard, Pusset et al. 2009). The accumulation of stress-responsive p21Waf1 protein was chosen as an end-point, and it occurred within the range of 100 μm from the irradiated cell; the mean propagation distance was 20–40 μm around the intranuclear α-particle impact point, encompassing a set of ≈30 cells (Gaillard, Pusset et al. 2009).

1.1.3. Media transfer experiments

Media transfer experiments originated from the premise that irradiated cells can secrete soluble factors into the culture media capable of inducing a DNA damage response in non-irradiated cells, compatible with the bystander effect model. Indeed, when human keratinocytes were irradiated with 0.5–5 Gy γ rays and propagated for

several passages, the media harvested at each passage caused a significant increase in apoptosis in non-irradiated cells (Lyng, Seymour et al. 2002). This increase was paralleled by rapid calcium fluxes, loss of mitochondrial membrane potential, and generation of ROS in cells subjected to the conditioned media (Lyng, Seymour et al. 2002). Importantly, these effects were independent of the dose and the cell-culture passage, indicating that bystander signals were still emitted by distant progeny of the irradiated cells (Lyng, Seymour et al. 2002; Lyng, Seymour et al. 2002). Induction of SCE was detected in non-irradiated cells cultured in media transferred from the irradiated cell culture, but not when cell-free media were irradiated, indicating that bystander signals are secreted by cells and are not a result of radio-chemical reactions in the media itself (Suzuki, Zhou et al. 2004). Bystander signals could be generated very rapidly up to 2.5 minutes after irradiation, and reach peak activity about 10 min post-exposure (Han, Wu et al. 2007).

An increase in DSBs, a decrease in clonogenic survival, as well as an increase in mitochondrial mass, were observed in media transfer experiments (Maguire, Mothersill et al. 2005; Chen, Zhao et al. 2009). These effects were diminished when the cells receiving radiation were depleted of mitochondria, and when the conditioned medium was treated with ROS scavengers (Chen, Zhao et al. 2009). The oxidative stress signaling observed in media transfer experiments may be mediated by production of the tumor necrosis factor α (TNFα) (Natarjan and Gibbons et al. 2007), and/or tumor necrosis factor β-1 (TNFβ-1) (Burdak-Rothkamm et al. 2006). Induction of the bystander response depended on the genetic background, since it was completely suppressed in cells with mutated DNA damage sensing kinase Atr (Burdak-Rothkamm et al. 2006); also, the extent of the

bystander effect seemed to depend on the DNA-repair phenotype of the cells receiving but not producing the stress signal (Kashino and Suzuki et al. 2007).

1.1.4. Microbeam experiments

Microbeam irradiation is extremely suitable for bystander studies, as it allows the delivery of an exact number of charged ionizing particles to a specific cell or even to a specific cell compartment. Microbeam dose delivery is gaining popularity and has been used extensively to study bystander effects in cell lines and 3-D tissue models. Microbeam experiments have confirmed excessive induction of strand breaks and mutations in cells adjacent to those receiving radiation exposure. The bystander response could be suppressed completely by the combination of ROS scavengers and gap-junction inhibitors, and the yield of bystander cells was shown to be independent of the number of particles traversing the target cells (Zhou, Randers-Pehrson et al. 2000; Belyakov, Malcolmson et al. 2001). Furthermore, bystander cells could undergo malignant transformation in vitro, providing a direct link to oncogenesis (Sawant, Randers-Pehrson et al. 2001). Microbeam exposure has provided strong and direct evidence that cytoplasmic exposure alone is sufficient to induce DNA damage both in the nuclei of a target cell and in bystander cells (Shao, Folkard et al. 2004). Nitric oxide scavenging (NO) and suppression of lipid rafts with filipine abrogated cytoplasmic response has demonstrated the importance of free radicals and membrane signaling in bystander effects (Shao, Folkard et al. 2004).

Distinct responses were detected in 3-D tissue models that attempt to combine the convenience of cell lines with realistic complexity of in vivo systems. Studies using porcine ureter explants showed a slight increase in micronuclei and apoptosis in

bystander cells, paralleled by significantly increased terminal differentiation resembling a protective response (Belyakov, Folkard et al. 2002). A study using a 3-D lung epithelium model showed that bystander DNA damage can occur as far as 1 mm from the targeted cell (Belyakov, Mitchell et al. 2005).

Recent work has demonstrated the induction of delayed genomic instability in bystander cells using a 3-D tissue model and cultured human lymphocytes (Sedelnikova, Nakamura et al. 2007; Kadhim, Lee et al. 2010). Genomic instability in bystander cells may be associated with the dysregulated expression of microRNAs and global genomic hypomethylation in recipient cells (Kovalchuk, Zemp et al. 2010; Sedelnikova, Nakamura et al. 2007).

1.1.5. Possible mechanisms

In vitro experiments have provided some important insights into the nature of bystander signaling. All experimental strategies used have demonstrated that irradiated cells transmit signals to neighboring cells, resulting in the accumulation of DNA damage. The signal can be communicated across cell-to-cell gap junctions and as extracellular soluble factors, and these two mechanisms are not mutually exclusive (Mitchell, Randers-Pehrson et al. 2004). The signals spread over a relatively short range, affecting a set of ≈30 adjacent cells, but the communication distance may be up to 1 mm in tissue models (Belyakov, Mitchell et al. 2005; Gaillard, Pusset et al. 2009). Cytoplasmic irradiation alone is sufficient to elicit a bystander response, therefore the whole cell and not only the nucleus has to be viewed as a radiation sensor (Shao, Folkard et al. 2004).

The generation of ROS and membrane signaling seem to mediate the induction of non-targeted DNA damage in many systems. The exact nature of bystander signals

remains unknown; however, stress-induced cytokines, including TGFα, TGFβ, and nitric oxide, were upregulated in a number of experiments (Dickey, Baird et al. 2009). Genetic and epigenetic background is important, since not all cell types can produce bystander signals and not all can serve as recipients (Mothersill, Seymour et al. 1997; Nagar, Smith et al. 2003; Mothersill, Rea et al. 2003). Furthemore, the end-points of bystander signaling in some 3-D tissue models manifested in increased differentiation of bystander cells (Belyakov, Folkard et al. 2002; 2006). Finally, bystander effects induce genomic instability that persists for several generations descending from recipient cells (Seymour and Mothersill 1997; Watson, Lorimore et al. 2000; Morgan, Hartmann et al. 2002).

1.1.6. Bystander effects in vivo

Bystander effects will become truly relevant for risk estimations associated with radiation exposure only when there is convincing experimental evidence of their action in animal models. To date, investigations of in vivo bystander effects remain scarce. Results of experiments in cell and tissue cultures raise several possibilities when extrapolated to intact organisms: (1) short-range communication of bystander signals through cell-to-cell gap junctions means that more cells are affected by a single traversing particle than predicted by the target model for low-dose exposure; (2) media transfer experiments suggest that soluble factors may be secreted into the bloodstream and cause bystander effects in distant parts of the body; (3) genomic instability triggered in bystander cells suggests that DNA damage induction may be persistent; and (4) the extent of bystander effect induction in vivo may depend on the tissue irradiated and the genetic background of the organism.

The first evidence of bystander effects in vivo came from the study of clastogenic (chromosome break inducing) factors in the serum of individuals subjected to therapeutic and accidental irradiation. The results of these studies showed that the incubation of test cells in the plasma of an irradiated donor causes a significant increase in chromosomal breakage as compared to plasma from non-irradiated controls (Emerit, Levy et al. 1994; Emerit, Quastel et al. 1997; Marozik, Mothersill et al. 2007). These effects were highly persistent, since they could be clearly detected even 20 years after the initial exposure (Marozik, Mothersill et al. 2007). Recent work has demonstrated that signals generated by in vivo irradiated bone marrow induced DNA damage and apoptosis in non-irradiated bone marrow cells (Burr, Robinson et al. 2010). The signaling mechanism involved FasL, Tnfα, NO, and superoxide, presumably generated by IR-induced macrophages; the signals were generated within 24 hours, but not immediately after radiation (Burr, Robinson et al. 2010).

An additional line of evidence is provided by shielding experiments, where part of the animal body is covered with a radio-protective screen, while a distant part receives radiation; the DNA damage end-points are then measured in the covered part. For instance, irradiation of the lung base showed a marked frequency of micronuclei in the shielded lung apex (Khan, Hill et al. 1998; Khan, Van Dyk et al. 2003). Interestingly, radiation of the lung apex did not lead to a substantial increase in DNA damage in the lung base, suggesting that even parts of the same organ may display different bystander competency (Khan, Van Dyk et al. 2003). ROS and NO scavengers reduced bystander effects in the unexposed part of the lung, suggesting the involvement of inflammatory process (Khan, Van Dyk et al. 2003).

It is of great interest whether radiation injury in one organ can be communicated to another. Unilateral exposure of one side of the body, while the other was protected by medical-grade shield, caused increased DNA damage in protected skin. DNA hypomethylation was detected only in directly exposed tissue, but there was a marked decrease in de novo DNA methylatrasferases (Dnmts) and methyl-CpG-binding proteins detected in bystander skin (Koturbash, Rugo et al. 2006). Cranial exposure to 20 Gy of X-rays resulted in a substantial accumulation of DNA double-strand breaks in a shielded spleen, paralleled by a decrease in DNA methylation at long interspersed nuclear element 1 (LINE1) and down-regulation of DNA methyltransferases. This effect could be mediated by a bystander-induced increase in microRNA-194, a putative regulator of Dnmt3a (Koturbash, Boyko et al. 2007). DNA hypomethylation effects persisted in bystander tissue for seven months post-exposure (Koturbash, Boyko et al. 2007). Cranial irradiation of mice resulted in the accumulation of p53 in the bystander spleen as well as an increase in apoptosis and cellular proliferation (Koturbash, Loree et al. 2008). Recent work showed that bystander signals induced persistent changes in the expression of microRNAs in the shielded spleen of male and female animals (Koturbash, Zemp et al. 2008). Bystander-induced changes in microRNA expression appeared to be sex-specific and were dramatically altered by sterilization of the animals, suggesting modulation by sex hormones (Koturbash, Zemp et al. 2008).

Until very recently, it was unclear whether bystander effects were relevant to the induction of carcinogenesis in vivo. This question was resolved when Mancuso and colleagues (2008) demonstrated the induction of malignancy in the shielded head (specifically the cerebellum) of radio-sensitive mice by exposure of the remainder of the

body to X-rays. Furthermore, the induction of malignancy was paralleled by the accumulation of DSBs and increased apoptosis; cell-to-cell gap junctions were necessary for the induction of tumorgenesis in the bystander brain (Mancuso, Pasquali et al. 2008).

Overall, in vivo studies have demonstrated that distant IR exposure results in DNA damage, apoptosis, and altered cellular proliferation. Changes in DNA methylation and microRNA expression may be vital for the maintenance of genomic instability in bystander tissue. Furthermore, bystander effects are carcinogenic in vivo, at least in the case of the radio-sensitive model.

1.2. RADIATION INDUCED GENOMIC INSTABILITY

1.2.1. Definition and manifestations

Radiation-induced genomic instability (RIGI) refers to the increased rate of de novo mutational events in the progeny of irradiated cells. Manifestations of RIGI include delayed reproductive death (Chang and Little 1991), chromosomal aberrations (Chang and Little 1992), increased apoptosis (Lyng, O'Reilly et al. 1996), micronuclei formation (Jamali and Trott 1996), gene expression changes (Tsai, Chen et al. 2006), and aberrant DNA methylation (Kaup, Grandjean et al. 2006).

Since an increased rate of mutations and chromosomal aberrations is observed in a variety of cancers (Loeb and Loeb 2000), RIGI was suggested as one of the driving forces behind radiation-induced carcinogenesis (Streffer 2010). Little is known about the mechanisms involved in the maintenance of RIGI across multiple cell divisions in affected clonal populations; however, existing data strongly implicate heritable epigenetic dysregulation paralleled by persistent changes in reactive oxygen species metabolism (Aypar and Morgan et al. 2010; Lorimore and Wright 2003). Furthermore, RIGI cannot

be understood in isolation from bystander effects, since it can be induced in cells never traversed by radiation but allocated to the same radiation environment (Kadhim, Macdonald et al. 1992; Watson, Lorimore et al. 2000; Lorimore, Coates et al. 2001). In this section we review experimental evidence of RIGI in vitro and in vivo, with an emphasis on the mechanistic aspects.

1.2.2. Experimental evidence of radiation-induced genomic instability

RIGI was first demonstrated by Kadhim and Macdonald et al. (1992) after irradiation of bone marrow stem cells with PU238 α-particles. The main outcome of this seminal study was the detection of non-clonal chromosomal aberrations in clonal descendants of irradiated cells, a phenomenon incompatible with target-hit theory. In the framework of classical radiation biology, chromosomal damage is induced due to direct interaction with ionizing particles or radio-chemically induced free radicals at the time of irradiation, and upon misrepair can be transmitted to the clonal progeny. In a number of follow-up experiments, non-clonal aberrations were induced in a variety of cellular systems after irradiation with both high-LET and low-LET ionizing particles (Holmberg, Falt et al. 1993; Durante, Grossi et al. 1996). RIGI was assessed using a broad spectrum of end-points, including delayed reproductive death (Chang and Little 1991), delayed apoptosis (Jamali and Trott 1996), mutation frequency (Grosovski and Parks et al. 1996), double-strand breaks (Desai, Durante et al. 2005), and altered gene expression (Snyder and Morgan 2005).

RIGI can be induced not only by IR but also by radiomimetic chemicals, causing the formation of complex DSBs; however, other DSB-inducing agents, such as restriction enzymes and hydrogen peroxide, were ineffective (Limoli, Kaplan et al. 1997). This data

indicates that the quality of DNA damage is important for the initiation of RIGI, and that the presence of DSBs per se is insufficient for induction.

One of the key features of persistent radiation-induced stress was the dysregulation of oxidative metabolism. Increased oxyradical activity and changes in signal-transduction proteins were detected in the progeny of X-ray exposed human lymphoblasts after 55 generations (Rugo and Schiestl 2004). The accumulation of superoxide was detected in bone marrow cell culture seven days after exposure, and the extent of this accumulation was dependent on the initial dose (Clutton, Townsend et al. 1996). Increased oxidative stress in the progeny of irradiated cells may be attributed to persistent mitochondrial damage. Limoli and Giedzinski (2003) showed a decreased mitochondrial potential and an increase in the number of mitochondria in unstable clones. The mitochondria from unstable clones had impaired respiration (Kim, Chandrasekaran et al. 2006); in addition, manganese superoxide dismutase (mnSOD), a mitochondrial antioxidant scavenger, was decreased in destabilized cells (Miller, Jin et al. 2008).

Recent work has suggested that a radiation-induced mutator phenotype is maintained by an increased steady-state level of hydrogen peroxide, paralleled by a decreased expression of catalase and glutathione peroxidase (Dayal, Martin et al. 2008). A follow-up study identified the dysfunction as originating from complex II of the respiratory chain as a source of hydrogen peroxide accumulation (Dayal, Martin et al. 2009). Hypoxia (2% oxygen) inhibited X-ray-induced delayed instability as compared to “normal” 20% oxygen conditions (Roy, Kodama et al. 2000). It has to be noted that a number of experiments have failed to detect dysregulation of ROS production in unstable

cellular populations (McIlrath, Lorimore et al. 2003; Suzuki, Kashino et al. 2009); therefore ROS may not be a definitive factor in the development of RIGI.

RIGI can be induced not only in the direct descendants of an irradiated cell, but also in the descendants of the cells subjected to a medium filtrated from the radiation-destabilized clones, indicating the involvement of factors secreted from destabilized cells, and implicating the bystander effect in the propagation and maintenance of genomic instability (Nagar, Smith et al. 2003; Nagar and Morgan 2005). An effort to identify this secreted factor pointed to interleukin-8 (Il-8) as a possible culprit (Laiakis, Baulch et al. 2008). However, the addition of Il-8 to the stable parental cell line induced immediate DNA damage but not persistent genomic instability (Laiakis, Baulch et al. 2008). An alternative candidate for the secreted bystander factor is Tnfα. Secretion of this pro-inflammatory cytokine was induced by direct radiation exposure; Tnfα influences a number of cellular processes, including the generation of ROS and apoptosis (Natarajan, Gibbons et al. 2007). In this experiment, ectopic addition of Tnfα caused persistent genomic instability in the progeny of treated cells (Natarajan, Gibbons et al. 2007).

An attempt to gain insight into the molecular mechanism of RIGI relied on the use of microarray gene expression profiling (Snyder and Morgan 2005). This analysis revealed lower levels of the transcripts of copper/zinc (Cu/Zn) superoxide dismutase 1 (Sod1) and nuclear-factor-kappaB (NF-kappaB) in unstable clones (Snyder and Morgan 2005). Sod1 is a ROS scavenger while NF-kappaB regulates diverse cellular processes, including inflammatory responses and apoptosis; these expression changes, however, were not confirmed by Northern and Western blots (Snyder and Morgan, 2003 and 2005).

Elegant experiments conceived by Watson et al. (2000) convincingly demonstrated that RIGI started in vitro can be maintained for a long time in vivo, and that it is present in the progeny not only of directly irradiated cells, but also of bystander cells. In this experiment, bone marrow stem cells irradiated with neutrons were mixed with non-irradiated cells and then transplanted into a bone marrow ablated congenic mouse. Three-way identification systems made it possible to prove that genomic instability is induced in transplanted non-irradiated cells by a bystander mechanism and can persist for up to 13 months in the host organism (Watson, Lorimore et al. 2000). The authors came to the conclusion that the cells responsible for the production of chromosomal instability in unirradiated cells are likely to be pro-inflammatory stromal macrophages that generate ROS in a bone microenvironment (Coates, Robinson et al. 2008; Lorimore, Chrystal et al. 2008). Therefore, RIGI may be in part attributed to radiation-induced inflammatory responses altering the bone-marrow microenvironment and contributing to the persistence of the effect (Lorimore, Coates et al. 2001; Lorimore, Chrystal et al. 2008).

1.3. TRANSGENERATIONAL EFFECTS OF RADIATION IN MICE AND HUMANS

1.3.1. Transgenerational genomic instability

Targeted effects of radiation are caused by the direct interaction of ionizing particles with genetic material. If misrepaired DNA damage is fixed as a mutation it may lead to cancer in the exposed organism if it occurs in soma, or to heritable phenotypic change if it occurs in the germ line. Direct germline mutations are transmitted to subsequent generations in accordance with the laws of classical genetics, and may lead to a whole spectrum of deleterious effects, including but not limited to dominant lethality and congenital malformations (Mole 1979; Goldman 1982).

However, a growing body of data shows that the heritable effects of radiation exposure are not limited to those caused by targeted mutations. A number of studies have shown de novo generation of mutations in the non-exposed progeny of an irradiated parent, resembling radiation-induced genomic instability (Luning, Frolen et al. 1976; Luke, Riches et al. 1997; Carls and Schiestl 1999; Shiraishi, Shimura et al. 2002; Slovinska, Elbertova et al. 2004; Streffer 2006). Early work, focused on the study of radiation-induced dominant lethal mutations in mice, unexpectedly showed an excess of intrauterine deaths of fetuses pre-conceptually irradiated at the spermatogonial stage, and the continuation of this effect to the F2 generation (Luning, Frolen et al. 1976).

An embryo chimera assay showed that the cells in embryos conceived by sperm irradiated at the type B spermatogonial stage were proliferating at a slower rate than cells in the normal embryo, and that this effect was transmitted to the F2 generation with no sign of suppression (Wiley, Baulch et al. 1997). Subsequent experiments demonstrated altered activities of receptor tyrosine kinase, protein kinase C, and MAP kinase, and the accumulation of p53 and p21Waf1 proteins in the F3 progeny of mice paternally irradiated at the spermatogonial stage (Baulch, Raabe et al. 2001; Vance, Baulch et al. 2002).

The discovery of hypermutable minisatellite loci (short tandem repeats in human and mouse genomes) provided an invaluable biomarker for the study of radiation-induced germline mutations (Dubrova, Jeffreys et al. 1993). Pioneering work by Dubrova et al. showed an increase in the germline mutation rate in the progeny of fathers exposed to radiation as a result of the Chernobyl accident (Dubrova, Nesterov et al. 1996; Dubrova, Nesterov et al. 1997). The same methods were used to detect an increased frequency of

germline mutations in multigenerational families living close to the Semipalatinsk nuclear test site and among residents of the Techa River population (Dubrova, Bersimbaev et al. 2002; Dubrova, Ploshchanskaya et al. 2006; Akleev, Dubrova Iu et al. 2007).

Animal studies conducted by the same group showed that germline mutations at minisatellite loci were induced at a frequency an order of magnitude higher than expected in the case of direct mutations (Dubrova, Plumb et al. 1998; Dubrova, Plumb et al. 2000). Importantly, increased rates of minisatellite mutations were still observed in the F2 progeny of irradiated male mice, and this trait was exhibited by most of the offspring, in violation of Mendelian laws (Barber, Plumb et al. 2002). The induction of transgenerational genomic instability was influenced by the dose, type of irradiation, and stage of germ cell development (Fan, Wang et al. 1995; Niwa, Fan et al. 1996; Dubrova, Plumb et al. 1998).

1.3.2. Transgenerational carcinogenesis

Transgenerational genomic instability may be linked to transgenerational radiation-induced carcinogenesis that manifests in an increased cancer risk in the unexposed progeny of irradiated parents. And increased predisposition to tumor formation in the progeny of irradiated mice was first demonstrated by Nomura (1982; 1983; 1989; 2004). This effect, although slightly carcinogenic, was transmitted to subsequent generations in a non-Mendelian manner, and was greatly enhanced upon the application of secondary carcinogens (Vorobtsova and Kitaev 1988; Vorobtsova, Aliyakparova et al. 1993). A number of experiments were designed to confirm and expand the initial findings of Nomura. Daher et al. (1998) reported a marginally

significant increase in leukemia incidence in the progeny of X-ray irradiated N5 mice, as well as the earlier onset of leukemia development in the progeny of mice injected with tritium.

Similarly, modulation in leukemia/lymphoma development was observed in BDF1 and CBA mice after pre-conceptual treatment with plutonium 239 followed by secondary exposure to methylnitrosourea (Lord, Woolford et al. 1998). A lifetime study that included 4,279 mice showed that pre-conceptual treatment with a high acute dose of X-rays leads to increased hematopoietic malignancy in female and bronchioloalveolar adenocarcinomas in male progeny. On the other hand, the same study failed to show increased tumor induction by secondary exposure to urethane (Mohr, Dasenbrock et al. 1999). The exact manifestations of radiation-induced transgenerational carcinogenesis were shown to depend on the dose, type of irradiation, stage of germ cell development, and genetic background of the model organism (Draper 1989; Lord 1999; Nomura 2006).

1.3.3. Possible mechanisms

To date, the exact molecular mechanisms of radiation-induced transgenerational effects remain unclear. The high frequency of induction and the fact that these effects are present in most if not all offspring are incompatible with the involvement of genetic mutations. Radiation-induced free radicals could be considered; however, their short-lived nature and negligible cytoplasmic component in sperm make them a very unlikely candidate. The manifestations and mode of transmission of this effect suggests that it has to be fixed across multiple loci in most if not all germ cells produced by an affected gonad. It is tempting to hypothesize that these changes are memorized in the germ cell through epigenetic mechanisms. DNA methylation marks at imprinted loci and certain

repeat elements were shown to be resistant to epigenetic reprogramming events in the developing embryo (Hajkova, Erhardt et al. 2002; Lane, Dean et al. 2003). A series of recent studies described the ability of sperm-bound small RNAs to trigger developmental changes in the embryo that could be transmitted to subsequent generations in a non-Mendelian manner (Rassoulzadegan, Grandjean et al. 2006; Rassoulzadegan, Grandjean et al. 2007; Grandjean, Gounon et al. 2009). No data is available on the ability of histone marks to trigger similar responses; however, histones were shown to be present in sperm, covering the genomic areas responsible for embryonic development (Brykczynska, Hisano et al. 2010).

A preliminary study conducted by Kovalchuk's group found a significant loss of cytosine DNA methylation in the thymus of progeny after paternal exposure to X-rays. The loss of DNA methylation was paralleled by a significant decrease in the levels of maintenance (DNMT1) and de novo (DNMT3a and DNMT3b) methyltranferases and methyl-CpG-binding protein (MECP2). They also noted a significant accumulation of DNA strand breaks in the thymus (Koturbash, Baker et al. 2006). Localized cranial irradiation of male rats led to the accumulation of DNA lesions and loss of global DNA methylation in mature sperm (Tamminga, Koturbash et al. 2008). Loss of DNA methylation along with dysregulation of DNA methylation enzymes was also found in the progeny of cranially exposed male rats (Tamminga, Koturbash et al. 2008). Microarray expression profiling revealed altered miRNA levels in the thymus of adult male mice pre-conceptually exposed to 2.5 Gy of X-rays (Filkowski, Ilnytskyy et al. 2010). Changes in the miRNAome were paralleled by dysregulation in miRNA processing machinery and decreased expression of Lsh (Filkowski, Ilnytskyy et al. 2010), a chromatin remodeling

factor implicated in the regulation of DNA methylation in repeat elements (Muegge 2005). DNA methylation profiling of repeats in the same samples revealed a loss of CpG methylation in LINE1 (long interspersed nuclear element 1) and SINE B2 (short interspersed nuclear element B2) of retrotransposable elements (Filkowski, Ilnytskyy et al. 2010).

Cumulative evidence points to the epigenetic origins of radiation-induced transgenerational genomic instability and cancer predisposition; however, the exact mechanisms remain unclear. Future research in this area has to rely on the use microarray technology, next-generation sequencing, and bioinformatic approaches in order to extract functionally relevant causal changes influencing epigenetic reprogramming and genomic stability across generations. Such research has both practical and fundamental value, as it may offer an understating of how genotoxic factors contribute to complex disease by altering our epigenome across generations.

1.4. EPIGENETIC DETERMINANTS OF NON-TARGETED EFFECTS OF RADIATION

1.4.1. Epigenetic modifications

Although all types of cells in a multicellular organism contain the same genome, the properties of the cells themselves are stunningly different due to the selective expression of different sets of genes in different cell types. In order for the tissue to maintain its physiological identity, such gene expression signatures are faithfully transmitted across cellular divisions. However, they also have to be plastic enough to respond to intrinsic and extrinsic physiological stimuli and to adapt to a changing environment (Jirtle and Skinner 2007). Such features are provided by chemical

modifications of DNA, histone proteins bound to DNA, and expression of non-coding RNAs (Bonasio, Tu et al. 2010; Gibney and Nolan 2010). Epigenetic traits acquired during development under the influence of adverse environmental factors may be stabilized across mitotic cellular divisions and cause an altered molecular phenotype, contributing to pathology (Bollati and Baccarelli 2010).

A number of investigators have suggested that radiation-induced epigenetic changes contribute to the maintenance and propagation of RIGI (Dubrova 2003; Kovalchuk and Baulch 2008; Aypar and Morgan, 2010). Here we present a brief overview of known epigenetic determinants and their responses to radiation exposure.

1.4.2. DNA methylation in mammals

DNA methylation was historically the first epigenetic modification discovered in mammals. It is a covalent modification found almost exclusively in cytosine bases in cytosine-phosphate-guanines (CpG)-dinucleotides (Bird 2009). Extensive non-CpG methylation was detected in embryonic stem cells (Ramsahoye, Biniszkiewicz et al. 2000). DNA methylation in mammals is generally associated with the repressed state of chromatin and silenced gene expression (Bird and Wolffe 1999).

Effector enzymes that directly transfer a methyl group to CpG cytosines are called DNA methyl transferases. Three bona fide DNA methyl transferases have been indentified in mammals to date: Dnmt1, Dnmt3a, and Dnmt3b. Dnmt1 is thought to copy the pre-existing methylation pattern onto the newly synthesized strand during replication, and is therefore considered maintenance Dnmt (Bestor 2000). Dnmt3a and Dnmt3b function as de novo methyltranferases primarily during embryonic development and in the germ line. The expression of de novo methyltransferases is high in the embryo, germ

line, and stem cells, and decreases in adult tissue. Still, the expression Dnmt3a remains ubiquitous in all tissues, while the expression of Dnmt3b is very low in all tissues except for testes, thyroid, and bone marrow (Xie, Wang et al. 1999). De novo methylation activity is required for the establishment of parental imprints and the methylation of repeat elements (Kato and Kaneda et al. 2007). Loss-of-function mutations of any of the active methyltransferases are embryonically or postnataly lethal (Li, Bestor et al. 1992; Okano, Bell et al. 1999).

A fourth DNA methyltransferase, Dnmt2, showed weak DNA methyltransferase activity in vitro (Hermann, Schmitt et al. 2003), but loss-of-function mutations of this protein had no effect on global methylation (Okano, Xie et al. 1998). Dnmt3l is a Dnmt-related protein without intrinsic methyltransferase activity; however, it was shown to directly associate with de novo methyltrasferases and enhance their function (Suetake, Shinozaki et al. 2004). Dnmt3l knockouts lead to transposon overexpression and meiotic arrest in the male germ line (Bourc'his and Bestor 2004).

The influence of DNA methylation on chromatin structure and gene silencing is modulated by methyl-CpG-binding proteins (MBPs) that attract transcriptional co-repressor molecules to silence transcription and modify the surrounding chromatin (Fujita, Watanabe et al. 2003; Kondo, Gu et al. 2005).

In normal cells, DNA methylation plays a critical role in controlling tissue-specific gene expression (Nagase and Ghosh 2008), parental imprinting (Bestor 2003), X-chromosome inactivation (Reik, Santos et al. 2003), silencing of repeat elements (Huang, Fan et al. 2004; Kato, Kaneda et al. 2007), and maintenance of genomic integrity (Chen, Pettersson et al. 1998). Most of the CpGs (60–90%) in the mammalian genome are

methylated, and certainly most of them are allocated to repeat elements. The methylation pattern of transcriptional units is more complex and tends to depend on many factors. CpG sites are not distributed randomly in the genome, but seem to form certain patterns with significant clustering in the vicinity of transcription start sites that define the core promoter area (Bird 2009). Such clusters are called CpG islands (CGI), and their hypermethylation leads to transcriptional silencing. Normally, only 5% of CGIs in the human or mouse genome are hypermethylated (Weber and Schubeler 2007). Hypermethylated high-CpG genes detected in normal differentiated tissues are enriched in the functional categories responsible for pluripotency, spermatogenesis, oogenesis, and meiosis (Weber and Schubeler, 2007). DNA methylation of promoter areas with low CpG content does not lead to their transcriptional silencing (Weber and Schubeler, 2007). Significant numbers of CGIs are located outside of promoter areas, within exons, introns, and intergenic areas. Their functional significance is unclear, but they may indicate positions of yet unidentified transcripts. Recent work showed that a large fraction of intragenic CGIs were methylated in a tissue-specific manner, thus regulating the expression of alternative transcripts (Maunakea, Nagarajan et al. 2010). Methylation of repeat elements appears to be necessary for silencing transposition, preventing the binding of transcriptional factors to cryptic promoters, and stabilizing chromosomal structure (Klose and Bird 2006).

Dramatic changes in DNA methylation are common in cancer, and manifest primarily as global DNA hypomethylation paralleled by local hypermethylation at gene promoters (Feinberg and Vogelstein 1983; Rhee, Bachman et al. 2002). DNA methylation can influence tumorgenesis in several ways: (1) promoter hypermethylation

leads to silencing of the tumor-suppressors (Rhee, Bachman et al. 2002); (2) global genomic hypomethylation leads to chromosomal instability (Gaudet, Hodgson et al. 2003); (3) hypomethylation at repeat elements causes transposon-mediated mutagenesis (Hedges and Deininger 2007); (4) DNA hypomethylation induces the transcription of oncogenes (Wolff, Byun et al. 2010); and finally (5) spontaneous deamination of methyl-cytosine induces point mutations (Ketterling, Vielhaber et al. 1994).

1.4.3. Influence of radiation exposure on DNA methylation

A number of reports suggest that both direct and bystander radiation exposure lead to changes in DNA methylation (Kalinich, Catravas et al. 1989; Tawa, Kimura et al. 1998; Kovalchuk, Burke et al. 2003; Raiche, Rodriguez-Juarez et al. 2004). γ-ray exposure of hamster and human cell lines led to global hypomethylation, paralleled by the relocation of Dnmt1 from the nucleus to the cytoplasm (Kalinich, Catravas et al. 1989). On the other hand, Tawa et al. (1998) detected dramatic DNA hypomethylation after X-ray irradiation in mouse liver, but not in mouse spleen or brain tissue (Tawa, Kimura et al. 1998). Radiation-induced hypomethylation was detected already at 8 hr post-exposure with no clear dose-response (Tawa, Kimura et al. 1998).

X-ray exposure of male and female C57Bl mice within a 0.5–5 Gy range showed dose-dependent hypomethylation in the female liver as well as in the male and female spleen. In the same work, decreased levels of Dnmt1, Dnmt3a/b, and methyl binding proteins MeCP2 and MBD2 were observed in the spleen and liver, along with increased level of DSBs (Raiche, Rodrigez-Juares et al. 2004). Thymus tissue of mice exposed to a fractionated 0.5 Gy dose of X-rays also showed DNA hypomethylation and a decreased expression of DNA methyltransferases and methyl-CpG-binding proteins (Pogribny,

Raiche et al. 2004). Decreased DNA methylation in mouse bone marrow was linked to radiation-induced leukemia (Giotopoulos, McCormick et al. 2006).

A similar response on the level of DNA methylation and methyltransferase proteins was detected in rat mammary glands (Loree, Koturbash et al. 2006); observed changes were paralleled by altered intra-cell signaling and cell cycle regulation pathways. X-ray irradiation of mouse tissue also induced hypermethylation of the p16 tumor-suppressor gene in tissue in a sex-specific manner (Kovalchuk, Burke et al. 2004). Overall, these in vivo studies suggest that radiation-induced DNA hypomethylation is modulated by sex- and tissue-specific factors, and may correlate with the accumulation of DSBs and changes in the activity of DNA methylation enzymes and methyl-CpG-binding proteins.

One in vitro study showed DNA methylation changes in the progeny of irradiated HPV-G cells after 20 passages, following initial exposure (Kaup, Grandjean et al. 2006). In this experiment, RIGI manifested as reproductive cell death, increased apoptosis, and an increase in non-clonal chromosomal aberrations. The epigenetic changes observed were mostly DNA hypermethylation at repeat elements, namely SAT2 and MLT1A; some specific hypomethylation was also detected, but not identified with respect to the sequence context (Kaup, Grandjean et al. 2006).

A very recent in vitro study using mouse embryonic stem cells showed that targeted disruption of Dnmt1 and Dnmt3a genes completely eliminated the transmission of RIGI (Rugo, Mutamba et al. 2010). To date, this is the only work that provides direct experimental proof that mitotically transmitted memory of a genotoxic insult is mediated by DNA methylation.

The exact mechanism of the induction of DNA methylation changes in irradiated tissue is currently unknown. A large array of experimental evidence suggests the persistent induction of ROS as a result of radiation exposure (Wright 2007). Free radical injury can profoundly alter DNA methylation levels (Weitzman, Turk et al. 1994; Cerda and Weitzman 1997; Bhusari, Dobosy et al. 2010), providing evidence that DNA methylation changes and enhanced free radical production cooperate in the maintenance and propagation of RIGI.

1.4.4. Histone modifications and chromatin

Changes in DNA methylation are not isolated events; such changes accompany global chromatin deregulation and, sometimes, altered histone modification levels (Jenuwein and Allis 2001; Jaenisch and Bird 2003). Histone modifications, including acetylation, methylation, ubiquitination, and phosphorylation, are important in transcriptional regulation, and add an enormous amount of complexity to the yet unexplored histone code (Jenuwein and Allis 2001; Weidman, Dolinoy et al. 2007).

Acetylated histone tails lose their positive charge, reducing their affinity for the negatively charged DNA, and leading to a relaxed chromatin packaging. Therefore, histone acetylation is linked to transcriptional activation, while histone deacetylation is an opposite repression event (Jenuwein and Allis 2001). Methylation of lysine 9 of histone H3 is associated with chromatin compaction and gene silencing, while methylation of lysines 4 and 27 of histone H3 results in transcription activation and chromatin relaxation. DNA methylation closely interacts with histone modifications in the setting of the transcriptional states of chromatin. Methyl-binding domains bind to methylated cytosines recruiting histone deacetylases (HDAC) and histone methyltransferases to hypermethylated sites (Gronbaek,

Hother et al. 2007). Combinations of different histone modifications and other chromatin-binding proteins define the structural and functional state of the chromatin (Verdone, Agricola et al. 2006).

It was recently shown that tumors undergo a massive loss of tri-methylation at lysine 20 of histone H4 (Fraga, Ballestar et al. 2005; Tryndyak, Kovalchuk et al. 2006). This loss occurs along with DNA hypomethylation and is linked to chromatin relaxation and aberrant expression. It has been suggested as a universal marker for malignant transformation (Sanders, Portoso et al. 2004; Fraga, Ballestar et al. 2005).

Phosphorylation is another important histone modification (He, Dunn et al. 2008). One of the best-studied modifications is the phosphorylation of histone H2AX. H2AX is a member of the H2A histone family. It becomes phosphorylated at serine 139 (γH2AX), possibly as one of the earliest cellular responses to double-strand breaks (DSBs) (Rogakou, Pilch et al. 1998; Pilch, Sedelnikova et al. 2003; Sedelnikova, Pilch et al. 2003). γH2AX accumulates in the nucleus at DSBs forming the γH2AX foci, and a direct correlation has been found between H2AX phosphorylation and the number of DSBs resulting from radiation (Celeste, Fernandez-Capetillo et al. 2003). γH2AX is crucially important for the repair of DNA strand breaks and for the maintenance of genome stability (Celeste, Difilippantonio et al. 2003).

1.4.5. Influence of ionizing radiation on histone modifications

Radiation-induced phosphorylation of γH2AX was extensively studied as a measure of double-strand break accumulation in irradiated cells (Bonner, Redon et al. 2008). The influence of radiation exposure on histone modification as an epigenetic mechanism is practically unexplored.

X-ray irradiation in a mouse model led to decreased tri-methylation of histone H4K20 in the thymus, which, according to the authors, could result in an overall relaxation of chromatin organization (Pogribny, Koturbash et al. 2005). Histone modifications in the direct vicinity of DSBs appear to be dynamic. γ irradiation results in relaxation of the chromatin structure around the DSB immediately after exposure; however, within 40 min these changes were replaced by increased methylation at H3K9, leading to the condensed state of the chromatin (Falk, Lukasova et al. 2008).

Irradiation can affect histone modifications at gene promoters. For instance, UV irradiation induced H3K9/14 acetylation at the promoter regions of several genes, including IL-8 and SOD, previously implicated in the bystander effect (Pollack, Sapkota et al. 2009). This change appeared to be transient, as they returned to their previous epigenetic state 72 hr post-irradiation (Pollack, Sapkota et al. 2009).

1.4.6. Small non-coding RNAs

Functional non-coding RNAs (ncRNAs), including transport, ribosomal, small cytoplasmic, nuclear RNAs, and ribozymes, have been known for a long time. Recently, an altogether different type of non-coding RNA has emerged as an extremely powerful regulator of gene expression and genomic stability (Chu and Rana 2007). Here we refer to small non-coding RNAs (sncRNAs), which, unlike other classes of non-coding RNAs, rarely exceed 33 nt in size, perform a variety of regulatory functions, and have different biogenesis pathways. So far, two classes of sncRNAs have been described in mammals, including mice and humans: microRNA (miRNA) and Piwi RNA (piRNA) (Costa 2010).

MiRNAs are evolutionarily conserved small RNAs (18–24 nt) involved primarily in post-transcriptional regulation of gene expression. The synthesis of miRNAs requires

several pre-processing steps. First, long primary RNA (pri-RNA) is transcribed by RNA polymerase II, polyadenylated at the 3' end, and capped at the 5' end (Cai, Hagedorn et al. 2004; Lee, Kim et al. 2004). Pri-miRNA is then processed by the RNAseIII enzyme Drosha, in cooperation with the RNA-binding factor Pasha. Drosha processing results in production of a 70–90 nt miRNA precursor (pre-miRNA), which is transported to the cytoplasm by exportin-5 (Lund, Guttinger et al. 2004). Once in the cytoplasm, it is further processed by another RNAseIII enzyme, Dicer (Lee, Nakahara et al. 2004). Dicer processing results in the production of a double-stranded miRNA 18–23 nt in size. One of the strands is then loaded into an RNA-induced silencing complex (RISC) in tight association with Ago2 protein, while the other strand is bound for degradation. Mature miRNAs display partial or complete complementarity to 3'-untranslated regions (3'-UTRs) in a large number of transcripts. The binding of the miRNA loaded into RISC to 3'-UTR, with full complementarity, results in transcript degradation, while binding with partial complementarity leads to translational attenuation (Khvorova, Reynolds et al. 2003; Schwarz, Hutvagner et al. 2003). Partial complementarity is more common in mammals, while full complementarity is found almost exclusively in plants (Tang, Reinhart et al. 2003).

Proteomic analysis showed that the knockout of single miRNAs leads to expression changes, albeit slight, in thousands of proteins (Selbach, Schwanhausser et al. 2008). However, their main functions appear to be linked to the few transcripts with the best binding properties, which serve as "true" targets and mediate the biological function of miRNAs. MiRNAs regulate cellular differentiation, embryonic development, stem cell maintenance, cell cycle regulation, apoptosis, and a number of other processes (Bartel

2004). Not surprisingly, dysregulation of miRNA expression is common in cancer. A number of miRNAs are deleted or silenced in cancer and therefore identified as onco-suppressors, while others, inversely, are overexpressed and oncogenic (Hwang and Mendell 2006; Aqeilan, Calin et al. 2010).

While miRNAs are ubiquitous gene expression regulators expressed in all tissues during every stage of animal development, piRNAs are detected strictly in the germ line. PiRNAs are a 24–33 nt long small RNA species extremely abundant in testes and also detected in oocytes (Ro, Park et al. 2007; Ro, Song et al. 2007). They were isolated from immunoprecipitates of Piwi proteins: PAZ/PIWI domain family proteins of the Argounaute superfamily. Piwi proteins are conserved across a variety of animal species, including *C. elegans*, *Drosophila*, zebrafish, mice, and humans (Klattenhoff and Theurkauf 2008). Expression of Piwi proteins is evolutionarily conserved and restricted to the germ line. Knockout of any of the Piwi members leads to mitotic arrest and failure of spermatogenesis, usually accompanied by a very dramatic increase in transposon activity (Carmell, Girard et al. 2007; Aravin, Sachidanandam et al. 2008). One of the Piwi proteins (Mili) was identified in the female germ line, although only in prenatal ovaries, and its function is yet to be understood (Watanabe, Totoki et al. 2008).

Mature piRNAs map to various regions of the genome, and their populations are dynamically altered during male germline development (He, Kokkinaki et al. 2009). In *Drosophila* they appear to be transcribed primarily from transposon-enriched clusters that function as transposon traps; piRNAs are generated from these clusters in large quantities and are complementary to the sequence of various mobile elements (Brennecke, Aravin et al. 2007). Knockout studies strongly suggest that the main function of piRNAs in flies

is the silencing of mobile elements during critical stages of spermatogenesis, and the maintenance of genomic integrity in the germ line (Brennecke, Aravin et al. 2007). Recent mechanistic studies have provided direct proof that piRNA-mediated silencing relies on sequence-specific targeting of heterochromatin formation factors to the mobile elements in the genome (Todeschini, Teysset et al. 2010).

The biogenesis of piRNAs is poorly understood. Analysis of piRNAs from *Drosophila* has led to the suggestion of a ping-pong model, in which piRNAs processed from a sense strand of transposons and bound to Ago3 protein recognize the antisense-transcript-producing 5'-end of an antisense product, which then associates with Auberin (Aub) or Piwi and undergoes nucleolitic cleavage, producing a mature 24–30 nt antisense piRNA. Antisense piRNAs associated with Aub or Piwi can recognize sense transcripts and cleave them with the production of 5'-sense product-bound Ago3, thus completing the cycle (Brennecke, Aravin et al. 2007).

The biogenesis and functions of piRNAs in the mouse male germ line are less clear. Similar to *Drosophila*, three Piwi proteins are operational during mouse spermatogenesis: Miwi, Mili, and Miwi2 (Kuramochi-Miyagawa, Kimura et al. 2001; Carmell, Girard et al. 2007). Each has distinct functions and expression patterns during germline development. Miwi2 is expressed in fetal testes and up to day 4 postpartum, along with Mili (Kuramochi-Miyagawa, Watanabe et al. 2008). PiRNA populations isolated from Mili and Miwi2 immunoprecipitates from fetal testes appear to be enriched in transposable elements processed from specific genomic clusters and sequence features, compatible with ping-pong cycle type processing. Miwi2 and Mili knockouts lead to the activation of mobile elements, hypomethylation at repeat elements, DNA damage, and

arrest of spermatogenesis at the pachytene stage of meiosis (Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008). PiRNA populations from adult testes isolated 10 days postpartum, when all spermatogenic cells were in the pre-pachytene stage, comprised primarily 24–27 nt long piRNA species typically associated with Mili (Aravin, Gaidatzis et al. 2006). Only 17% were derived from repeat elements (LINE, LTR, and SINEs), while the rest mapped to exons, introns, and intergenic regions, and their functions are not understood (Aravin, Gaidatzis et al. 2006). Miwi expression reaches its peak at the pachytene stage of meiosis and is primarily associated with the population of longer 29–31 nt piRNAs, which are depleted in repeat elements and map almost exclusively to gene bodies and intergenic regions (Grivna, Pyhtila et al. 2006). Miwi knockout caused the failure of spermatogenesis due to massive apoptosis at the round spermatid stage. Miwi bound piRNAs were shown to co-precipitate with polysomes and stabilize complementary transcripts, which led to a hypothesis that at least one of their functions is the stabilization and sequestration of transcripts produced during the pachytene stage but required for spermiogenesis at later stages when chromatin is condensed and transcription shut down (Grivna, Pyhtila et al. 2006).

1.4.7. Influence of radiation on the expression of non-coding RNAs

In spite of the tremendous importance of microRNAs for the regulation of various cellular functions, their involvement in radiation responses and in stress responses in general remains practically unexplored. The first report about the functional significance of microRNA in radiation responses came from a *Drosophila* study (Jaklevic, Uyetake et al. 2008). The microRNA termed bantam binds to the 3'-UTR of the proapoptotic protein Hid, thus suppressing its expression. IR caused overexpression of bantam and

suppression of Hid-mediated apoptosis, increasing the survival of larval cells (Jaklevic, Uyetake et al. 2008).

Studies on the effects of whole-body rodent IR radiation exposure on microRNA levels have been conducted by the Kovalchuk laboratory, using tissues and organs that are known targets of IR, namely hematopoietic and germline tissues. IR induced deregulation of miRNA expression after whole-body X-ray exposure in the spleen and thymus of male and female mice. These expression changes were modulated by sex- and tissue-specific factors (Ilnytskyy, Zemp et al. 2008). Among the differentially expressed miRNAs, tumor-suppressors miR-34a and miR-7 were most significantly changed. A significant increase in the expression of the tumor-suppressor miR-34a was paralleled by a decrease in the expression of its target oncogenes NOTCH1, MYC, E2F3, and cyclin D1. MiR-7 was proven to target the lymphoid-specific helicase LSH, a key regulator of DNA methylation and genome stability. Decreased levels of miR-7 allowed increased expression of LSH, potentially preventing radiation-induced hypomethylation. These microRNAome changes may constitute a cellular protective effect.

IR exposure was also shown to have a profound effect on microRNA expression in the murine germline. Specifically, analysis of miRNA expression in the testes of whole-body irradiated mice revealed that a large number of miRNAs were significantly changed after irradiation. Among these, miRNA-709 expression was significantly increased in the testes of whole-body irradiated mice (Tamminga, Kathiria et al. 2008). This miRNA, controlled through the DNA damage response ATR/Rfx1 pathway, was proven to target the brother of the regulator of imprinted sites (BORIS), a testes-specific

gene that directs epigenetic reprogramming and DNA methylation during male germ cell differentiation.

IR-induced upregulation of miR-709 led to decreased levels of BORIS. It was suggested that the irradiation DNA-damage-induced and ATR/Rfx1-mediated increase in miR-709 expression in exposed testes may be a protective mechanism aimed to prevent a massive aberrant erasure of DNA methylation (Tamminga, Kathiria et al. 2008).

IR effects on microRNAome were also analyzed in murine brains. Kovalchuk and colleagues analyzed the effects of X-ray irradiation on microRNA expression in the hippocampus, frontal cortex, and cerebellum of male and female mice (Koturbash et al. 2010a). Analysis revealed a number of tissue-, time-, and sex-specific brain radiation responses, as well as an important interplay between miRNAs and their targets.

Overall, whole-body in vivo radiation exposure of mice significantly affected the miRNAome expression of different tissues and organs. IR-induced miRNAome alterations were detected as early as several hours after exposure, and persisted for days, weeks, and even months after irradiation.

An analysis of IR-effects on the human microRNAome was conducted using cell lines or artificial human 3-D tissues. Real-time PCR analysis of miRNA expression in Jurkat and TK6 cells showed upregulation of let-7 family members that target the Ras oncogene (Chaudhry 2009). Also, miRNAs responsible for targeting of the cMyc oncogene involved in radiation-induced cancers were upregulated in both cell types. A recent study has demonstrated a connection between radiation exposure, chromatin structure, and miRNA expression (Lal, Pan et al. 2009). In this study, miR-24 was shown to directly target γH2AX, a histone variant specifically accumulated at the site of DSBs

and necessary for efficient DNA repair. γH2AX induction is reduced in terminally differentiated cells by a miR-24-mediated mechanism, rendering them DNA repair deficient. MiRNAs were strongly dysregulated in human fibroblasts exposed to 1 or 10 Gy of radiation, and the number of miRNAs showing a statistically significant change of more than two-fold was 73 in the 1 Gy and 33 in the 10 Gy exposure groups. Gene enrichment analysis among putative targets showed these genes to be responsible for the regulation of apoptosis, the cell cycle, and DNA repair (Cha, Shin et al. 2009).

A significant overlap was detected between miRNAs with altered expression detected after ionizing radiation and after oxidative radical exposure (Simone, Soule et al. 2009), pointing to the involvement of oxidative stress in radiation responses at the miRNA level. The same study directly showed the induction of ROS in the irradiated human fibroblast (Simone, Soule et al. 2009). Most miRNAs that were changed, after both peroxide and IR exposure, belonged to the let-7 family, which is involved in regulation of the Ras oncogene. Exposure of the human lymphoblast line IM9 to a range of doses between 1 and 40 Gy caused dose-dependent regulation of a number of miRNAs (Cha, Seong et al. 2009). Analysis of the targets of differentially expressed miRNAs showed that they were involved in apoptosis, regulation of the cell cycle, and DNA repair.

IR exposure significantly affected the microRNAome profile of artificial human 3-D EpiAirway (Air-112) tissue (MatTek Corporation, Ashland, MA). These artificial tissues were designed to maintain normal tissue architecture and preserve in vivo cell differentiation patterns. As a result, these tissues are mitotically and metabolically active and closely resemble the epithelial tissue of the respiratory tract (Boelsma et al. 2000).

The IR-induced miRNA expression profiles of human 3-D model tissues were compared to time-matched mock controls at 30 min, 8 hr and 7 days post-irradiation with 0.2 or 2 Gy of γ rays. At 30 min post-irradiation, there were 46 and 39 significantly regulated miRNAs ($p<0.05$) in the 0.2 and 2.0 Gy treatments, respectively. At 8 hr post-exposure, these numbers fell to 37 and 28 significantly regulated miRNAs for the two doses, respectively. Finally, at 7 days post-irradiation (dpi), 34 and 42 miRNAs, respectively, were significantly regulated (Dickey et al. 2010).

MiRNAs also play a key part in indirect bystander IR effects. Indeed, miRNAome changes occur in bystander tissues in vivo. The first experiments were conducted using a rat cranial irradiation model (Koturbash et al. 2007) and showed that miR-194 was significantly upregulated in bystander rat spleen tissue 24 hr and 7 months after exposure (Koturbach et al. 2007). Furthermore, IR-induced bystander effects in human 3-D tissues were associated with significant changes in the microRNAome (Kovalchuk et al. 2010). Key bystander end-points, such as apoptosis, cell cycle deregulation, and DNA hypomethylation, were mediated by the altered expression of miRNAs. Specifically, cMyc-mediated upregulation of the miR-17 family was associated with decreased levels of E2F1 and RB1, suggesting a switch to increased proliferation and suppressed apoptosis in bystander tissues. Upregulation of the miR-29 microRNA family led to decreased levels of DNMT3a and MCL1, and therefore affected DNA methylation and apoptosis. Notwithstanding, it was recently shown that even though miRNAs play key roles in the manifestation of bystander-effect end-points, they appear not to be the primary bystander signaling molecules in the formation of bystander effect-induced DNA

strand breaks. Their roles as signaling messengers for other bystander end-points still need to be established (Dickey et al. 2010).

Currently there is no information about the influence of IR or any other environmental stressors on piRNA levels. The roles of miRNAs and piRNAs in IR-induced transgenerational effects have yet to be studied.

RATIONALE AND HYPOTHESIS

Epigenentic changes are pivotal regulators of gene expression, genome stability, and cellular homeostasis. IR is a potent damaging agent that causes genomic instability in directly exposed cells, in unexposed bystander cells and even in the progeny of exposed animals. These phenomena have not been fully explored in the epigenetic domain. The role of epigenetic DNA methylation and miRNA changes in the effects of in vivo whole-body irradiation needs to be defined, and the tissue specificity of IR-induced epigenetic effects needs to be established. While bystander effects were studied using *in vitro* and *in vivo*, their mechanism and tissue specificity *in vivo* needs to be further elucidated.

Furthermore, there is well-documented evidence that IR exposure leads to damage in the male germline and, consequently, to transgenerational genome instability in the offspring of exposed parents. The exact molecular mechanisms of this phenomenon have yet to be defined; however recent evidence suggests that they may be epigenetic in nature.

Based on evidence from the literature, we hypothesized that direct and indirect IR-induced effects and transgenerational effects were epigenetically regulated through changes in DNA methylation and small RNAs. We proposed that direct and indirect IR exposure affected epigenetic changes in a tissue-specific manner. We predicted that IR-induced epigenetic changes were more pronounced in IR target organs such as hematopoietic tissue and the germ line. Furthermore, we suggested that transgenerational effects were epigenetically mediated via action of small RNAs and methylation.

Several experiments were designed to test the proposed hypothesis. They include:

1) analysis of tissue specificity of IR-induced changes in global DNA methylation and microRNAome in skin and spleen of animals subjected to whole-body and cranial IR exposure;
2) investigation of the involvement of miRNA in radiation responses of IR sensitive hematopoietic lymphoid tissues of males and females;
3) investigation of the role of DNA methylation and miRNAs in the germ line and the role of piRNAs in transgenerational genome and epigenome instability.

Overall, the chapters of this thesis are united by common theme of epigenetic reprogramming that occurs in irradiated cells or cells that received and IR-induced stress signal.

1. In the Chapter II (Ilnytskyy, Koturbash et al. 2009), we examine whether fractionated doses of radiation induce bystander effects in mouse model. We discovered that both acute and fractionated doses induced persistent bystander effects in spleen, but not in skin tissue of cranially irradiated mice. DNA hypomethylation observed in spleen was paralleled by reduction of MeCP2 level and changes in miRNA expression. Bystander responses were distinct between examined skin and spleen, with latter being considerably more sensitive.

2. In the Chapter III (Ilnytskyy, Zemp et al. 2008) we investigated early response to acute dose of radiation in the spleen and thymus of C57Bl mice to a single dose irradiation. Furthermore, we compared radiation-induced miRNA expression patterns in male and female mice. We found significant sex differences in the IR-induced

miRNA levels and in the levels of miRNA target proteins. Furthermore, we confirmed that miR-7 targeted LSH an important epigenetic modifier regulating DNA methylation.

3. In Chapter IV (Filkowski, Ilnytskyy et al. 2010) [equal author contribution] we examined the influence of paternal exposure on levels of DNA methylation and miRNA expression in the un-exposed progeny as a signature of radiation induced transgenerational epigenome instability. We found that radiation-induced genomic instability in the germ line of exposed parents and in sometic tissues of their unexposed progeny was associated with the altered microRNAome expression and DNA methylation. We suggested that miRNA-mediated epigenome regulation played a central role in transgenerational effects of radiation.

4. Finally, in Chapter V, we used Illumina deep sequencing to characterize small RNA trascriptome in adult mouse testes after direct and paternal radiation exposure. The analysis revealed astonishing diversity of small RNAs in adult mouse testes. The predominant class was represented by repeat-derived small RNAs, other abundant categories mapped to transcripts, intergenic regions and known miRNAs. Both paternal and direct exposures resulted in a decline in repeat-derived small RNAs and very dramatic upregulation of miRNAs. A detailed analysis and discussion of functional implications of these changes are presented in Chapter V.

2. RADIATION-INDUCED BYSTANDER EFFECTS IN VIVO ARE EPIGENETICALLY REGULATED IN TISSUE SPECIFIC MANNER[1]

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

Ilnytskyy, Y., I. Koturbash, et al. (2009). "Radiation-induced bystander effects in vivo are epigenetically regulated in a tissue-specific manner." Environ Mol Mutagen **50**(2): 105-113.

1.2. ABSTRACT

Exposure of animal body parts to ionizing radiation (IR) can lead to molecular changes in distant shielded ‘‘bystander’’ tissues and organs. Nevertheless, tissue specificity of bystander responses within the same organism has not been examined in detail. Studies on in vivo bystander effect conducted so far analyzed changes induced by single dose exposure. The potential of fractionated irradiation to induce bystander effects in vivo has never been studied. We analyzed changes in global DNA methylation and microRNAome in skin and spleen of animals subjected to acute or fractionated, whole-body or cranial exposure to 0.5 Gy of X-rays. We found that IR-induced DNA methylation changes in bystander spleen and skin were distinct. Acute radiation exposure resulted in a significant loss of global DNA methylation in the exposed and bystander spleen 6 hr, 96 hr, and 14 days after irradiation. Fractionated irradiation led to hypomethylation in bystander spleen 6 hr after whole-body exposure, and 6 hr, 96 hr, and 14 days after cranial irradiation. In contrast, changes in the skin of the same animals were seen only 6 hr after acute whole body and head exposure. DNA hypomethylation observed in spleen was paralleled by a reduction of methyl-binding protein MeCP2 expression. Irradiation also induced tissue-specific microRNAome alterations in skin and spleen. For the first time, we have shown that IR-induced epigenetic bystander effects that occur in the same organism are triggered by both acute and fractionated exposure and are very distinct in different bystander organs. Future studies are clearly needed to address organismal and carcinogenic repercussions of those changes.

2.2. INTRODUCTION

It is now well accepted that ionizing radiation (IR) can elicit molecular and cellular responses in cells that were not hit directly by IR but rather received a distress signal from irradiated cells (Morgan 2003; Mothersill and Seymour 2004; Morgan and Sowa 2007). These responses are termed bystander effects, and they are very well studied using cell cultures (Lyng, Maguire et al. 2006; Maguire, Mothersill et al. 2007), tissue explants (Belyakov et al., 2003, 2006), and reconstructed 3D tissue models (Belyakov et al., 2005; Sedelnikova et al., 2007).

Bystander effects also manifest themselves in the whole-organism context. IR leads to a release of soluble ‘‘clastogenic’’ factors into circulating blood. These factors were capable of inducing chromosome damage in cultured cells (Emerit, Levy et al. 1994; Emerit, Quastel et al. 1997). Clastogenic activity was found in the plasma of patients receiving a high dose of radiotherapy, as well as individuals who were accidentally exposed to radiation (Emerit, Levy et al. 1994; Marozik, Mothersill et al. 2007). Also, during partial-organ irradiation, significant molecular and cellular damage is seen in shielded organ parts (Brooks 2004). Evidence of bystander effects has also been found in humans in the form of radiotherapy-induced abscopal ‘‘out of field’’ effects (Sgouros, Knox et al. 2007). Abscopal effects were initially determined by Mole in 1953 and defined as ‘‘action at a distance from the irradiated volume but within the same organism’’ (Mole 1953). The relevance of abscopal events to bystander effect is a matter of debate, however some researchers proposed to use the two terms interchangeably.

Recently, the existence of bystander effects in somatic organs was confirmed using whole animal models (Koturbash et al., 2006, 2007, 2008). IR exposure of one side of the animal’s body caused profound epigenetic changes in the shielded bystander parts

(Koturbash et al., 2006, 2007, 2008). Although bystander effects have been noted in different animal tissues, the nature and magnitude of bystander changes differ. Notwithstanding, tissue specificity of bystander responses within the same organism has never been examined in detail. Elegant in vitro studies showed that fractionated IR exposure can induce bystander effects in cultured cells (Mothersill and Seymour, 2002). Yet, the capacity of fractionated irradiation to induce bystander effects in vivo needs to be analyzed in detail. Bystander effects are associated with epigenetic changes such as altered DNA methylation (Koturbash et al., 2006, 2007, 2008; Kaup et al., 2006) and microRNAome (Koturbash, Boyko et al. 2007).

Cytosine DNA methylation is crucially important for the normal development, cell proliferation and proper maintenance of genome stability (Klose and Bird 2006; Jirtle and Skinner 2007; Weber and Schu¨beler 2007). In mammals, DNA methylation occurs predominantly in the context of CpG dinucleotides which are methylated at 60–80% of CpG sites (Weber and Schubeler 2007). DNA methylation is known to be associated with an inactive chromatin state and repressed gene expression activity. In mammals, the association of DNA methylation with transcriptional repression is thought to be mediated by the MBD (methyl CpG-binding domain) family of proteins that includes MeCP2, MBD1, MBD2, and MBD3 (Klose and Bird 2006; Weber and Schubeler 2007). Loss of global DNA methylation at CpG dinucleotides has been linked to altered gene expression, activation of transposable elements, elevated chromosome breakage, aneuploidy, increased mutation rates and, thus to the phenomenon of global genomic instability (Weidman, Dolinoy et al. 2007).

MicroRNAs (miRNAs) are evolutionally conserved, small, single-stranded, nonprotein-coding RNA molecules which function as key regulators of an RNA interference pathway and are presently recognized as major regulators of gene expression (Bernstein and Allis 2005; Griffiths-Jones, Grocock et al. 2006). They can reduce the level of their target transcripts as well as the amount of protein encoded by their transcripts (Bartel 2004). To control the translation of target mRNAs, miRNAs associate with RNA-induced silencing complexes (RISC) (Khvorova, Reynolds et al. 2003). After association with RISC complexes, miRNAs bind to the 3'UTR of mRNAs and serve as translational suppressors, thereby regulating the production of proteins and affecting many cellular functions including proliferation, differentiation, and cell death (Bernstein and Allis 2005). Importantly, both DNA methylation (Bogdanovic and Veenstra 2009) and regulatory microRNAs (Brennecke, Hipfner et al. 2003; Carrington and Ambros 2003) govern tissue-specific gene expression and function.

Here, we analyzed and compared changes in DNA methylation and microRNAome induced by the whole body and cranial, acute and fractionated IR exposure in mouse skin and spleen.

2.3. MATERIALS AND METHODS

2.3.1. Animal model and radiation treatment

Fifty-day-old male mice (C57BL6) were randomly assigned to different treatment groups (18 animals per cohort). Handling and care of animals were conducted in accordance with recommendations of the Canadian Council for Animal Care and Use. Procedures have been approved by the University of Lethbridge Animal Wefare Committee. Animals were housed in pathogen free controlled facility and given food and water *ad libitum*.

The first cohort received 0.5 Gy of X-rays (5 cGy/s, 90 kV, 5mA) delivered to the entire body as a single acute dose. The second cohort received 0.5 Gy of acute X-ray exposure to scull only, while the rest of the animals body was protected by the medical grade led shield (Koturbash, Rugo et al. 2006). Protection of the shielded "bystander" tissue was complete, as verified by careful dosimetry using RAD-CHECK^{tm} monitor (nuclear Associates div. of Victoreen, FL).

The third cohort received whole body exposure to 0.5 Gy of X-rays delivered as five equal daily fractions of 0.1 Gy a day over 5 days. The fourth cohort received cranial exposure to 0.5 Gy of X-rays delivered as five equal daily fractions of 0.1 Gy a day for 5 days, while the rest of the body was protected by the led shield. Control mice were sham treated.

Animals were humanely sacrificed 6, 96 and 14 days post irradiation (6 animals/time point/cohort). These time points were chosen to characterize initial (6 hours) and persistent (96 hours and 14 days) responses. Spleen and skin tissue located above spleen were sampled for further molecular studies. These tissues are radiation sensitive targets linked to radiation carcinogenesis (Boulton, Cleary et al. 2002).

2.3.2. DNA methylation analysis

Total DNA was prepared from spleen and skin tissue using Qiagen DNAeasy™ Kit (Qiagen, Valencia, CA) according to manufacturer's protocol. The well-established radiolabeled cytosine extension assay was employed to evaluate global DNA methylation levels (Pogribny, Yi et al. 1999). The assay measures the proportion of CCGG sites that lost methyl groups on both DNA strands. Hpa II is the methylation sensitive restriction enzyme that cleaves CCGG sites when internal cytosine is unmethylated on both strands. It leaves a 5'-guanine overhang after DNA cleavage. This overhang can be used to prime subsequent single nucleotide extension with labeled [^{3}H]dCTP. The extent of [^{3}H]dCTP incorporation is directly proportional to the number of cleaved and thus unmethylated HpaII sites in the genome. The assay was performed with 0.5 ug of genomic DNA digested with 20 U of the methylation-sensitive HpaII restriction endonuclease (a 20-fold enzyme excess) (New England Biolabs, Beverly, MA) for 18 hr at 37 °C. A second 0.5 ug DNA aliquot was treated in exactly the same way in the absence of restriction enzyme and served as the background incorporation control. Finally, a third 0.5 ug aliqout was digested with 20 U of MSPI, which cleaves CCGG site regardless of methylation status.

A single nucleotide extension was performed in a 25 ul reaction mixture containing 0.25 ug of DNA, 1X PCR buffer II, 1 mM $MgCl_2$, 0.25 U AmpliTaq DNA polymerase and 0.1 ul of [^{3}H]dCTP (57.4 Ci/mmol) (Perkin Elmer, Boston, MA), and incubated at 56 °C for 1 hr. samples were applied to Whatman DE-81 ion exchange filters and washed three times with 0.5 sodium phosphate buffer (pH 7.0) at room temperature. Filters were dried and processed for counting using a scintillation counter (Beckman LS 5000CE, Fullerton, CA). The incorporation of [^{3}H]dCTP into DNA was expressed as

mean disintegrations per minute (dpm) per ug of DNA after subtraction of background incorporation from undigested samples.

2.3.3. miRNA microarray expression analysis

MicroRNA microarray analysis was conducted using skin and spleen of mice exposed to acute irradiation to the whole body and skull 6 hours after exposure. Total RNA was extracted from mouse spleen and skin tissues using TRIzol Reagent (Invitrogen, Burlington, ON) according to manufacturer's instructions. Tissue from 4 control, 3 whole body and 3 head exposed animals was used for the analysis, miRNA microarray was performed by LC Sciences (Houston, TX). Ten micrograms of total RNA were size fractionated (<200 nucleotides) by using mirVana kit (Ambion, Austin, TX). Poly-A tails were added to RNA sequences at 3'-ends using poly(A) polymerase, and nucleotide tags were than ligated to poly-A tails. Tagged RNAs were then hybridized to dual-channel miacroarray µParaFlo microfluidic chips (LC Sciences) containing probes for 439 miRNA's for rat and mouse and then labeled with tag specific dendrimer Cy3 and Cy5 flurescent dyes. Dye switching was performed to eliminate dye bias. The melting temperature of detection probes was balanced by incorporating varying numbers of modified nucleotides with increased binding affinities. Hybridization images were collected using a GenePix 4000B laser scanner (Molecular Devices, Sunnyvale, CA), and then digitized using Array-Pro image analysis software (Media Cybernetics, Silver Spring, MD). Maximum signal level of background probes was 180. A miRNA detection signal threshold was set as twice the maximum background signal. Normalization was performed with the cyclic LOWESS (locally weighted regression) method to remove system-related variations, as previously described (Pogribny, Tryndyak et al. 2007). Data

adjustments included data-filtering, log 2 transformation, gene centering and normalization. The t-test analysis was conducted between control, whole body exposed and bystander sample groups. MicroRNAs with P-values <0.05 were selected for cluster analysis.

2.3.4. Quantitative Real-Time PCR

Quantitative real-time PCRs (qRT-PCRs) were performed with skin and spleen samples from all groups by using SuperTaq Polymerase (Ambion) and mirVana qRT-PCR miRNA detection kit (Ambion) following the manufacturer's instructions. Reactions contained mirVana qRT-PCR primer sets specific for miR-194. 5S rRNA served as a positive control. qRT-PCR were performed with Smart-Cycler (Cepheid, Sunnyvale, CA). End-point reaction products were resolved on 3.5% agarose gel stained with etidium bromide.

2.3.5. Western immunoblotting

Western immunoblotting for MeCP2 and actin (loading control) was conducted using spleen and skin samples from all of the experimental groups. Tissue samples were sonicated in 0.4-0.8 ml of ice-chilled 1% SDS and boiled for 10 minutes. Small aliquots of homogenate were reserved from protein determination using protein assay reagents from Bio-Rad (Hercules, CA). Equal amounts protein (20 µg) were resolved in 8-12% polyacrylamide gels and electro-transferred to PVDF membranes (Amersham, Baie-d'Urfe, Quebec). Each gel run was performed in duplicate.

Membranes were incubated with antibodies against MeCP2 (1:1000, Abcam, Cambridge, MA) and actin (1:2000; Abcam). Antibody binding was revealed by incubation with horse radish peroxidase-conjugated secondary antibodies (Amersham)

and the ECL plus immunoblotting detection system (Amersham). Chemiluminescence was detected (Eastmen Kodak, New Heaven, CT). Unaltered PVDF membranes were stained Coomasie Blue (Bio-Rad, Hercules), and the intensity of a Mr 50,000 protein band was assessed as a loading control. Signals were quantified using the NIH ImageJ 1.63 Software and normalized to both actin and the Mr 50,000 protein band (values relative to Mr 50000 are plotted). Protein levels in tissue of exposed animals were related to controls.

2.3.6. Statistical analysis

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

2.4. RESULTS

2.4.1. Tissue-specificity of IR- and bystander-induced DNA methylation changes

We set out to analyze the tissue and exposure regime specificity of epigenetic bystander changes using a well established animal model, whereby the animal's head was exposed to IR, while the body was protected by a medical grade lead shield (Koturbash, Rugo et al. 2006). We analyzed and compared two different tissues sampled from the same animal — spleen and skin (Figure 2.1). Skin tissue was sampled from an area directly above the spleen projection; therefore it was the same distance from the irradiation field.

Global cytosine DNA methylation was studied in spleen and skin of control, completely irradiated and head-exposed mice 6 hr, 96 hr, and 14 days after acute or fractionated irradiation. We employed a cytosine extension assay, which measures a proportion of unmethylated CCGG sites in genomic DNA (Koturbash, Rugo et al. 2006). We found that IR-induced DNA methylation changes in exposed and bystander spleen and skin were distinct. Acute radiation exposure resulted in a signicant loss of global DNA methylation in the exposed and bystander spleen 6 hr, 96 hr, and 14 days after irradiation. Fractionated irradiation led to hypomethylation in the bystander spleen and skin were distinct.

Acute radiation exposure resulted in a significant loss of global DNA methylation in the exposed and bystander spleen 6 hr, 96 hr, and 14 days after irradiation. Fractionated irradiation caused hypomethylation in the bystander spleen 6 hr after whole-body exposure, and 6 hr, 96 hr and 14 days after cranial irradiation. In contarst, changes in skin of the same animals were seen only 6 hr after acute whole-body and head exposure (Figure 2.2).

Upon detection of distinct DNA methylation changes in exposed and bystander skin and spleen, we decided to analyze the level of MeCP2, an important methyl DNA binding protein. MeCP2 was shown to interact with CpG rich methylated DNA. It selectively recognizes symmetrically methylated CpG dinucleoties through the contact with a major groove, and plays a key role in methylation mediated chromatin remodeling and gene silencing (Jones, Veenstra et al. 1998; Nan, Cross et al. 1998).

We found that MeCP2 was significantly ($P<0.05$) downregulated in mouse spleen upon acute and fractionalted whole-body and head irradiation 6 hr, 96 hr, and 14 days after exposure. In contrast, no MeCP2 changes were noted in skin, except the acute head exposure group. There we noted a slight but significant increase of MeCP2 levels 6 hr after exposure (Figure 2.2).

2.4.2. Tissue-specificity of IR-induced microRNAome changes

As a next step in our study we analyzed the expression patterns of known microRNAs in spleen and skin using micorarray technology. Since we previously noted that both direct irradiation and bystander effects in spleen were pronounced at 6 and 96 hr after exposure and even persisted for 14 days, we decided to use 6 hr time-point for initial analysis of radiation-induced changes in microRNA expression in spleen and skin subjected to single acute dose of 0.5 Gy of X-rays. Significantly changed microRNAs were selected for further qRT-PCR analysis to confirm micoarray results and establish their expression at other time-points.

We found that IR exposure led to very different patterns of microRNA expression in skin and spleen (Figure 2.3A). Overall microRNAs were affected more in spleen than in skin. Interestingly, we identified that miR-194 was significantly ($P<0.01$) upregulated

6 hr after acute irradiation in bystander spleen of head exposed mice. This miRNA was previously shown to be elevated in bystander rat spleen 24 hr and 7 month after cranial irradiation (Koturbash et al, 2007). To confirm microarray results and extend our dataset, we carried out miRNA specific RT-PCR of miR-194 in spleen of animals subjected to acute or fractionated head exposure. We analyzed changes seen 6 hr, 96 hr, and 14 days after head irradiation. RT-PCR was a solid validation of the microarray results obtained. We found that miR-194 was persistently upregulated in bystander spleen 6 hr, 96 hr, and 14 days after irradiation (Figure 2.3B).

2.5. DISCUSSION

This is the first study to compare epigenetic in vivo bystander effects in different organs of the same organism that were located the same distance from the exposure field. Here, for the first time, we describe distinct epigenetic bystander effects in the lead shielded spleen and skin after acute and fractionated X-ray irradiation.

In spleen we detected a pronounced loss of global DNA methylation that was paralleled by a decrease in the level of methyl-CpG-binding protein MeCP2. On the contrary, DNA methylation changes in skin of the same animals were much less pronounced than in spleen and were observed only 6 hr after acute whole body and head exposure. Furthermore, no MeCP changes were noted in skin, except an increase of MeCP2 level 6 hr after acute whole-body exposure. This data is in agreement with previously observed global genomic DNA hypomethylation and elevated MeCP2 levels in bystander skin of head exposed mice (Koturbash et al, 2006).

MeCP2 selectively recognizes and binds to symmetrically methylated cytosine throughout genome, and it is an important transcriptional silencer and chromatin regulator (Jones, Veenstra et al. 1998; Nan, Cross et al. 1998). A recent study revealed high levels of DNA methylation at repetitive satellite DNA sequences, retro- and DNA transposons (Martens, O'Sullivan et al. 2005). It was also shown that reduced DNA methylation levels lead to transposon reactivation and genome instability (Xu, Bestor et al. 1999; Jaenisch and Bird 2003). Similarly, downregulation of MeCP2 was also shown to predispose cells to transposon reactivation, mutation and genome instability, thus creating favorable conditions for carcinogenesis (Yu, Zingler et al. 2001). In the light of these findings, we can speculate that observed loss of global methylation and decreased MeCP2 levels occur preferentially in transposons and satellite DNA, thus underlying

genome instability in spleen tissue. In the bystander skin of exposed mice increased levels of MeCP 6 hr after acute irradiation may offset the loss of DNA methylation and contribute to gene silencing. However biological consequences and the exact nature of DNA sequences affected by altered CpG methylation and MeCP2 binding remain unclear.

The most pronounced loss of DNA methylation was observed in the spleen of animals that received whole-body acute exposure. Extensive loss of DNA methylation may result from radiation-induced DNA damage. DNA lesions were previously reported to interfere with the methylation ability of DNA methyltranferases (Weitzman, Turk et al. 1994; Turk, Laayoun et al. 1995). Furthermore during repair DNA synthesis, cellular DNA polymerases incorporate cytidine, but not methyl-cytidine. Consequently, the presence and repair of radiation induced lesions may result in DNA hypomethylation. Whole body acute exposure usually leads to higher DNA damage levels than cranial exposure (Koturbash, Rugo et al. 2006; Tamminga, Koturbash et al. 2008). Also it induces more damage than fractionated radiation (Koturbash, Pogribny et al. 2005). Further studies are needed to elucidate causes of DNA demethylation resulting from IR exposure. An important outcome of this study was that fractionated radiation exposure can also induce pronounced and persistent epigenetic bystander effect in spleen but not in skin. Further studies are needed to dissect the detailed nature, biological outcomes, and dose response of bystander effects in spleen and their links to carcinogenesis.

Another important novel finding is tissue-specificity of IR-induced direct and bystander miRNA expression changes. Similarly to DNA methylation, miRNA expression changes were more pronounced in spleen than in skin. Among upregulated

miRNAs, miR-194 appears to be most relevant with regard to genomic instability and carcinogenesis. MiR-194 is predicted by TARGETSCAN 4.0 and by Sanger Databases (http://microrna.sanger.ac.uk/sequences/) to target wide range of genes, including the adapter related protein complex 1 gamma 1 subunit, suppressor of tumorgenicity 18, proto-oncogene AF4, retinoblastoma-binding protein 4, breast cancer type 1 susceptibility protein, DNA methyltransferase 3A, methyl-CpG-binding protein 2, protein kinase Akt-3, monocytic leukemia zinc-finger protein related factor, and other genes involved in cell cycle control, proliferation, DNA repair and other cellular processes.

Interestingly, among the variety of targets, miR-194 is presumed to affect MeCP2 (Koturbash, Boyko et al. 2007). Indeed, upregulation of miR-194 observed in spleen may be a factor underlying persistent MeCP2 suppression. Therefore, miR-194 may be one of the key players in the maintenance epigenetic bystander response in spleen. Our previous studies have shown that miR-194 was the only miRNA that was significantly and reproducibly upregulated in bystander rat spleen tissue 24 hr and 7 month after exposure. Moreover, upregulation of miR-194 was significant down-regulation of MeCP2 (Koturbash, Boyko et al. 2007). Therefore, radiation-induced upregulation of miR-194 in spleen is a cross-specie phenomenon.

In skin, IR and bystander-induced miRNAome changes were distinct from those detected in spleen and much less pronounced.

Although the nature of bystander signal in vivo remains to be elucidated, our study proves that the bystander signal, which most probably is carried with the blood stream, can reach organs distant from irradiated site and exert distinct tissue specific responses on epigenetic level.

Overall, our findings provide a road map for the further analysis of epigenetic and miRNA expression alterations in direct and bystander IR-induced responses in the context of the whole organism.

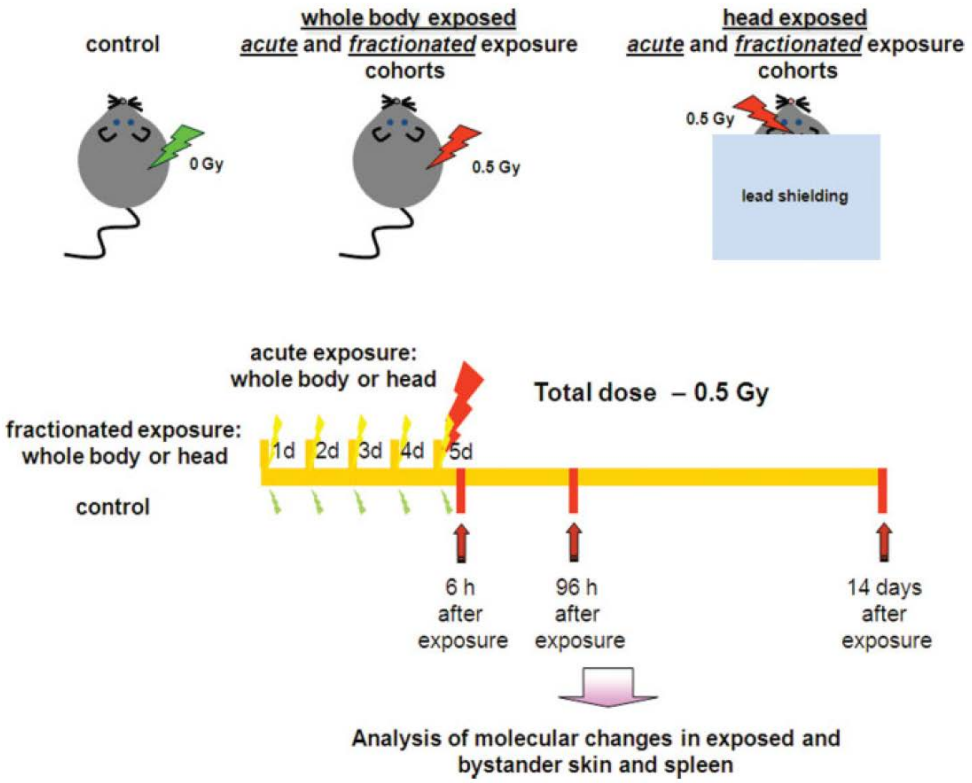


Figure 2.1. Induction of bystander effects in vivo. Animal cohorts: Whole body exposed animals received 0.5 Gy of X-rays delivered to the entire body as a single acute dose, or as five equal daily fractions of 0.1 Gy/day over 5 days. Head exposed animals received 0.5 Gy of X-rays delivered to the scull only as a single acute dose, or as five equal daily fractions of 0.1 Gy/day over 5 days to skull only, while the rest of the animal body was protected by a 3 mm-thick medical-grade lead shield. Control animals were sham treated. Animals were humanely sacrificed 6 hr, 96 hr, or 14 days after completion of the exposure protocols.

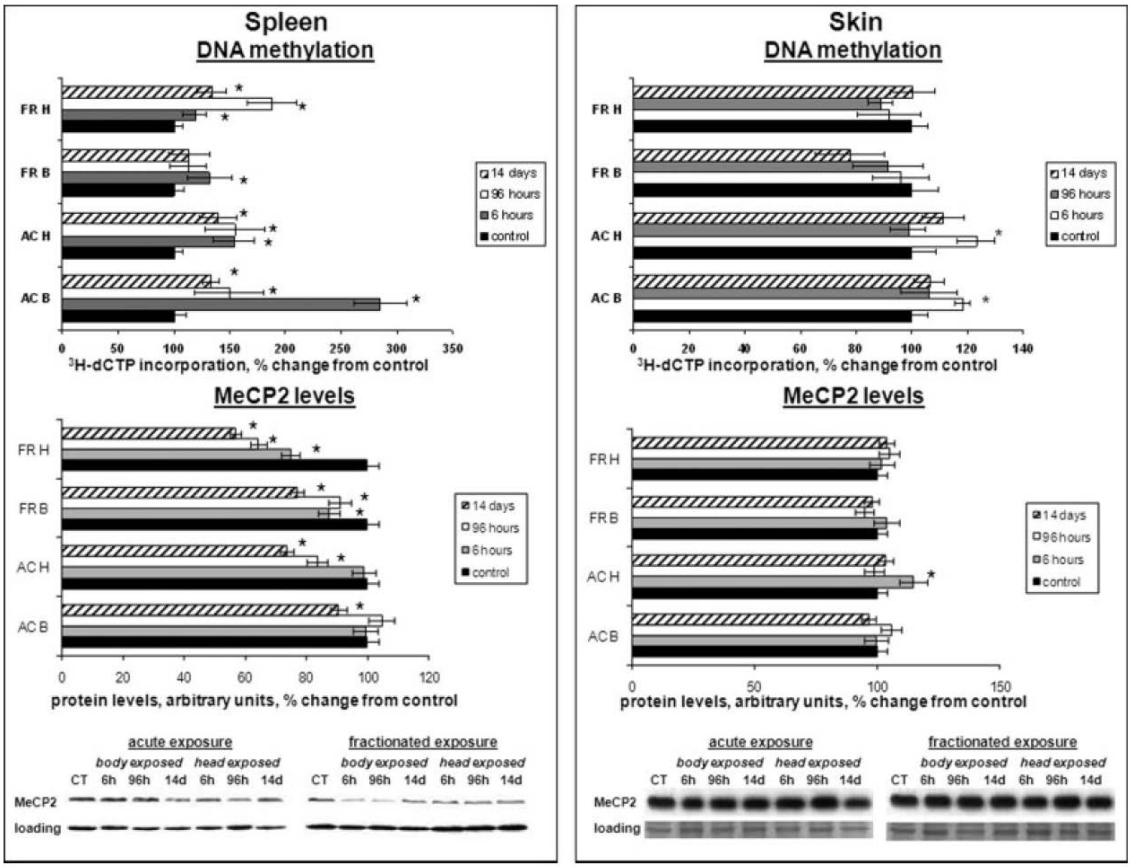


Figure 2.2. Tissue-specific epigenetic effects of radiation exposure. The levels of global genome DNA methylation in spleen and skin tissue of irradiated mice were measured by the cytosine extension assay based on the treatment of DNA with a methylation-sensitive restriction enzyme HpaII. This enzyme cleaves CCGG sequences in case if internal cytosine residues are unmethylated on both strands. The cleavage results in the formation of a 50-guanine overhang that is used for the subsequent single nucleotide extension with the labeled [3H]dCTP. The extent of [3H]dCTP incorporation opposite the exposed guanine is directly proportional to the number of cleaved and thus unmethylated CpG sites and inversely proportional to the levels of methylation (i.e., the higher the methylation, the lower the incorporation of [3H]dCTP). Levels of MeCP2 protein were

analyzed by western immunoblotting. DNA methylation: Cytosine extension assay results. The levels of [3H]dCTP incorporation relative to control are presented as mean values +/- standard deviation, n = 6; (*P < 0.05), and they are significantly different from control, Students' t-test, Tukey-Kramer test and Dunnett's test. MeCP2 levels: Levels of MeCP2 protein as analyzed by western immunoblotting analysis. Lysates from spleen and cutaneous tissue were immunoblotted using antibodies against MeCP2. Protein levels relative to loading controls were compared and the statistically significant changes relative to untreated sham controls are shown as mean values 1/2 standard deviation, (*P < 0.05), Students' t-test, Tukey-Kramer test, and Dunnett's test. Representative blots from three independent experiments; each experiment included pooled lysates from three animals for each exposure condition, with equal representation of each animal. Animal cohort abbreviations: AC B, acute body exposure group; AC H, acute head exposure group; FR B, fractionated body exposure group; FR H, fractionated head exposure group.

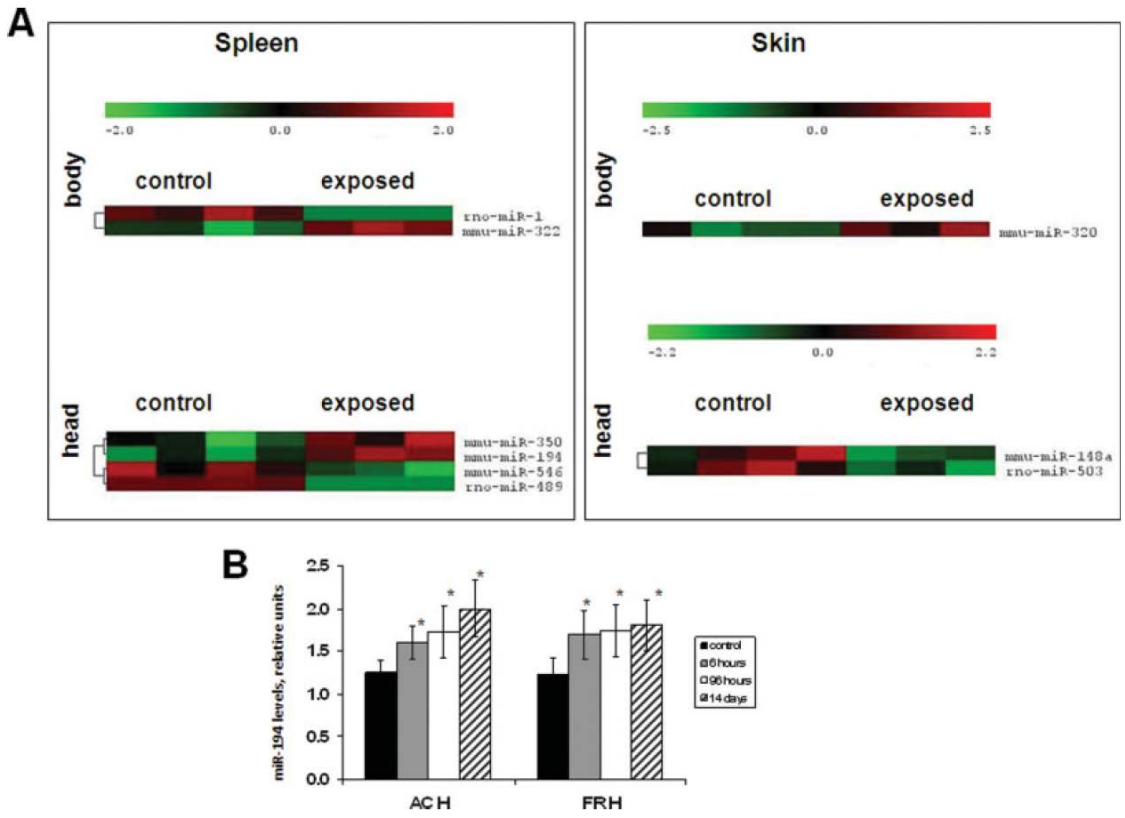


Figure 2.3. MiRNAs that were differentially expressed in the spleen and skin of mice 6 hr after acute whole-body or head-only exposure to 0.5 Gy of X-rays. A. Hierarchical clusters of differentially expressed miRNA genes in spleen and skin (as determined by ANOVA). Each miRNA listed is differentially expressed between control and treatment groups ($P < 0.05$). B. Expression of miR-194 in bystander spleen upon acute and fractionated head irradiation as studied by the qRT-PCR (mean values +/- standard deviation). AC H, acute head exposure group; FR H, fractionated head exposure group; *$P < 0.05$, Students' t-test, Tukey-Kramer test and Dunnett's test. Each of the array hybridization represents separate animal and corresponds to single line on the heat map, transition from green to red color spectrum represents expression level relative to the average value normalized between arrays. Microarray experiment utilized 3 animals per

experimental group. We compared array data for 4 animals in control group with 3 animals in exposed.

3. ALTERED MICRORNA EXPRESSION PATTERNS IN IRRADIATED HEMATOPOIETIC TISSUES SUGGEST A SEX-SPECIFIC PROTECTIVE MECHANISM[1]

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

Ilnytskyy, Y., F. J. Zemp, et al. (2008). "Altered microRNA expression patterns in irradiated hematopoietic tissues suggest a sex-specific protective mechanism." Biochem Biophys Res Commun **377**(1): 41-45.

3.1. ABSTRACT

To investigate involvement of miRNAs in radiation responses we used microRNAome profiling to analyze the sex-specific response of radiation sensitive hematopoietic lymphoid tissues. We show that radiation exposure resulted in a significant and sex-specific deregulation of microRNA expression in murine spleen and thymus tissues. Among the regulated miRNAs, we found that changes in expression of miR-34a and miR-7 may be involved in important protective mechanisms counteracting radiation cytotoxicity. We observed a significant increase in the expression of tumor-suppressor miR-34a, paralleled by a decrease in the expression of its target oncogenes Notch1, Myc, E2f3 and cyclin D1. Additionally, we show that miR-7 targets the lymphoid–specific helicase Lsh, a pivotal regulator of DNA methylation and genome stability. While miR-7 was significantly down-regulated Lsh was significantly up-regulated. These cellular changes may constitute an attempt to counteract radiation-induced hypomethylation. Tissue specificity of miRNA responses and possible regulation of miRNA expression upon irradiation are discussed.

3.2. INTRODUCTION

Ionizing radiation (IR) is an important diagnostic and treatment modality. Conversely, it is a potent DNA damaging and cancerinducing agent (Little 2000). Lymphoid and myeloid hematopoietic tissues are the main targets for radiation carcinogenesis in both humans (Gluzman, Imamura et al. 2005) and animal models (Covelli, Di Majo et al. 1989; Boulton, Cleary et al. 2002). IR-induced leukemia in humans was first reported early in the 20th century (Little 2000). Since then numerous studies of atomic bomb survivors, people working or living near nuclear power plants, radiologists, and radiotherapy patients have proved that IR exposure results in a high risk of leukemia and lymphoma (Shimizu 1966; Parkin, Cardis et al. 1992; Gluzman, Imamura et al. 2005). However, the exact mechanisms by which IR exposure may result in a predisposition to the development of IR-induced leukemia and lymphoma remains to be determined.

In recent years, the role of epigenetics in the etiology of disease, including cancer, has been increasingly recognized (Egger, Liang et al. 2004; Plass, Oakes et al. 2008). Epigenetic changes are stable alterations in gene expression that include DNA methylation, histone modification, and small RNA-mediated effects. Amongst the small RNAs in mammals, microRNA (miRNAs) are the most abundant and well understood. MiRNAs are small non-coding regulatory RNAs that act as translational inhibitors to down-regulate the expression of a wide variety of genes (Hwang and Mendell 2006). MiRNAs originate from various areas of the genome, including repetitive regions, transposons, and the intronic sequences of coding genes. To regulate the expression of target mRNAs, mature miRNAs associate with the RNA-induced silencing complex (RISC) proteins to bind to target mRNAs in a sequence-specific manner (Hwang and

Mendell 2006). In doing so, the RISC associated miRNAs can impact various processes such as cellular differentiation, proliferation, and apoptosis, therefore potentially affecting an individual's predisposition to malignancy (Hwang and Mendell 2006). Indeed, aberrant levels of miRNAs have been reported in a variety of human cancers (Hwang and Mendell 2006), including leukemia and lymphoma (Croce 2008; Garzon and Croce 2008). In carcinogenesis, miRNAs can act as tumor-suppressors or oncogenes by regulating other tumour-suppressor or oncogene mRNAs. For example, over-expression of the oncogenic miR-17- 92 cluster facilitates the development of small-cell lung cancer and chronic myeloid leukemia (Venturini, Battmer et al. 2007). Mir-34a is a potential tumor suppressor that induces apoptosis in neuroblastoma cells (Welch, Chen et al. 2007). Similarly, tumor-suppressors miR-15 and miR-16 induce apoptosis by targeting the anti-apoptotic B cell lymphoma 2 (BCL2) transcript and have been found to be either absent or down-regulated in the majority of chronic lymphocytic leukemia (CLL) cases (Calin and Croce 2006).

Despite the growing evidence of the importance of miRNAs in the development of hematopoietic tissue malignancies, fairly little is known about the radiation-induced miRNA response in these tissues. Further, as IR is a potent inducer of these types of cancer, dissecting microRNAome changes in IR-exposed hematopoietic tissues may help us in the identification of the initial, pre-malignant radiation induced changes that may further lead to carcinogenesis. With this in mind, we analyzed the IR-induced changes to the microRNAome in the radiation-sensitive lymphatic tissues of the hematopoietic system in a well-established murine model. Here we show that whole body IR exposure resulted in a significant and sex-specific deregulation of microRNA expression in mouse

spleen and thymus tissue. MicroRNAome changes were paralleled by altered expression of microRNA target proteins.

3.3. MATERIALS AND METHODS

3.3.1. Model

In this study, we utilized an in vivo mouse model to study microRNAome changes in the thymus and spleen of male and female animals following IR exposure. The C57BL/6 mouse model is widely used, well characterized, and generally accepted for studies of IR-induced changes in hematopoietic lymphoid tissue (Boulton, Cleary et al. 2002). Handling and care of animals was in accordance with the recommendations of the Canadian Council for Animal Care and Use.

3.3.2. Radiation exposure

Fifty-day-old C57BL/6 mice (10 males and 10 females) were randomly assigned to different treatment groups. All animals had comparable body weights, were kept in a virus-free facility, and given food and water ad libitum. Five male and five female mice received 2.5 Gy of X-ray exposure to the entire body (90 kV, 5 mA). Ten animals (5 male and 5 female mice) were sham treated and served as controls. No signs of pain or distress were observed during the experiment. Animals were humanly sacrificed 6 h after exposure. Thymus and spleen tissues were harvested immediately following sacrifice, divided into two equal parts, frozen in liquid nitrogen, and stored at -80C for further analysis. This experiment was independently replicated once according to the same experimental scheme using a cohort of 12 C57/BL6 mice each time (3 animals per group/per sex). Both experiments yielded congruent data.

3.3.3. miRNA microarray expression analysis

Total RNA was extracted from mouse spleen and thymus tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions. Microarray analysis was conducted using the tissue from control and exposed animals. Tissue from 3 animals per group per sex was used for the analysis. The miRNA microarray analysis was performed by LC Sciences (Houston, TX)(Pogribny, Tryndyak et al. 2007). Normalization was performed with a cyclic LOWESS (locally weighted regression) method to remove system-related variations, as previously described (Pogribny, Tryndyak et al. 2007). The t-test analysis was conducted between the control, whole-body exposed and bystander sample groups. MicroRNAs with p-values <0.05 were selected for cluster analysis. Quantitative real-time PCR (qRT-PCR) analysis.

Quantitative realtime PCRs (qRT-PCRs) were performed by using SuperTaq Polymerase (Ambion) and a mirVana qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained mirVana qRT-PCR primer sets specific for miR-34a. The 5S rRNA served as a positive control. Quantitative real-time PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA).

3.3.4. Western immunoblotting

Western immunoblotting was conducted using spleen tissue of both groups using a well-established protocol (Pogribny, Koturbash et al. 2005). Membranes were incubated with antibodies against Notch1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D1 (1:2000; Cell Signalling), Myc (1:500; Santa Cruz Biotechnology), Lsh (1:1000, Abcam, Cambridge, MA), E2f3 (1:500, Lab Vision) and actin (1:2000; Abcam). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated

secondary antibodies (GE Healthcare) and the ECL Plus immunoblotting detection system (GE Healthcare). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Unaltered PVDF membranes were stained with Coomassie Blue (Bio-Rad, Hercules) and the intensity of the Mr 50,000 protein band was assessed as a loading control. Signals were quantified using NIH ImageJ 1.63 Software and normalized to both actin and the Mr 50,000 protein band, which gave consistent results (values relative to Mr 50,000 are plotted). The protein levels in the tissues of exposed animals were related to controls. Two technical replicates were used for each immunoblotting. Luciferase reporter assay for targeting LSH-3'-UTR. For the luciferase reporter experiments, a 3'-UTR segment of LSH gene corresponding to a region of 366 nucleotides (nt) (from 2642 nt through 3008 nt of the total transcript) for LSH (Acc. No.NM_008234) was amplified by PCR from mouse genomic DNA and cloned into the pGL3-control vector (Promega, Madison, WI). The HEK293 cells were transfected with the firefly luciferase UTR-report vector, control Renilla luciferase pRL-TK vector (Promega), transfection controls and precursor miR-7 using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), as previously described (Kovalchuk, Filkowski et al. 2008). Twenty-four hours after transfection, cells were lysed with a 1X passive lysis buffer and the activity of both Renilla and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions, as previously described (Kovalchuk, Filkowski et al. 2008). Statistical difference between the group's means was determined by Student's t-test using JMP 5.0 and Excel software (Microsoft Corporation, Redmond, WA).

3.4. RESULTS AND DISCUSSION

In this study, we utilized an in vivo murine model to analyze and compare microRNAome changes induced by exposure to X-rays in the lymphoid hematopoietic spleen and thymus tissue of male and female mice. Equal cohorts of male and female experimental animals were divided in two experimental groups, 'exposed' animals (2.5 Gy of whole-body X-ray irradiation) and sham treated 'control' animals. Analysis of the murine spleen microRNAome revealed a number of intriguing patterns. In male spleen samples, 15 miRNA were significantly regulated ($p < 0.05$; 7 up- and 8 down-regulated; Figure 3.1A), and in female spleen samples 9 miRNAs were significantly regulated ($p < 0.05$; 6 up- and 3 down-regulated; Figure 3.1A). Interestingly, between these groups, only two miRNAs were similarly regulated in male and female samples, miR-346 and miR-34a. These miRNAs were 5- and 2.3-fold up-regulated in females and 3- and 2-fold up-regulated in males, respectively. The upregulation of miR-34a was independently confirmed by qRT-PCR (Figure 3.1B).

MiR-34a is a known tumor-suppressor miRNA, as it targets the Myc (Wei, Song et al. 2008) (Sun, Fu et al. 2008), cyclin D1 (Sun, Fu et al. 2008) and Notch1 (Lewis, Shih et al. 2003) oncogenes, as well as the potent transcriptional inducer of cell-cycle progression E2f3 (Welch, Chen et al. 2007). Indeed, we observed a significant down-regulation of Myc, cyclin D1, Notch1 and E2f3 in the spleen tissue of exposed male and female mice (Figure 3.1B), suggesting that the radiation-induced up-regulation of miR-34a may be viewed as a protective mechanism aimed to suppress oncogene overexpression after IR exposure. Further, miR-34a up-regulation and concurrent E2f3 down-regulation were recently shown to lead to increased apoptosis (Welch, Chen et al. 2007). Overall, miRNAs 34a and 346 were the only miRNAs to have similarly changed

in response to irradiation in both male and female spleen, suggesting that these miRNAs are radiation responsive regardless of sex. The miR-34 family is highly conserved among animals and invertebrates and has been previously shown to be regulated via the p53 transcription factor (He, He et al. 2007). These data correspond with our previously reports of p53 and miR-34 up-regulation of in the spleen of male and female mice exposed to X-rays (Koturbash, Loree et al. 2008; Koturbash, Zemp et al. 2008).

Interestingly, it has previously been shown that the radioresistant model of C57BL/6 has an earlier p53 response than other radio-sensitive strains, resulting in a 2-fold higher apoptotic response in the spleen of irradiated animals (Lindsay, Coates et al. 2007). Indeed, this may be a similar mechanism as seen with miR-34 up-regulating apoptosis neuroblastoma cells (Welch, Chen et al. 2007), and further asserts miR-34a role as a protective miRNA in response to radiation. MiR-346 was previously shown to be significantly up-regulated in follicular thyroid carcinoma, where in vitro overexpression of miR-346 caused increases in cellular proliferation (Lindsay, Coates et al. 2007). Similarly to leukemia's and lymphoma, thyroid carcinomas are frequently induced by radiation exposure (Rubino, Cailleux et al. 2002). Therefore, the analysis of functional consequences of miR-346 activation in the spleen of irradiated mice deserves future attention. Activation of both of miR-34a and miR-346 in a similar manner suggests their like regulation. As it has been previously established that miR-34a is regulated by p53 (He, He et al. 2007), we searched the area surrounding miR-346 for putative p53 binding elements; however, no strong p53 binding sites could be found within 30 kB of the miRNA. miR-346 is an intronic miRNA, and, as such, could be under the transcriptional regulation of its host gene glutamate receptor, ionotropic, delta 1 (GRID1).

Unfortunately, p53 binding elements could not be found within 30 kB of the GRID1 transcription start site. Yet, when the 12 kB regions surrounding miR-34a and miR-346 GRID1 putative promoters were analyzed in silico for transcription factor binding sites using MatchTM, 26 and 17 consensus sequences were found for the miR-34a and GRID1 promoter, respectively, with 11 and 15 different transcription factor (TF) binding sites in each.

Interestingly, 5 common TF binding site types were found between the two putative promoters: Pax-4, Oct-1, MyoD, Hnf-4, and Elk-1. Of particular interest, Oct-1 is a well known DNA damage- induced response element (Zhao, Jin et al. 2000). Future studies are needed to dissect the details of the IR-induced transcriptional regulation of these microRNA loci, the biological repercussions of their up-regulation, and their roles, if any, in the predisposition to IR-induced genome instability and carcinogenesis. Amongst the other miRNAs, miR-7 was significantly downregulated in the spleen tissue of exposed males. Computational analysis (TargetScan 4.2) revealed that miR-7 targets murine lymphoid-specific helicase (Lsh), with conserved binding sites in the human, rat, and dog Lsh UTRs. This prediction was further confirmed by the miRGen software. LSH belongs to the SNF2 family of chromatin-remodeling ATPases and is required for the proper establishment and maintenance of DNA methylation in mammals (Dennis, Fan et al. 2001; Zhu, Geiman et al. 2006). DNA methylation was shown to be profoundly affected in radiation-exposed hematopoietic tissue (Koturbash, Pogribny et al. 2005), and recent data suggest the involvement of Lsh-induced DNA hypomethylation in the development of erythroleukemia (Fan, Schmidtmann et al. 2008).

Therefore, to examine whether Lsh is indeed functionally targeted by miR-7, the segment of LSH-3'-UTR containing the miR-7 complementary site was cloned into the 3'-UTR of a luciferase reporter system (Figure 3.2A). The resulting reporter vector was transfected into the HEK293 cells together with transfection controls and miR-7. The luciferase reporter construct that did not contain the Lsh-UTR was used as a negative control. Figure 3.2B shows that miR-7 inhibited the luciferase activity from the construct with the Lsh-3'-UTR segment in a concentration-dependent manner (Figure 3.2B). There was no change in the luciferase reporter activity when the cells were co-transfected with negative control (scrambled oligonucleotides). No luciferase expression changes were seen when the cells were transfected with the plasmid lacking the Lsh-3'-UTR fragment (data not shown). In order to further confirm that miR-7 indeed affects Lsh in vivo, we analyzed Lsh protein levels in the spleen tissue of control and exposed animals.

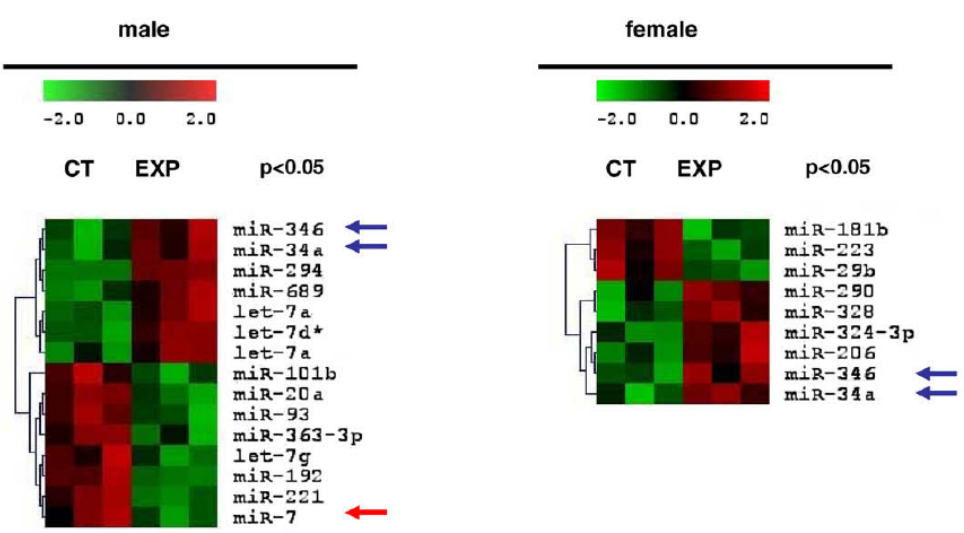
We observed a negative correlation between levels of miR-7 and Lsh. While the expression of miR-7 was decreased in the splenic tissue of the exposed male mice, the levels Lsh protein increased (Figure 3.3). Furthermore, no Lsh changes were seen in the spleen of exposed female mice, where miR-7 levels remained unchanged. The male-specific up-regulation of Lsh through miR-7 in response to ionizing radiation constitutes an intriguing find. Lsh is essential for correct DNA methylation patterning, as it has been shown that loss-of-function mutations in the murine Lsh gene result in dramatic hypomethylation of the murine genome and reactivation of transposable elements (Huang, Fan et al. 2004). Lsh overexpression, in contrast, leads to re-establishment of methylation marks and is viewed as a genome stabilizing event (Myant and Stancheva 2008). Radiation is well-known to induce a significant loss of DNA methylation, which is

much more pronounced in the male spleen tissue when compared to female spleen tissue (Raiche, Rodriguez-Juarez et al. 2004). Therefore, one could reason that the down-regulation of miR-7 with the consequent Lsh up-regulation in male spleen may constitute a cellular attempt to counteract the radiation-induced hypomethylation.

Interestingly, in female spleen, where the IR-induced hypomethylation was far less pronounced than in males, no miR-7 down-regulation and Lsh activation was seen. The mechanism of miR-7 expression and the roles of miR-7 and Lsh in genotoxic stress responses need to be further dissected in the future. Another interesting outcome of this study is the fact that IR exposure-induced microRNAome changes in the spleen that were distinct from those in the thymus. Changes in the thymus microRNAome were much less pronounced, especially in the female thymus where only 1 microRNA was significantly changed upon irradiation (Figure 3.4). In male thymus, 1 miRNA was upregulated, and 6 miRNAs were down-regulated (Figure 3.4). Both the spleen and thymus are radiation target organs, and are important components of lymphoid hematopoietic tissue; yet, their physiological roles in the organism are distinct. The spleen is involved in the destruction of redundant erythrocytes, and holds a reservoir of red blood cells and B-lymphocytes. The thymus holds a large number of apoptotic lymphocytes, and is involved in T-lymphocyte generation and maturation. Therefore, it is quite logical that these highly specialized parts of the hematopoietic system exhibit distinct microRNAome responses upon irradiation. The molecular mechanism and physiological consequences of radiation response of the spleen and thymus of male and female animals needs to be further addressed. This will help in further understanding the sex-specific changes to hematopoietic tissue under genotoxic stress conditions.

Overall, future studies are needed to understand the transcriptional regulation of microRNA loci upon irradiation, and other genotoxic stresses. Additionally, the changes to DNA methylation patterns under the influence of radiation may itself lead to the changes in the expression of a number of miRNA species, further contributing to aberrant miRNA expression patterns (Giotopoulos, McCormick et al. 2006). The link between DNA methylation and microRNAome expression upon genotoxic stress exposure needs to be established and studied in a tissue- and sex-specific context. This analysis is especially important given that approximately half of all known miRNA gene promoters are associated with CpG islands (Weber, Stresemann et al. 2007), and about 10% of known miRNAs are regulated by DNA methylation (Han, Witmer et al. 2007). Our microRNAome datasets may provide a roadmap for further analysis of the role of the microRNAome in radiation responses of hematopoietic tissues of males and females. The radiation-induced microRNAs identified in our study may further be explored as sex-specific biomarkers of radiation exposure in hematopoietic tissues.

A



B

MicroRNAlevel, Fold change from control	male spleen		female spleen	
	array	qRT-PCR	array	qRT-PCR
miR-34a	2.0	3.7	2.4	3.3
Level of target protein/ Fold change from control	**male spleen**		**female spleen**	
NOTCH1	-1.5		-1.4	
cyclin D1	-1.5		-1.5	
MYC	-1.3		-1.2	
E2F3	-1.6		-1.5	

Figure 3.1. Differentially expressed miRNAs in the spleen tissue of male and female mice subjected to whole-body exposure to 2.5 Gy of X-rays. (A) Hierarchical clusters of differentially expressed miRNA genes in the spleen of exposed male and female mice (as determined by t-test). Each miRNA listed is differentially expressed between control and exposed groups ($p < 0.05$). Arrows denote microRNAs discussed in the text. (B) Fold changes of miR-34a as measured by both microarray and qRT-PCR. These changes in miRNA expression correlate to protein level changes found in their targeted oncogenes Notch1, cyclin D1, Myc and E2f3. Each of the array hybridization represents separate animal and corresponds to single line on the heat map, transition from green to red color spectrum represents expression level relative to the average value normalized between arrays. Microarray experiment utilized 3 animals per experimental group.

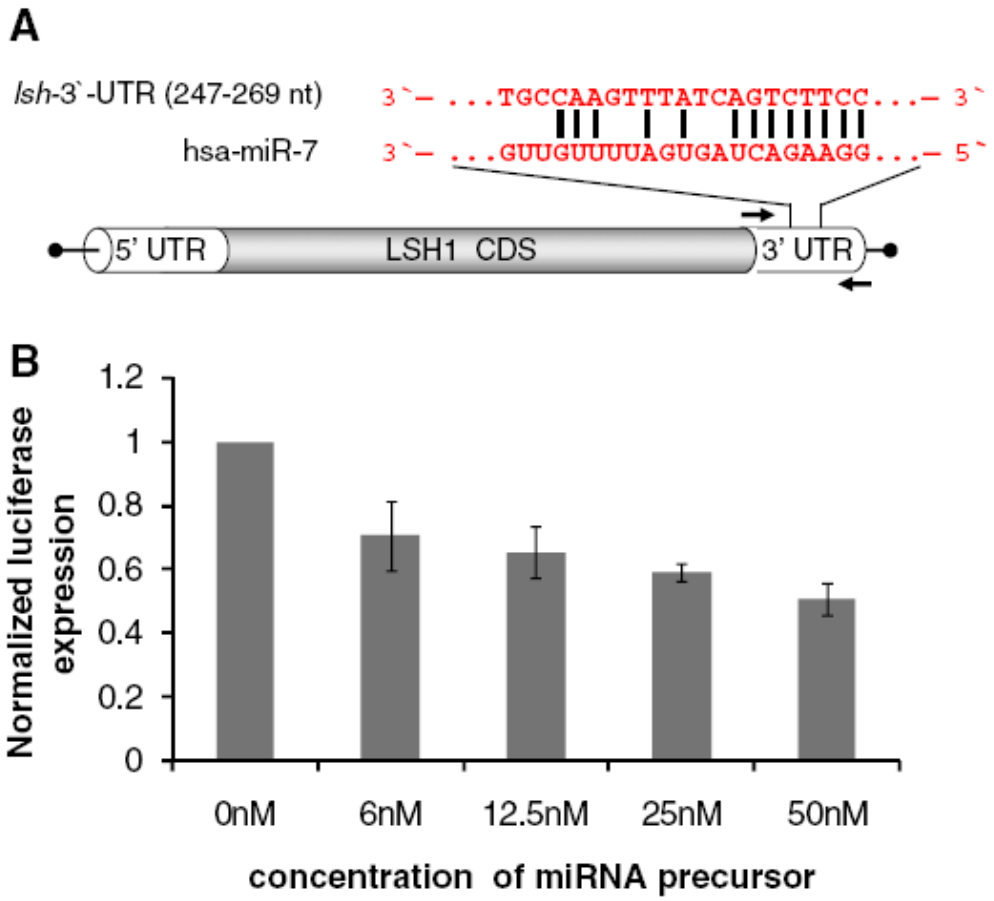


Figure 3.2. Mir-7 directly targets Lsh. (A) Complementary site for miR-7 in the 3'-UTR of LSH. (B) Dose-dependent inhibition of Lsh expression in the luciferase assay after transfection of the HEK293 cells with miR-7 or a negative control.

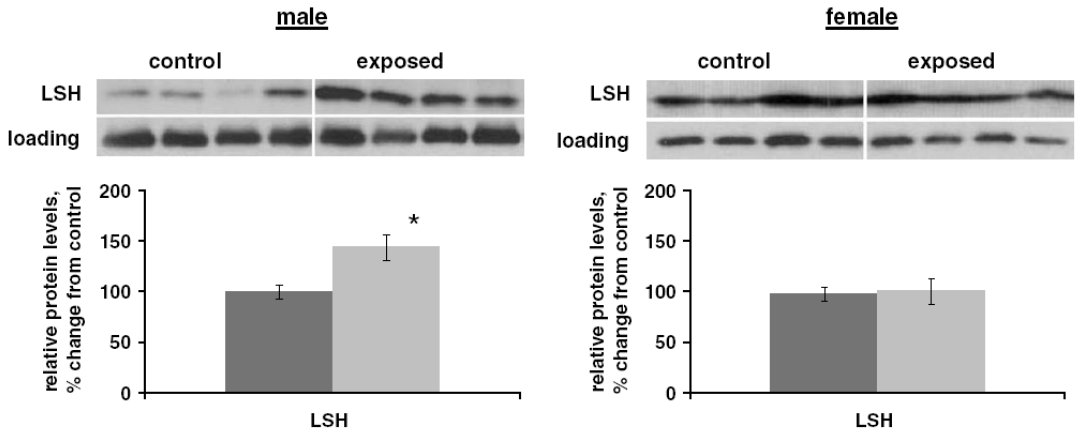


Figure 3.3. Altered expression of Lsh in murine spleen after IR exposure. Protein levels relative to those of control animals are shown as the means ± SD, *significant, 95% confidence limit, $p < 0.05$, Student's t-test. Representative western blots from among three independent technical repeats of the experiments are shown.

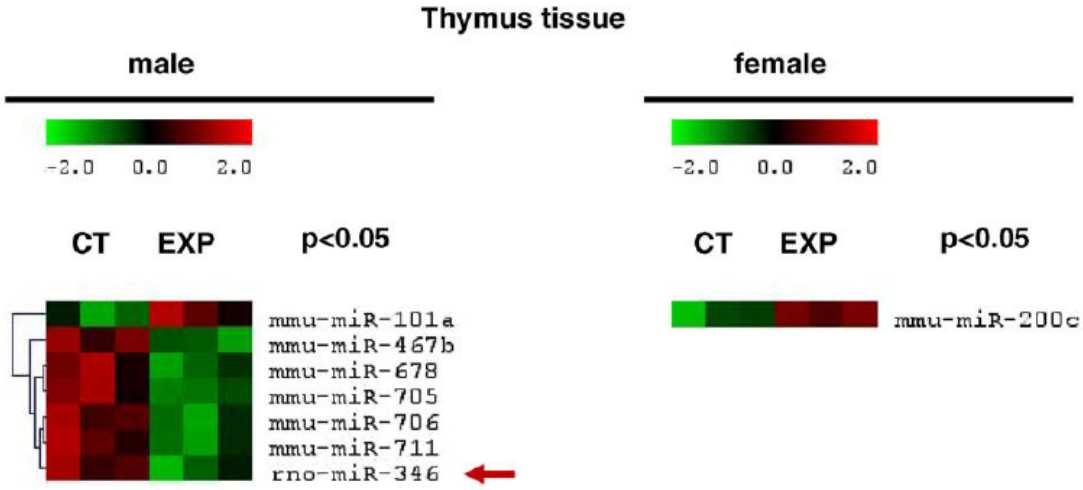


Figure 3.4. Differentially expressed miRNAs in the thymus tissue of male and female mice subjected to whole-body exposure to 2.5 Gy of X-rays. (A) Hierarchical clusters of differentially expressed miRNA genes in thymus of exposed male and female mice. Each miRNA listed is differentially expressed between control and exposed groups ($p < 0.05$). Each of the array hybridization represents separate animal and corresponds to single line on the heat map, transition from green to red color spectrum represents expression level relative to the average value normalized between arrays. Microarray experiment utilized 3 animals per experimental group.

4. HYPOMETHYLATION AND GENOME INSTABILITY IN THE GERMLINE OF EXPOSED PARENTS AND THEIR PROGENY IS ASSOCIATED WITH ALTERED MIRNA EXPRESSION[1]

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

Filkowski, J. N., Y. Ilnytskyy, et al. (2010). "Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression." Carcinogenesis **31**(6): 1110-1115.

4.1. ABSTRACT

Recent studies suggest that transgenerational genome instability may be epigenetic in nature and mediated via altered DNA methylation and microRNAome. Here, we investigated the nature and mechanisms underlying the disruption of DNA methylation and microRNA expression status in the germline and progeny of exposed parents. We have found that paternal irradiation leads to upregulation of the miR-29 family in the exposed male germline, which causes decreased expression of de novo methyltransferase, DNA methyltransferase 3a, and profound hypomethylation of long interspersed nuclear elements 1 (LINE1) and short interspersed nuclear elements B2 (SINE B2). Epigenetic changes in the male germline further resulted in deleterious effects in the somatic thymus tissue from the progeny of exposed animals, including hypomethylation of LINE1 and SINE B2. Hypomethylation of LINE1 and SINE B2 in the thymus tissue from the progeny was associated with a significant decrease in the levels of lymphoid-specific helicase (Lsh) that is crucial for the maintenance of methylation and silencing of repetitive elements. Furthermore, we noted a significant upregulation of miR-468 that targets Lsh and leads to its decreased expression in thymus in the progeny of exposed parents. We suggest that miR-468-mediated suppression of LSH leads to aberrant methylation of LINE1 and SINE B2. In summary, altered microRNAome and hypomethylation of retroelements constitute deleterious effects that may significantly influence genome stability of the parental germline and consequently cause genome instability in the progeny.

4.2. INTRODUCTION

Paternal exposure to genotoxic agents such as ionizing radiation (Dubrova 2003; Morgan 2003; Barber and Dubrova 2006; Niwa 2006), environmental toxicants (Dubrova, Hickenbotham et al. 2008; Nomura 2008) and chemotherapeutic drugs (Hales, Barton et al. 2005) poses a great threat to the progeny of exposed parents by inducing transgenerational genome instability. While the occurrence of transgenerational genome instability, especially radiation-induced genome instability, has been well documented, the mechanisms by which it arises remain elusive. It has been suggested that transgenerational genome instability may be epigenetic by its nature (Baulch, Raabe et al. 2001; Dubrova 2003; Dubrova 2003).

Epigenetic changes are alterations in gene expression that include DNA methylation, histone modification and RNA-associated silencing (Egger, Liang et al. 2004; Feinberg 2004). Epigenetic changes also include the best-known and most studied epigenetic mechanism—DNA methylation, the covalent addition of a methyl group to the cytosine residue at CpG sequences that affects gene expression and genome stability (Jaenisch and Bird 2003). Amongst short RNAs, microRNAs (miRNAs) deserve special attention. MiRNAs are evolutionally conserved, small, single-stranded RNA molecules that are recognized as major regulators of gene expression, cellular functions and genome stability (Huppi, Volfovsky et al. 2008). To fully understand transgenerational genome instability, it is important to define what happens in the germline of exposed parents and in the progeny. In our previous study, we demonstrated that paternal X-ray irradiation leads to a significant accumulation of DNA damage, loss of global methylation and altered global expression of miRNAs in the paternal germline (Tamminga, Kathiria et al. 2008; Tamminga, Koturbash et al. 2008). We found that it also influences global DNA

methylation in bone marrow, and thymus in unexposed offspring (Koturbash, Baker et al. 2006; Tamminga, Koturbash et al. 2008).

4.3. MATERIALS AND METHODS

4.3.1. Model

In this study, we utilized an in vivo murine model to analyze the role of epigenetic alterations in transgenerational radiation effects. The murine model is widely used, well characterized and generally accepted for studies of radiation-induced changes and transgenerational effects (Boulton, Cleary et al. 2002; Baulch and Raabe 2005; Barber, Hardwick et al. 2009). Mice (mature 60-day-old male C57BL/6J animals) were randomly assigned to different treatment groups. Handling and care of animals were performed in accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures were approved by the University of Lethbridge Animal Welfare Committee. Animals were housed in a virus-free facility and given food and water ad libitum. The exposed cohort (10 animals) received 2.5 Gy (3 Gy/min) of X-rays (90 kV, 5 mA) to the whole body. In our previous studies, this dose led to significant deleterious effects in the progeny (Koturbash, Baker et al. 2006). For the irradiation procedure, animals were placed in small plastic vented containers. These containers limit the movement of animals and insure the dose uniformity. Control mice (10 animals) were sham treated. For sham treatment, containers with animals were placed into the irradiator machine, but X-rays were not turned on. Four days (96 h) after exposure, mice were humanly killed, and testes were sampled and processed for further analysis. To analyze the effects of exposure on the progeny, 4 days after irradiation control (10 mice) and exposed (10 mice) animals were mated with unexposed females. Both sets of progeny were killed 6 months after birth. The thymus tissues were extracted, immediately frozen and stored at 80C until the analysis.

4.3.2. miRNA microarray expression analysis

Total RNA was extracted from mouse testes and thymus tissues using TRIzol Reagent (Invitrogen, Burlington, Ontario, Canada) according to the manufacturer's instructions. Tissues from two animals per group (paternal germline analysis) or eight animals per group (progeny analysis) were used for the analysis. The miRNA microarray analysis was performed by LC Sciences (Houston, TX) and was confirmed by quantitative real-time polymerase chain reaction (PCR) as described before . Quantitative real-time-PCR was conducted using tissues of six animals per group (paternal germline analysis) or eight animals per group (progeny analysis).

4.3.3. Immunohistochemistry

Paraffin embedding and sectioning were conducted at Histoprobe Consulting (Surray, British Columbia, Canada). The sections were stained with hematoxylin and eosin for the histopathological examination. Following the pathological examination, the tissues were assembled into tissue microarrays with 2.5 mm cores by Pantomics (www.pantomics.com; Richmond, CA). Immunohistochemical staining was conducted using the antibodies against Dicer (Santa Cruz Biotechnology, Santa Cruz, CA) in accordance with the manufacturer's recommendations, as described previously (27).

4.3.3. Luciferase reporter assay for targeting lymphoid-specific helicase-3'-untranslated region

For the luciferase reporter experiments, a 3'-untranslated region (UTR) segment of lymphoid-specific helicase (LSH) gene corresponding to a region of 366 nt (from 2642 nt through 3008 nt of the total transcript) for LSH (Acc.# NM_008234) was amplified by PCR from mouse genomic DNA and cloned into the pGL3-control vector (Promega,

Madison, WI). In parallel, the miR-468 seed sequence-binding region in the 3'-UTR segment (5'-tcagttatg-3') of the LSH gene was mutated to 5'-gagagggga-3'. The HEK293 cells were transfected with the firefly luciferase UTR-report vector, control Renilla luciferase pRL-TK vector (Promega), transfection controls and precursor miR-468 using lipofectamine 2000 reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA), as described previously. Twenty-four hours after transfection, cells were lysed with a 1X passive lysis buffer and the activity of both renilla and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions, as described previously (Kovalchuk, Filkowski et al. 2008). To check the suppression of the native mouse Lsh protein by miR-468, mouse NIH3T3 cells were transfected with miR-468 precursors (100 nM). Twenty-four hours following transfection, the cellular levels of Lsh were detected by immunofluorescence or western immunoblotting using anti-Lsh antibodies (Santa Cruz Biotechnology) according to manufacturer's instructions.

4.3.4. Western immunoblotting

Western immunoblotting was conducted as described previously, using antibodies against LSH (1:500; Abcam, Cambridge, MA), DNA methyltransferase DNMT3a (1:500; Santa Cruz Biotechnology) and actin (loading control) (1:2000; Abcam).

4.3.5. DNA extraction and DNA methylation analysis

DNA was extracted from thymus using a Qiagen DNAeasy kit (Qiagen), according to the manufacturer's instructions. Methylation analysis of long interspersed nuclear elements 1 (LINE1) and short interspersed nuclear elements B2 (SINE B2) retrotransposons was determined by the methylation-sensitive McrBC-qPCR assay as

described previously (Kovalchuk, Filkowski et al. 2008). Genomic DNA (1 ug) was digested overnight with the methylation-specific restriction enzyme McrBC (New England Biolabs,Ipswich, MA) and then analyzed by quantitative PCR using primers described in Martens et al. (Martens, O'Sullivan et al. 2005). Two-step quantitative PCR was performed using a SYBR GreenERTM SuperMix (Invitrogen, Carlsbad, CA) for Cycler (Bio-Rad, Hercules, CA) with 40 cycles of 45 s at 95C and 90 s at 58C. After the final cycle, melting curve analysis of all samples was conducted within the range of 55–95C. All reactions were run in triplicate. The threshold cycle (Ct) is defined as the fractional cycle number that passes the fixed threshold. Ct values for each repetitive element were converted into the absolute amount of input DNA using the absolute standard curve method. An increased amount of input DNA after digestion with McrBC is indicative of hypomethylation, whereas a decreased amount of input DNA is indicative of hypermethylation. Global DNA methylation was analyzed using a well-established HpaII based cytosine extension assay as described previously (Tamminga, Koturbash et al. 2008). Statistical analysis Statistical analysis was performed using MS Excel 2007 and JMP5 software packages.

4.4. RESULTS

4.4.1. Altered miRNA expression leads to decreased levels of de novo methyltransferases in the germline of exposed male mice

In the present study, we investigated the exact nature and mechanisms underlying the disruption of microRNAome and DNA methylationstatus in the germline of exposed fathers and in the radiation target thymus tissue of their 6-month-old progeny that was conceived 4 days after paternal exposure to 2.5 Gy of X-rays. The paternal radiation exposure resulted in profound miRNA changes in the germline of exposed fathers (Tamminga, Kathiria et al. 2008), particularly in a significant upregulation of miR-29a and 29b (Figure 4.1A). These changes were confirmed by real-time PCR. The miR-29 family influences the de novo Dnmts, Dnmt3a and Dnmt3b. Fabbri et al. (2007) showed that miR-29 expression could directly affect methylation patterns. Here, we noted that miR-29a and miR-29b upregulation was paralleled by a significant downregulation of Dnmt3a in the testes of exposed animals (Figure 4.1B).

These data are in good agreement with a significant loss of global DNA methylation and altered miRNAome changes in the exposed mouse testes tissues, previously reported by our group (Tamminga, Kathiria et al. 2008). Interestingly, irradiation did not cause any significant changes in the levels of maintenance Dnmt1. Furthermore, it did not affect the cellular levels of Lsh, a chromatin remodeling protein that is thought to be important for the maintenance of genome-wide methylation (Supplementary figure I). Thus the main changes were seen only in the levels of de novo methyltransferases.

4.4.2. Loss of DNA methylation of transposable elements in the germline of exposed mice

It is well known that DNMT3a partakes in methylation and silencing of transposable elements and safeguarding genome stability. Therefore, we determined the DNA methylation status of LINE1 and SINE B2, two of the most prevalent classes of repetitive sequences that compose 20% of the mouse genome (Waterston, Lindblad-Toh et al. 2002). The status of LINE1 and SINE B2 methylation was determined by methylation-sensitive McrBC quantitative PCR analysis (Martens, O'Sullivan et al. 2005). The methylation-sensitive McrBC endonuclease is a restriction enzyme that digests only methylated DNA sequences but does not cleave unmethylated DNA (Sutherland, Coe et al. 1992). Importantly, we found a significant loss of LINE1 methylation and a tendency of SINE B2 hypomethylation upon radiation exposure in the male germline (Figure 4.1C). This is a very interesting finding because hypomethylation of certain repetitive elements, including LINE1, is considered to be a hallmark of genomic instability (Roman-Gomez, Jimenez-Velasco et al. 2005).

Paternal exposure leads to altered LINE1 and SINE B2 methylation in the offspring. Genome instability in the paternal germline may exert a negative influence on fertilized eggs and therefore cause deleterious effects in the offspring (Aitken and De Iuliis 2007). Our next step was to investigate the extent of inherited epigenetic changes in the thymus tissue of unexposed progeny of exposed male mice. Thymus is an important lymphoid hematopoietic organ that is a target of radiation carcinogenesis. Furthermore, in our previous studies, we observed a loss of global genome-wide methylation in the thymus tissue of the progeny of exposed parents (Koturbash, Baker et al. 2006). One of

the main functions of DNA methylation in normal mammalian somatic tissues is the suppression of transposable repetitive elements (Yoder, Walsh et al. 1997; Miranda and Jones 2007).With this in mind, we determined the DNA methylation status of LINEs and SINEs, particularly LINE1 and SINE B2, in thymus tissues of the progeny of exposed parents. Figure 4.2A demonstrates a profound level of hypomethylation of LINE1 and SINE B2 sequences in the thymus tissue of the progeny of exposed male mice as evidenced by a significantly ($P < 0.05$) greater recovery of LINE1 and SINE B2 PCR products (by 3.8 and 2.2 times, respectively) after pretreatment of DNA with McrBC endonuclease. This may be indicative of genomic instability in the thymus tissue of the progeny (Roman-Gomez, Jimenez-Velasco et al. 2005; Howard, Eiges et al. 2008).

4.4.3. Decreased levels of LSH in the progeny of exposed parents

In order to determine whether or not loss of DNA methylation in the thymus of the offspring of irradiated male mice is associated with the dysregulated function of the DNA methylation machinery, we measured the protein levels of Dnmts. Surprisingly, we did not detect changes in protein levels of either the maintenance Dnmt1 or the de novo Dnmt3a and Dnmt3b (data not shown). However, we detected a substantial decrease in the levels of the Lsh protein in the thymus tissue of the offspring of exposed parents as compared with controls (Figure 4.2 B). Lsh, a member of the SNF2 family of chromatin remodeling proteins, is thought to be crucial for the maintenance of genome-wide CpG methylation (Dennis, Fan et al. 2001; Huang, Fan et al. 2004), especially for methylation and silencing of repetitive elements such as LINEs and SINEs (Huang, Fan et al. 2004). Recently, it has been demonstrated that Lsh is directly involved in the control of de novo methylation of DNA (Zhu, Geiman et al. 2006), and LSH deficiency leads to aberrant

upregulation of retroviral repetitive elements in the genome, abnormal mitosis with amplified centrosomes and genomic instability (Muegge 2005). Therefore, LINE1 and SINE B2 hypomethylation in the thymus tissue of the progeny of exposed mice may be related to the decreased levels of Lsh.

4.4.4. Lsh expression is mediated by a miRNA

To further explore molecular mechanisms underlying hypomethylation of repetitive elements in the progeny of irradiated male mice driven by Lsh downregulation, we studied the regulation of LSH expression mediated by short noncoding RNAs. It has been demonstrated that miRNAs participate in the regulation of a variety of cellular processes in mammals, including DNA methylation (Carrington and Ambros 2003; Bartel 2004). In total, miRNAs control the activity of 30% of all protein-coding genes (Filipowicz, Bhattacharyya et al. 2008), including proteins that control methylation of DNA (31). Additionally, the results of recent studies have shown that deficiency of Dicer, an RNAse III family nuclease that generates miRNAs, is associated with decreased DNA methylation (Filipowicz, Bhattacharyya et al. 2008). Indeed, Figure 2.3 A demonstrates a decrease in Dicer expression in the thymus tissue of the offspring.

Next, we analyzed miRNA expression profiles using miRNA microarrays in the thymus tissue of the progeny of control and ex-posed parents. Cluster analysis revealed that pre-conceptional paternal exposure led to significant changes in miRNA expressions in the thymus tissue of the offspring. We identified 25 miRNA genes (17 upregulated and 8 downregulated) that were differentially expressed ($P < 0.05$) in the progeny of exposed animals as compared with controls (Figure 4.3 B). Interestingly, we did not detect changes in the expression of two miRNA families, miR-29 and miR-290, that regulate

levels of DNMTs (Fabbri, Garzon et al. 2007; Benetti, Gonzalo et al. 2008). However, computational analysis revealed that miR-468, which was upregulated in the progeny of exposed parents, targets murine Lsh (Figure 4.4 A). This prediction was further confirmed by the comprehensive microRNA.org data resource (Betel, Wilson et al. 2008). Therefore, to examine whether or not LSH is indeed functionally targeted by miR-468, a segment of Lsh-3'-UTR containing the miR-468 complementary region was cloned into the 3'-UTR of the luciferase reporter system.

Additionally, we have mutated the miR-468 seed-interacting sequence in the Lsh-3'-UTR. The resulting reporter vectors were then transfected into HEK293 cells together with transfection controls and miR-468. The luciferase reporter construct that did not contain the Lsh UTR was used as a negative control. Figure 4.4 B shows that miR-468 inhibited the luciferase activity of the construct containing the Lsh-3'-UTR segment (Figure 4.4 B). There was no change in the luciferase reporter activity if cells were cotransfected with a negative control (scrambled oligonucleotides) or with a vector harboring mutated Lsh-3'-UTR. No luciferase expression changes were observed if cells were transfected with the plasmid lacking a Lsh-3'-UTR fragment (Figure 4.4 B).

To further confirm that miR-468 indeed affects the protein levels of Lsh in mouse NIH3T3 cells, these cells were transfected with miR-468, and the level of Lsh was determined by immunocytochemistry and western immunoblotting 24h after transfection. Transfection of NIH3T3 cells with miR-468 resulted in a decrease of Lsh levels (Figure 4.5 A). Therefore, we propose that miR-468-mediated suppression of Lsh leads to aberrant methylation of LINE1 and SINE B2 in the thymus tissue of the progeny of exposed parents. The mechanistic link between miR-468-mediated Lsh downregulation

and the DNA hypomethylation was confirmed by transfection of mouse NIH3T3 cells with miR-468 or scrambled oligonucleotides.Cells were harvested 48 h later and the levels of global DNA methylation were analyzed. Interestingly, we noted a 9% decrease global genomic DNA methylation in the miR-468-transfected cells that was statistically significant at 90% confidence. Furthermore, transfection of NIH3T3 cells with miR-468 led to a significant ($P<0.05$) 24% loss of methylation of SINE elements. Methylation levels of LINE1 elements also tended to decrease 48 h after transfection of the cells with miR-468 precursors (Figure 4.5).

4.5. DISCUSSION

Approximately 5% of human live births today have a birth defect, a de novo genetic disease or chromosomal abnormality (Wyrobek, Mulvihill et al. 2007). Even having this knowledge, we still have little understanding of the mechanisms of genome instability. Recent studies suggest that transgenerational genome instability may be epigenetic in nature and mediated via altered DNA methylation and microRNAome. To fully understand transgenerational genome instability, it is important to define what happens in the germline of exposed parents and in the progeny. Here, we for the first time analyzed the nature and mechanisms underlying the disruption of DNA methylation and miRNA expression status in the germline and progeny of exposed parents. We demonstrate that paternal irradiation leads to upregulation of the miR-29 family in the exposed male germline, which correlated with decreased expression of de novo methyltransferase DNMT3a and profound hypomethylation of transposable LINE1 and SINE B2 sequences. Therefore, radiation-induced hypomethylation in the male germline may be explained, at least in part, by the radiation-induced miR-29 changes.

In our previous studies, we have shown that radiation-induced deregulation of another important miRNA, miR-709, also partakes in regulation of DNA methylation in the male germline (Tamminga, Koturbash et al. 2008). Importantly, epigenetic DNA methylation and microRNAome changes observed in the male germline coincided with molecular effects in the somatic thymus tissue from the progeny of exposed animals, including hypomethylation of LINE1 and SINE B2. Hypomethylation of LINE1 and SINE B2 in the thymus tissue from the progeny was associated with a significant decrease in the levels of Lsh. Lsh was previously reported to be crucial for the maintenance of methylation and silencing of repetitive elements (Dennis, Fan et al. 2001;

Fan, Schmidtmann et al. 2008; Myant and Stancheva 2008). Lsh cooperates with DNA methyltransfearses (Dnmts) in setting DNA methylation patterns and silencing (Myant and Stancheva 2008). Therefore, even though we have not seen any changes in the levels of Dnmts in the thymus tissue of the progeny of exposed parents, the changes observed in the levels of Lsh may have possibly affected the functioning of Dnmts. The role of Dnmt–Lsh inter-actions in the germline and transgenerational effects has still to be analyzed in detail. Additionally, the tissue-specific roles of Lsh and Dnmts need to be further analyzed.

Furthermore, our results demonstrate for the first time that paternal irradiation leads to microRNAome changes in the progeny. Specifically, we noted a significant upregulation of miR-468 that targets LSH and, possibly, leads to its decreased expression in thymus tissue of the progeny of exposed parents. We suggest that miR-468-mediated suppression of LSH leads to aberrant methylation of LINE1 and SINE B2 in thymus of the progeny of exposed parents. Transfection of mouse fibroblast NIH3T3 cells with miR-468 led to a significant decrease in the levels of Lsh and resulted in decreased global genomic DNA methylation levels. Mammalian genomes have high levels of DNA methylation at repetitive satellite DNA sequences, retro and DNA transposons. Repetitive sequences are the first targets of DNA methylation loss caused by the altered levels of the methylation machinery (Huang, Fan et al. 2004; Martens, O'Sullivan et al. 2005; Fan, Schmidtmann et al. 2008).

This regulation may be specifically required for the cells that were exposed to a genotoxic stressor, such as radiation, and for their descendants. Radiation induces DNA damage, which is known to cause significant DNA hypomethylation. In normal cells,

after DNA is repaired, DNA methylation machinery will most probably relatively quickly restore the methylation patterns. Yet, if miR-468 is overexpressed and LSH is suppressed such a restoration will not be as efficient due to the lack of LSH and will take longer time.

Further studies are needed to analyze the roles of mir-468-mediated LSH changes in normal conditions and upon stress exposure. Altered microRNAome levels, global DNA hypomethylation and reactivation of retroelements all constitute deleterious effects that may significantly influence genome stability and therefore lead to carcinogenesis. Importantly, changes were observed in radiation carcinogenesis of the target organ (thymus). Biological repercussions of these molecular and cellular changes and their etiological role in transgeneration carcinogenesis need to be further analyzed in detail. Additionally, further studies are needed to address epigenetic and miRNAome changes in the germline of progeny of exposed parents. Epigenetic changes in the progeny were linked to male parent exposure. The paternal genome is extremely sensitive to exposure to genotoxic agents (Anderson 2005; Barber and Dubrova 2006; Nomura 2006). Therefore, our data agree with the previous reports on radiation induction of transgenerational genomic instability upon paternal exposure. The epigenetic alterations observed may be associated with the transmission of altered DNA methylation, reactivation of transposons and DNA damage in parental sperm cells.

After fertilization, mammalian genomes undergo marked methylation reprogramming in order to establish correct parent-of-origin developmental programs (Hajkova, Erhardt et al. 2002; Lane, Dean et al. 2003; Anway, Memon et al. 2006). The epigenetic changes observed in sperm cells may interfere with postfertilization epigenetic

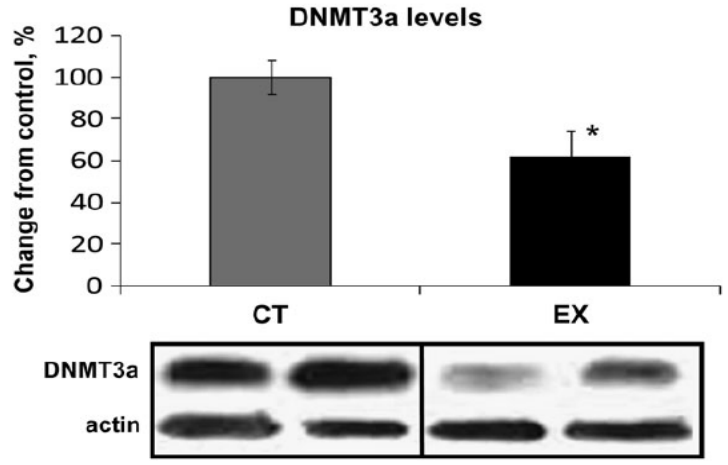
reprogramming, thus affecting fertilized eggs and leading to subsequent deleterious changes in the embryo (Aitken and De Iuliis 2007). Interestingly, some studies reported that transgenerational genome instability is more pronounced in mice than in humans. Our data may offer a plausible explanation for an apparent discrepancy in the magnitude of transgenerational effects in mice and humans. Detailed analysis of miRNA and genome databases revealed that miR-468 is a mouse-specific miRNA. While the 3'-UTR of human and mouse Lsh genes are 98% identical and the miR-468-binding sites are preserved, functional miR-468 does not exist in a human genome. The lack of miR-468 may explain, at least in part, why transgenerational genome instability is less pronounced in humans. Further in depth studies are needed to understand the roles of miRNAs in germline and trans- generational effects. In our previous study (Ilnytskyy and Zemp et al, 2009) LSH appeared to be induced in the spleen of males upon direct exposure, and this increase correlated with the increase of miR-7, which targeted LSH-3'UTR *in vitro*. We suggested this to be a sex-specific protective mechanism. Here we report decrease of LSH expression in the thymus of PPI mice, which correlated with the increase of miR-468. Such opposite regulation of LSH seem to be paradoxical, however direct irradiation cannot be compared directly with pre-conceptual exposure. Furthermore, it is unclear how altered miRNA expression is induced in unexposed progeny and to which extent miR-468 actually regulates LSH expression in vivo.

Therefore, this study may serve as a future roadmap for analyzing the role of methylation and microRNAome in transgenerational genomic instability.

A

miRNA	Fold induction	Target protein
miR-29a	2.33	DNMT3a, DNMT3b
miR-29b	1.23	DNMT3a, DNMT3b

B



C

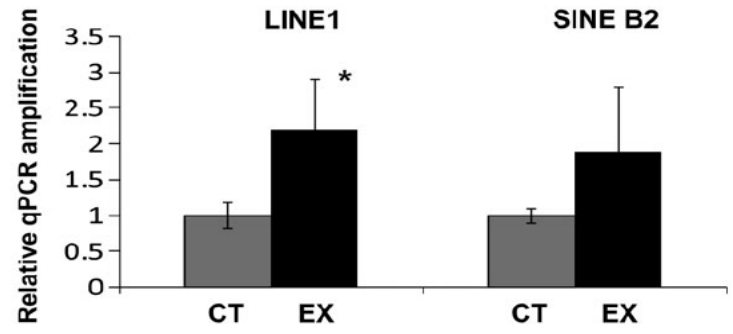


Figure 4.1. Radiation exposure alters miRNA expression and methylation of LINE1 and SINE B2 retrotransposons in male germline. Mice (mature 60-day-old male C57BL/6 animals) were randomly assigned to different treatment groups. The exposed cohort (10 animals) received 2.5 Gy (3 Gy/min) of X-rays (90 kV, 5 mA) to the whole body. In our previous studies, this dose led to significant deleterious effects in the progeny (Koturbash, Baker et al. 2006). Control mice (10 animals) were sham treated. Four days (96 h) after exposure, mice were humanly killed, and testes were sampled and processed for further analysis. (A). Altered expression of miR-29 family in germline of exposed male animals. Global miRNA expression was previously determined by the LC Sciences miRNA microarray platform (Tamminga, Kathiria et al. 2008). Levels of miR-29a and miR-29b were confirmed by quantitative real-time PCR using specific primers (Ambion) as described (Ilnytskyy, Zemp et al. 2008). (B). Decreased levels of DNMT3a in germline of exposed male mice. Lysates from testes tissue were subjected to immunoblotting using antibodies against DNMT3a. Protein levels relative to those of control animals are shown as the mean ± SD, significant, 95% confidence limit, P , 0.05, Student's t-test. Representative western blots. Each experiment included pooled lysates from two animals for each cohort, with equal representation of each animal. Western blots were repeated at least three times to ensure the reproducibility and robustness of the results. (C). Hypomethylation of LINE1 and SINE B2 in the germline of exposed animals as determined by methylation-sensitive McrBC-based quantitative real-time PCR analysis ($P < 0.05$, Student's t-test. CT, control animals; EX, exposed animals).

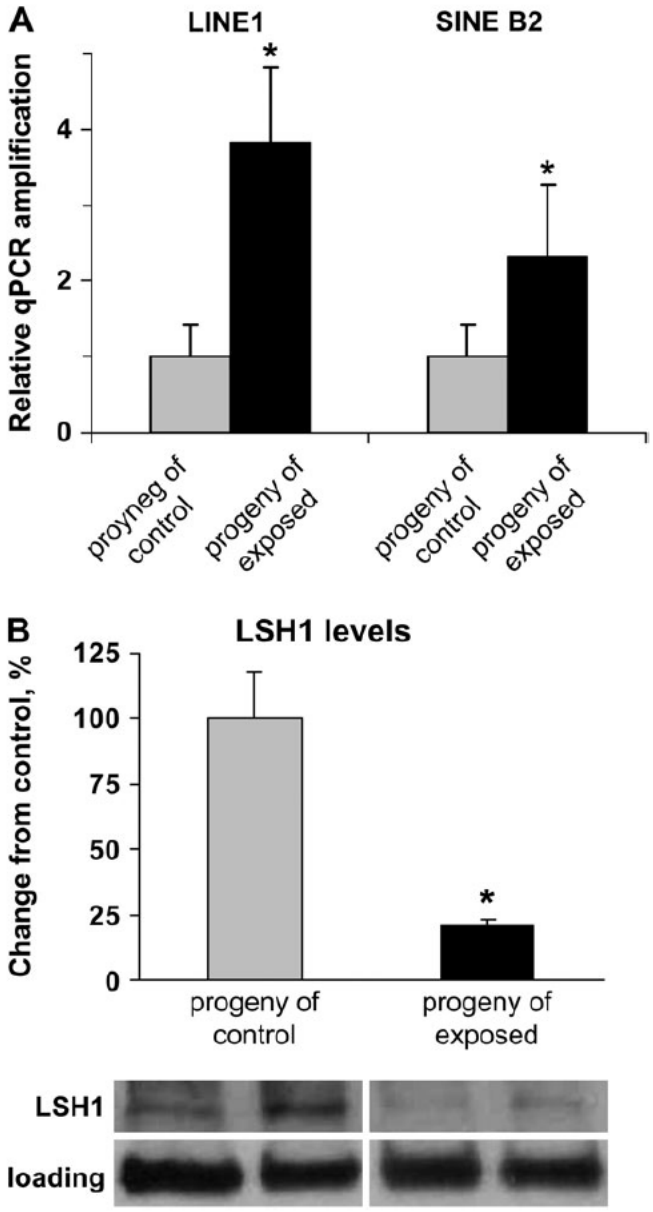


Figure 4.2. Paternal radiation exposure causes hypomethylation of LINE1 and SINE B2 retrotransposons and alters cellular levels of the methylation regulator LSH. To analyze the effects of exposure on the progeny, 4 days after irradiation control and exposed animals were mated with unexposed females. Both sets of progeny were killed 6 months after birth. The thymus tissues were extracted, immediately frozen and stored at -80C

until the analysis. (A). Hypomethylation of LINE1 and SINE B2 in the progeny of exposed parents as determined by methylation-sensitive McrBC-based quantitative real-time PCR analysi. ($P < 0.05$, Student's t-test). (B). Paternal radiation exposure decreases LSH protein levels in the thymus of the progeny in vivo. Lysates from thymus tissue were subjected to immunoblotting using antibodies against Lsh. Representative blots fromfour independent experiments are shown; each experiment included pooled lysates from four animals for each exposure condition with equal representation of each animal.

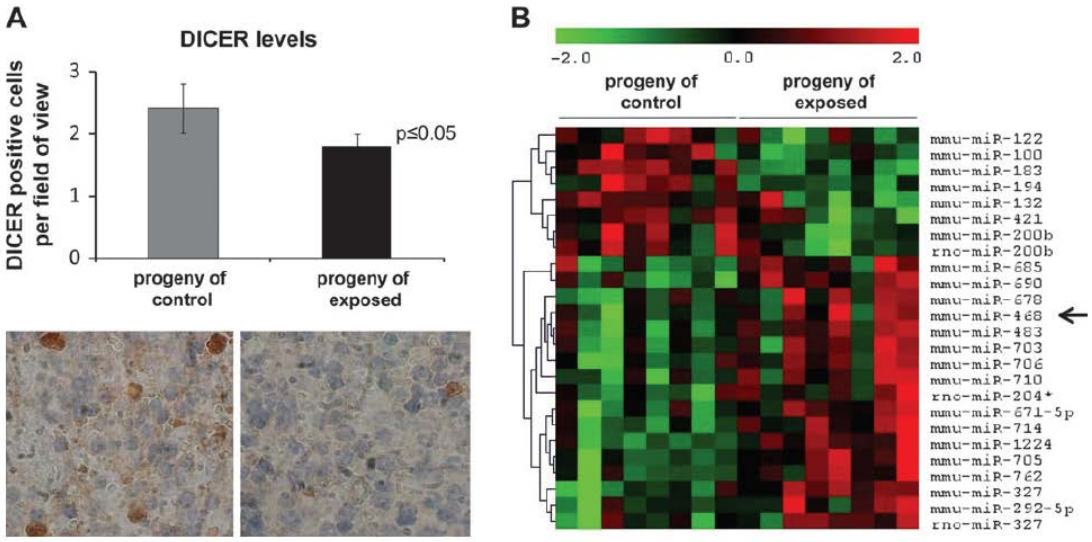


Figure 4.3. Paternal radiation exposure results in microRNAome deregulation in the thymus tissue of the unexposed progeny. (A) Decreased levels of Dicer in the thymus tissue of the progeny of exposed animals as determined by immunohistochemistry (IHC). IHC was conducted as described before (Koturbash, Kutanzi et al. 2008). Levels of Dicer positive cells per field of view are shown, mean values ± SEM, n=5, 10 fields per animals, 40 cells per field, $P<0.05$, Student's t-test. (B). Hierarchical clustering of differentially expressed miRNA genes in the thymus tissue of the progeny of control and exposed mice. Total RNAwas extracted from the thymus tissue using TRIzol Reagent (Invitrogen, Burlington, Ontario, Canada). miRNA microarray analysis was performed by LC Sciences (Houston, TX). Each of the array hybridization represents separate animal and corresponds to single line on the heat map, transition from green to red color spectrum represents expression level relative to the average value normalized between arrays. Microarray experiment utilized 8 animals per experimental group.

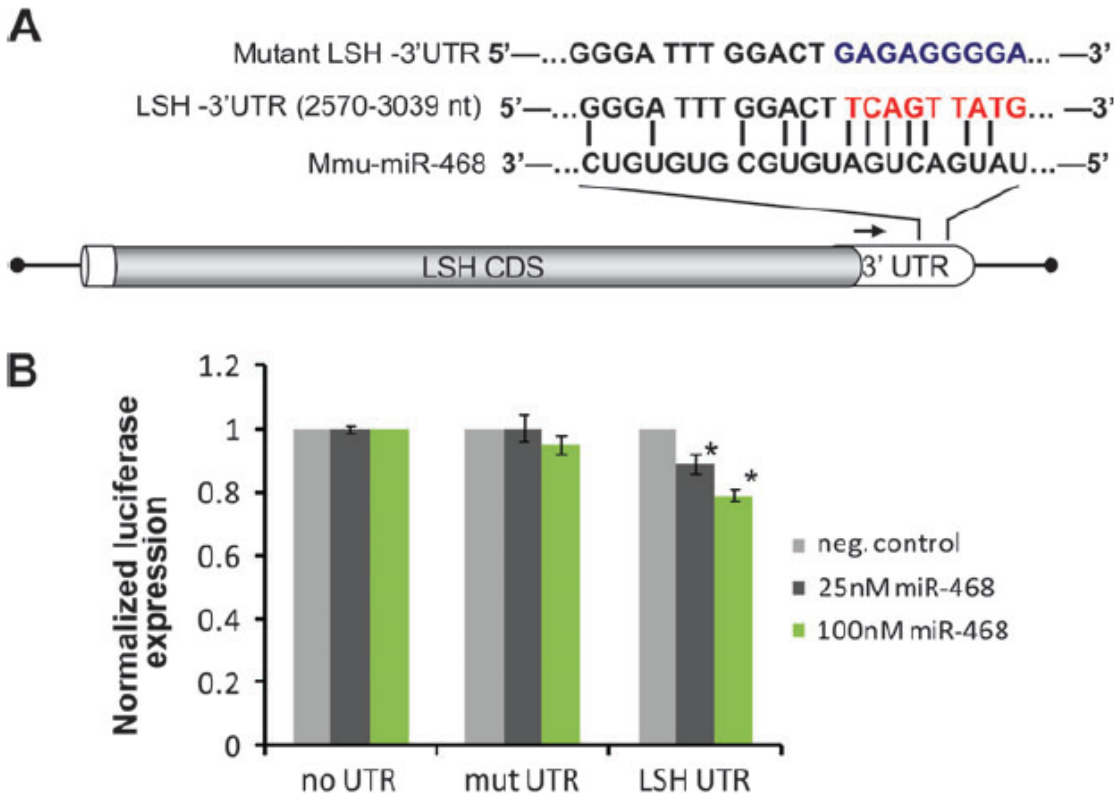


Figure 4.4. MiR-468 targets LSH. (A) Complementary site for miR-468 in the 3#-UTR of Lsh1. (B) A dose-dependent inhibition of LSH expression in the luciferase assay. A 3'-UTR segment of the LSH gene corresponding to a region of 366 nts (from 2642 nt through 3008 nt of the total transcript) of LSH (Acc. # NM_008234) was amplified by PCR from mouse genomic DNA and cloned into the pGL3-control vector (Promega, Madison, WI). HEK293 cells were transfected with the firefly luciferase UTR-report vector, the control Renilla luciferase pRL-TK vector (Promega), transfection controls either with precursor miR-468 or with miRNAs that do not have binding sites within the 3'-UTR of LSH (Ambion) as described previously (26). Twenty four hours after transfection, cells were lysed, and the activity of both renilla and firefly luciferases was assayed using the dual-luciferase reporter assay system (Promega) as described

previously. The graph depicts an inhibition of LSH expression in the luciferase assay after transfection of HEK293 cells with miR-468. RLU, relative luminescence units; $P < 0.05$,Student's t-test.

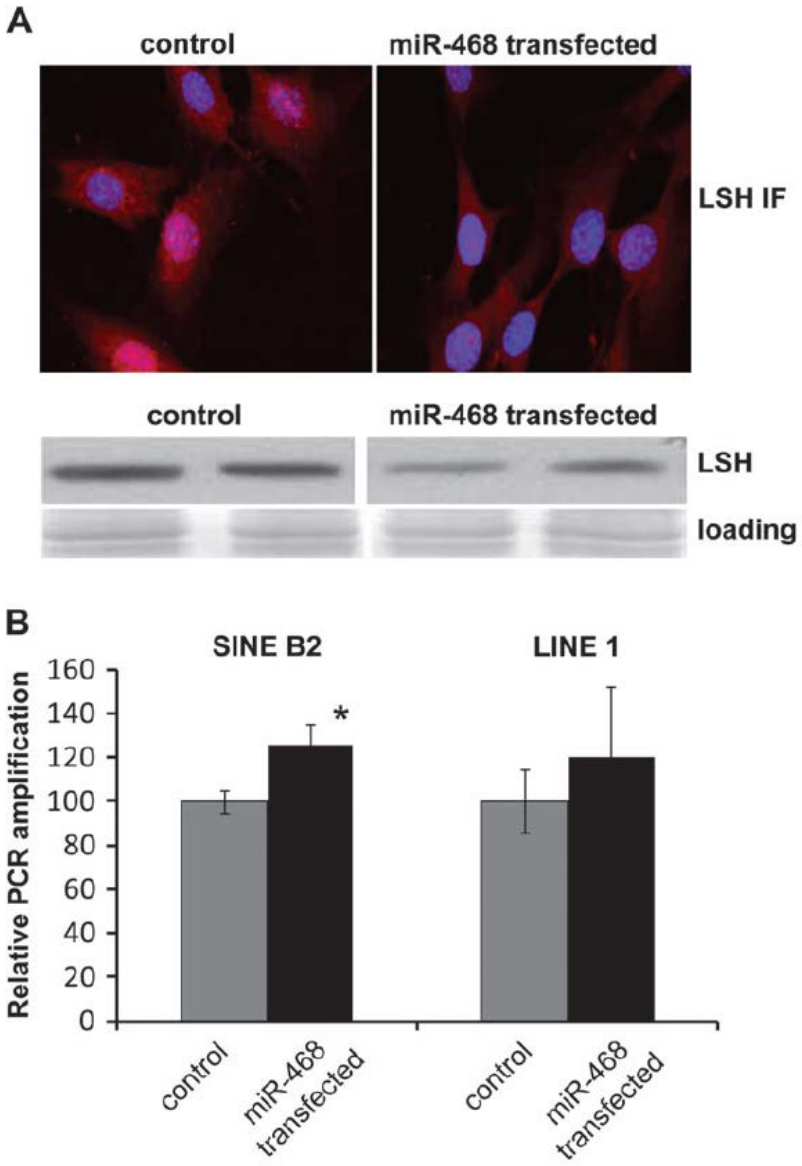


Figure 4.5. miR-468 reduces cellular levels of LSH and affects DNA methylation.(A) Transfection of mouse NIH3T3 cells with miR-468 effectively reduces cellular levels of LSH. Levels of LSH were detected by immunofluorescence and by western immunoblotting using anti-LSH antibodies. Red indicates LSH and blue indicates nuclear 4,6-diamidino-2-phenylindole stain. (B) Altered methylation of LINE1 and SINE B2 as determined by methylation-sensitive McrBC-based real-time PCR analysis in NIH3T3 cells 48 h after transfection with miR-468, $P < 0.05$, Student's t-test.

5. DYSREGULATION OF SMALL NON-CODING RNA TRANSCRIPTOMES IN MALE GERM LINE INDUCED BY DIRECT AND PATERNAL IRRADIATION.

5.1. ABSTRACT

A considerable body of evidence suggests the non-genetic transmission of radiation induced genomic instability and developmental abnormalities to the offspring of irradiated males. Molecular mechanisms underlying transgenerational effects of ionizing radiation are currently unknown. Detailed analysis of epigenetic changes induced by radiation in the male germ line is required to gain a better understanding regarding the molecular origins of these effects. Recently, small RNAs have emerged as key regulators of genomic integrity in the male germ line. Possible influences of radiation on these small RNA species have not yet been assessed.

In this paper, we report assembly of small RNA sequencing libraries of adult testes in control mice or following direct and paternal X-ray exposure. Analysis of deep sequencing libraries revealed an incredibly diverse profile of small RNA in testes, which was clearly dominated by 28-30 nucleotide long repeat derived small RNAs. Between-library comparisons showed small RNA transcriptome dysregulation after direct and paternal exposure that, in turn, may have influenced DNA methylation at repeat elements. The observed dysregulation was manifested as a decrease in repeat-derived small RNA pools and a dramatic up-regulation of the number of microRNAs following direct and paternal irradiation.

5.2. INTRODUCTION

Extensive research suggests that ionizing exposure is a major factor that results in deleterious developmental outcomes in the offspring of irradiated males (Vorobtsova 1989; Lowery, Au et al. 1990; Friedler 1996; Nomura 2003; Streffer 2006). These outcomes include increased cancer predisposition in the offspring of exposed fathers (Nomura 1989; Daher, Varin et al. 1998; Mohr, Dasenbrock et al. 1999), congenital abnormalities (Muller, Streffer et al. 1999; Pils, Muller et al. 1999; Nomura, Nakajima et al. 2004), increased rate of lethal mutations in subsequent generations (Luning, Frolen et al. 1976; Ryabokon and Goncharova 2006), transmissible genomic instability (Shiraishi, Shimura et al. 2002; Dubrova 2003; Barber, Hickenbotham et al. 2006; Niwa 2006), altered intracellular signaling (Baulch, Raabe et al. 2001; Vance, Baulch et al. 2002) and behavioral abnormalities (Lowery, Au et al. 1990).

A number of researchers have noted that the mode of transmission for detected end-points of male-mediated radiation induced abnormalities was different from that expected according to Mendelian laws (Vorobtsova 1989; Baulch, Raabe et al. 2001; Barber and Dubrova 2006). Experimental evidence suggests that de novo mutations occur at higher rates in all or most of the progeny and that this phenotype is transmitted to the F2 generation (Barber, Plumb et al. 2002), that altered DNA damage signaling is consistently observed in F3 progeny of irradiated male mice (Baulch, Raabe et al. 2001; Vance, Baulch et al. 2002), and that F2 generation offspring of irradiated fathers are more susceptible to secondary carcinogenic insults (Vorobtsova, Aliyakparova et al. 1993). These observed changes could not be explained by direct mutations induced by radiation exposure and transmitted to the progeny, since there was a lack of segregation and the frequency of induction was orders of magnitude higher than expected for single locus

mutations (Baulch, Raabe et al. 2001; Barber and Dubrova 2006; Nomura 2006). This elevated mutation rate and dysregulation of cellular signaling is compatible with a picture of persistent cellular reprogramming induced in the progeny conceived by irradiated sperm and then maintained across multiple cellular divisions. This type of reprogramming can be achieved by induction of epigenetic changes; i.e., by formation of heritable patterns of DNA methylation, chromatin modifications, and small RNA expression in paternally irradiated embryos. Global hypomethylation, hypomethylation of repeat elements, and altered miRNA expression were recently demonstrated in the progeny of irradiated mice (Koturbash, Baker et al. 2006; Tamminga, Koturbash et al. 2008; Filkowski, Ilnytskyy et al. 2010). Consequently, altered small RNA expression patterns were suggested as one of the central components of transgenerational genomic and epigenomic instability (Filkowski, Ilnytskyy et al. 2010).

Recently, small RNAs have emerged as a key regulator connecting extra- and intracellular signals to chromatin modifications in yeast, plants, and Drosophila (Zofall and Grewal 2006; Lin and Yin 2008; Martienssen, Kloc et al. 2008; Todeschini, Teysset et al. 2010). Similar small RNA mediated mechanisms may also operate in the mammalian germ line (Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008), which specifically expresses abundant novel class of small RNAs processed by the PIWI/PAZ domain proteins: Miwi, Mili, and Miwi2 (Aravin and Hannon 2008). Piwi associated RNAs (piRNAs) were shown to be key regulators of genomic integrity in the male germ line, primarily due to their ability to silence selfish transposable elements (TEs) in the genome by transcriptional and post-transcriptional mechanisms (Carmell, Girard et al. 2007; Aravin, Sachidanandam et al. 2008;

Kuramochi-Miyagawa, Watanabe et al. 2008). Loss of function in any of the Piwi proteins, or some other components of piRNA mediated silencing pathways, lead to reactivation of transposable elements, arrest of meiosis, induction of apoptosis, and failure of spermatogenesis (Klattenhoff and Theurkauf 2008; Soper, van der Heijden et al. 2008; van der Heijden and Bortvin 2009).

In the testes, the small RNA transcriptome is abundantly expressed and is comprised of high number of microRNA species and an extremely diverse population of piRNAs (Klattenhoff and Theurkauf 2008), which act together to regulate chromatin structure, transposon activity, and gene expression. Currently, little is known about the influence of adverse environmental factors, including radiation, on global small RNA transcriptome in the germ line, yet it may hold a key to the understanding of externally induced epigenetic reprogramming in the male germ line and in subsequent generations.

In this paper, we report the assembly of Illumina small RNA deep sequencing libraries from irradiated, paternally irradiated and control testes from adult mice. Analysis of these libraries revealed an extremely diverse landscape of small RNA expression in the male germ line. The main components were repeat derived small RNAs; however, a number of small RNAs were also mapped to transcripts, intergenic areas, and known and novel miRNAs. Both direct and paternal exposure affected library composition, resulting in decreases in repeat associated RNAs and dramatic up-regulation of several miRNA species. Changes in repeat derived small RNAs may be correlated with altered DNA methylation status observed at the some repeat loci in the progeny. Analysis of potential targets of up-regulated miRNAs provided some mechanistic clues about potential causes of observed effects.

5.3. MATERIALS AND METHODS

5.3.1. Animals and treatments

Six- to eight-week old male C57BL6 mice (Charles Rivers) were randomly assigned to control and treatment groups (10 animals per group). Handling and care of animals was provided according to the recommendations of the Canadian Council for Animal Care and Use. Procedures were approved by the University of Lethbridge Animal Welfare Committee. Animals were housed in a pathogen-free environment and were given food and water *ad libitum*. Mice were allowed to photo adapt for 24 hours upon arrival.

The first group of 10 animals received acute dose of 2.5 Gy of X-rays (5 cGy/s, 90 kV, 5 mA) delivered to whole body. The second group was sham-treated (handled exactly as the first group, but not irradiated) and served as a control. Mice from exposed and control groups were sacrificed by lethal injection 96 hours later and whole testes were surgically removed, snap-frozen in liquid nitrogen, and preserved at -80C for subsequent RNA analysis.

Young adult C57BL6 male mice (Charles Rivers) were randomly assigned to control and treatment groups. The treatment group received a 2.5 Gy acute dose of X-Ray to their whole body, while the control group was sham-treated. Irradiated and control mice were then mated to control virgin females of similar ages. The matings were performed at 1, 3 or 6 weeks after treatment in order to ensure that the progeny were derived from irradiated mature spermatozoa, early spermatids, and spermatogonia respectively. Progeny from the spermatid stage of exposure were interbred in order to obtain an F2 generation. The male progeny were sacrificed at the age of 45 days and spleens were surgically extracted, snap-frozen and preserved at -80C. Bone marrow was

washed out with PBS from femur bones, precipitated by 5 minute centrifugation at 3000g, snap-frozen in liquid nitrogen, and stored at -80 C for subsequent DNA extraction. Testes for RNA extraction and subsequent deep sequencing were harvested from spermatozoa from F1 animals.

5.3.2. RNA extraction

RNA was extracted with Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Concentration was measured by spectrophotometry and the quality assessed as absorption ratios at 260/280 and 260/230 nm. Agarose gel electrophoresis was performed in order to verify RNA sample integrity.

5.3.3. Deep sequencing

Total RNA from 10 control and 10 directly exposed male mice was pooled within experimental groups and delivered on dry ice to the sequencing provider, Fasteris (Geneva, Switzerland). Samples were sequenced as 2 bar-coded samples in 1 single-read channel. In each case, samples were processed using Illumina's small RNA sample preparation protocol. Briefly, small RNA fragments of 20-30 nucleotides were purified in acrylamide gel and ligated to single-stranded bar-coded 5' adapter. A single-stranded 3' adapter was ligated after a second acrylamide gel purification and then reverse transcribed and PCR amplified to generate DNA colony template library. Quality control of the DNA colony template library was performed by cloning an aliquot into a TOPO plasmid (Invitrogen) and capillary sequencing of 5-10 clones. Successful quality control was followed by flow-cell preparation on the Solexa Cluster Station and high-throughput sequencing on the Illumina Genome Analyzer GAII.

5.3.4. Read classification and mapping

Sequencing reads generated by Fasteris were further processed and mapped to the genome using a proprietary Mir-Intess^{TM} bioinformatics pipeline (Berezikov, Thuemmler et al. 2006; de Wit, Linsen et al. 2009). Parameters of the analysis are listed in table 5.1.

No adapter trimming was performed prior to insertion, since the sequence left after 4 nt barcode was insufficient for efficient recognition of 3'adapter. Instead 3'adapters were trimmed on the mapping stage, but the exact 3'ends of the sequences could be compromised. Only perfect and 3' trimmed matches were accepted for genome mapping. In case of perfect matches, a full read sequence comprised an exact match to the corresponding genomic sequence, while for 3' trimmed matches, at least an 18 nt perfect match was considered sufficient.

5.3.5. MicroRNA identification and mapping

Deep sequencing reads corresponding to mature miRNAs were identified and mapped using the miR-Intess^{TM} bioinformatic pipeline. Briefly, deep sequencing reads mapping to hairpin forming genomic loci were classified according to the features of hairpins. A balance of "good" and "bad" features served as a confidence level determinant for microRNA identification. The "good" features were defined by presence of DICER sites, the ability of the candidate region to fold into a thermodynamically stable hairpin according to Randfold software, (Bonnet, Wuyts et al. 2004) and a detection of level of more than 10 reads in at least 1 of the libraries. The "bad" features included the absence of Dicer sites, high nucleotide variation at the 5' end, a short stem of the candidate hairpin, mapping to repeats and RNA, and a high GC content. Candidate sequences were assigned to three confidence levels according to the presence of "bad"

and “good” features (1. confident: bad = 0, good >= 2; 2. candidate: bad = 0, good > 0, or bad = 1 and good >= 2; 3. other). The computational approach for microRNA identification used in this analysis was described elsewhere (Berezikov, Guryev et al. 2005; Houwing, Berezikov et al. 2008).

5.3.6. DNA isolation

Genomic DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen) according to manufacturer’s instructions. Sample concentrations were measured by UV spectroscopy; purity of preparations was determined according to 260/280 and 260/230 ratios. Samples with any of the ratios below 1.7 were additionally purified with DNA Clean & Concentrator™-5 (Zymo Research). Genomic DNA integrity was verified by agarose gel electrophoresis.

5.3.7. Methylation-sensitive McrBC-PCR assay

Methylation status of SINE B1 and LINE1 ORF2 elements in the testes tissue of PPI mice was evaluated with help of a McrBC-PCR assay. This assay is based on the use of a methylation sensitive restriction nuclease, McrBC, that is known to digest methylated templates at a site that consists of two half sites in the form of $(G/A)^{m}C$ separated by 40-3000 nucleotides. Under certain conditions, McrBC digestion, followed by PCR, allows comparison of the relative level of methylation at the selected region, depending on the amount of amplicon generated (Pogribny, Ross et al. 2006).

In our experiment, the McrBC-PCR assay was performed as described by Pogribny et al.(Pogribny, Ross et al. 2006). Briefly, 1 µg of DNA was digested with 20U of McrBC restriction endonuclease (New England Biolabs) for 2 hours at 37C. Another 1

µg aliquot was treated under exactly the same conditions, but without the enzyme, to serve as an undigested control. Enzyme was inactivated by a 5 minute incubation at 95ºC. McrBC digestions were performed for each of the individual progeny from both experimental groups. Forty ng of digested template were PCR amplified with 1U of ZymoTaq DNA Polymerase (Zymo Research) in the final volume of 25 µl under the following PCR conditions: 10 minutes initial denaturation at 95ºC, followed by 28 cycles of 30 seconds at 94ºC, 30 seconds annealing at 55ºC, and 30 seconds of extension at 70ºC. Two ng of undigested DNA was amplified in parallel under the same conditions. Primer sequences were as described by Martens et al. (2005).

The resulting PCR product was resolved on 2% agarose gel, stained with ethidium bromide, and photographed. The band intensity was measured by ImageJ software (NIH) and the methylation status was calculated as the ratio of digested to undigested band intensity for each of the samples. This experiment was repeated to verify the results and, statistical significance was calculated using a Student's t-test.

5.3.8. Bisulfite sequencing

Genomic DNA aliquot (500 ng) was bisulfite converted using an Epitech bisulfite conversion kit (QIAGEN) following the manufacturer's instructions. Bisulfite PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems) or ZymoTaq DNA Polymerase (Zymo Research). Bisulfite PCR primers were designed using Methprimer software (http://www.urogene.org/methprimer), making sure that at least one of the primers could bind to unique sequences flanking the repetitive region of interest. The primer sequences and the corresponding regions of interest (UCSC, mm9) were as follows: SI001, RMER17B, chr7:120071640-120072576 F-attgtgttttagttatggattagat, R-

ccaataattacaaattctcaatct; SI005, ID_B1, chr18:35903136-35903272: F-gttttagaaatttaaaataaatgaa, R-atttctaacaaaataccaaaaaaaa; B1_Mm, chr14:114469751-114469831, F-agtttgggaaaggatttttattatt, R-tttaatctctatacccctctatccc; SI025,B1_Mur3, chr11:101,472,302-101,472,499, F-ggtagagttttaatggaagggagtagt, R-aaccacaatatcataaacataccaaca; SI043, RLTR9B2, chr16:12,754,087-12,754,284: F-gttttattttaggtggttttatatt, R-acaaactacaaatattct ccttaa; SI049, B1_Mur, chr4:155091403-155091632, F-tttttgttttatttttttggttgttg, R-ctcccaattccccaaattacc. For each primer pair, the annealing temperature was determined experimentally. All primer pairs were tested with un-converted genomic DNA to ensure absence of amplification.

An amplified product of appropriate size was excised from the gel and extracted with QIAquik Gel Extraction Kit (Qiagen). The PCR product was cloned into a pGEM-T vector system (Promega). Around twenty clones, representing each sample, were isolated and sequenced using an SP6 sequencing primer. The resulting sequences were analyzed using a BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de) with quality thresholds set at default levels (at least 90% sequence identity and 80% conversion rate). Methylation levels were calculated as a percent of unconverted CpGs. Graphic diagrams were generated using BiQ Analyzer Diagrams, available at http://biq-analyzer.bioinf.mpi-inf.mpg.de/tools/MethylationDiagrams/index.php.

5.4. RESULTS AND DISCUSSION

5.4.1. Mapping and general composition of the libraries

Small RNA Illumina sequencing was performed to compare small RNA transcriptomes in the male germ line of mice exposed to direct and paternal doses of X-ray. This experiment generated 4 sequencing libraries: CT – sham treated; IR – whole body exposure to 2.5 Gy X-ray; PROG CT – progeny of sham treated; and PROG IR – progeny of IR exposed mice. The sequencing reads that passed initial quality control were adapter trimmed and mapped to the mouse reference genome (UCSC genome browser assembly mm9); only perfect matches and 3'trimmed sequences with at least 18 nucleotides of perfect match were accepted for mapping. The number of perfect matches was in the range of hundreds in direct exposure libraries, while it was in the range of hundreds of thousands for paternal exposure (Table 5.2). The vast majority of sequencing reads from direct exposure libraries were 3'trimmed at the mapping stage, resulting in shorter reads and, consequently, a shift in sequence length distribution (Figure 5.2. A, B, C). Direct exposure and progeny libraries were constructed and sequenced by Fasteris at different times and substantial differences in the fraction of perfectly mapping reads between them were noted. Therefore, we only conducted comparisons of CT/IR and PROG CT/PROG IR, since they were processed under the same experimental conditions.

Mapped reads in all 4 libraries matched a broad spectrum of sequences including repeats, known and novel miRNAs, transport and ribosomal RNAs, small interfering RNAs (siRNA), small nuclear (snRNA), small nucleolar (snoRNA), and small cytoplasmic RNA (scRNA), among others (Figures 5.1 A and B). Overall, read distribution was very similar across all of the libraries. More than half (57-62%) of the total small RNA pool was occupied by repeat associated small RNAs, a second most

abundant category was non-hairpin RNAs, comprising close to 30% of all reads. Reads that were mapped to known miRNAs occupied a smaller portion of sequencing libraries, at 3.4 – 8%. Sequencing reads that mapped to un-annotated hairpins and sense RNAs accounted for close to 3% each, regardless of the library. The rest of genomic sequence categories had much lower representation, at less than 1% of the reads mapped to the genome (Figure 5.1 B).

Length distribution analysis of sequencing reads that mapped to the more prominent genomic sequence categories showed that the majority of repeat associated small RNAs fell between 27 and 30 nucleotides, corresponding to the length of (pre-/pachytene piRNAs (Girard, Sachidanandam et al. 2006) (Figure 5.2. A, B, C). Since, in our experiment, small RNAs were not directly co-precipitated with PIWI proteins, we can only speculate about their biogenesis and support our conclusions by indirect evidence. We will refer to this class of small RNAs as piwi-like RNAs henceforth.

Similar piwi-like length distribution was observed in the case of sequence classes that mapped to un-annotated regions of the genome with no hairpin folding – i.e., non-hairpin RNA – and those that mapped to sense transcripts (Figure 5.2. C and E). The majority of piRNAs with these features is expressed during the pachytene and map to non-repetitive loci (Aravin, Sachidanandam et al, 2007). Length distribution analysis of sequence reads that map to known miRNAs yielded an expected Dicer dependent peak between 21 and 23 in all of the libraries. The frequency of non-hairpin miRNAs peaked at 26-30 nucleotides, which served as evidence of a non-Dicer dependent biogenesis characteristic of longer than normal intronic miRNAs, processed by splicing from mammalian introns (Berezikov, Chung et al. 2007). A pattern of random degradation

was observed for ribosomal RNAs (Figure 5.2. F), transport and small cytoplasmic RNAs (data not shown).

Piwi-like small RNAs from adult mouse testes libraries were reported to have bimodal length distribution, with the major peak at 30-31 nt and a smaller shoulder at 26-27 nt (Aravin, Gaidatzis et al. 2006). These features were clearly visible in the case of our progeny libraries; however, in direct exposure libraries we observed a shift towards shorter lengths, possibly due to 3' trimming prior to mapping (Figure 2). PiRNas within the range of 26-27 nt were primarily associated with MILI in adult murine testes (Aravin, Gaidatzis et al. 2006), while those in the range of 29-31 nt were co-precipitated with Miwi (Grivna, Pyhtila et al. 2006) and Msy2 (Xu, Medvedev et al. 2009). Since our libraries were derived from the testes of fertile adults, the majority of repeat-derived sequences in the range of 29-31 nt must have likely originated from spermatogenic cells in post-leptotene stage, as only 26-28 nt small RNAs were previously detected at the preceding stages of spermatogenesis (Aravin, Gaidatzis et al. 2006).

In contrast to previous sequencing efforts (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Pyhtila et al. 2006; Watanabe, Takeda et al. 2006) we report that the majority of non-coding small RNAs within the piRNA range in adult testes mapped to repeats. Earlier sequencing efforts reported that only 17% of sequences in piRNA length range mapped to repeats in adult testes (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006); similar results were obtained by Grivna et al (2006). Here, we report a comprehensive profile of small noncoding RNA transcriptomes in adult mouse testes, based on 4 Illumina sequencing libraries with close to a total of 14 million mapped reads; repeat derived reads consistently dominated, with close to 60% occupancy

in each of the libraries, while length distribution analysis showed a major peak at 29-31 nt, corresponding to piRNAs and possibly with preferential binding to Miwi (Grivna, Pyhtila et al. 2006). This discrepancy may be attributed to relatively low coverage of previous libraries: 52000 (Girard, Sachidanandam et al. 2006), 15000 (Aravin, Gaidatzis et al. 2006), close to 1000 (Watanabe, Takeda et al. 2006) and only 40 sequences (Grivna, Pyhtila et al. 2006), or alternatively, to differences in the cloning protocols, leading to capture bias (Linsen, de Wit et al. 2009).

Piwi-like RNAs mapping to intergenic and sense transcript sequences were also detected at high levels, albeit much lower than in case of repeats frequency (Figure 5.1 A). Chromosomal distribution analysis that excluded miRNA reads showed that the small RNA reads mapped across the entire length of the chromosome, including telomeric and centromeric regions. For most of the loci, the number of mapped reads was lower than 10; however, a few hot spots were noted that had a number of reads exceeding 1000 (data not shown). Therefore, a substantial fraction of the mouse genome is capable of generation of small non-coding RNA in the male germ line, albeit at a low level. At this point, inferring a functional significance of repeat-derived small RNA in adult testes is difficult, but their high abundance and the fact that they map to hundreds of thousands of loci across all of the chromosomes points to their functional importance in the process of spermatogenesis. Most of repeat associated small RNAs are 29-30 nt in length, which serves as an indication that they are expressed in association with Miwi during the pachytene stage of meiosis and until the stage of round spermatids (Grivna, Pyhtila et al. 2006).

A recent study showed that transient relaxation of transposable elements (TEs),

paralleled by a decrease in cytosine methylation, occurs at the onset of meiosis (van der Heijden and Bortvin 2009), while suppression of meiotically relaxed TEs started at mid-pachynema, thereby coinciding with expression of an abundant fraction of 29-30 nt piRNAs (Soper, van der Heijden et al. 2008). Expression of pachytene piRNA was shown to depend on the nuage component Maelstrom (Mael), as knockout of Mael caused dramatic up-regulation of TE transcripts, arrest of meiosis, and resultant male sterility (Soper, van der Heijden et al. 2008). The abundant 29-30 nt piRNA population also disappeared in Mael -/- (Soper, van der Heijden et al. 2008). A phenotype identical to Mael-/- was also induced by deletion of a putative DEAD-box helicase, MOV10-like-1(Mov10l) (Frost, Hamra et al. 2010). Deletion of these proteins did not affect pre-pachytene stages of spermatogenesis, showing that piRNA-mediated silencing of TEs operates primarily in pachytene spermatocytes (Frost, Hamra et al. 2010).

5.4.2. Impact of irradiation on the overall small RNA profile in the male germ line

Both direct and pre-conceptual irradiations had an impact on the general composition of the libraries. In the case of direct exposure, we observed a slight decrease from 62 to 58% for the repeat-derived piwi-like RNA pool in irradiated testes. A similar decrease was observed in the germ line of paternally exposed progeny (Figure 1, A). In contrast, the relative abundance of non-hairpin and sense-piwi-like RNAs remained un-changed in direct and progeny libraries (Figure 5.1. A). Examination of length distribution curves showed that repeat-derived RNA decline was mainly attributed to a decrease within the high length spectrum of piwi-like RNAs (29-31 nt) expressed in meiotic spermatogenic cells (Aravin, Gaidatzis et al. 2006) and possibly bound to Miwi protein (Grivna, Pyhtila et al. 2006). No change was noticed in case of reads that mapped

to non-hairpin- and sense RNAs (Figure 5.2). However, length distribution analysis showed a similar decline in the long range piwi-like RNAs in non-hairpin- and sense RNA categories in direct exposure libraries, but this was seemingly compensated by the increase in short range piwi-like RNAs. More strikingly, this simple compositional analysis showed an almost two-fold increase in miRNA pools after either direct or pre-conceptual irradiations. The frequency of reads that mapped to other sequence categories remained un-changed, apart from substantial increase in rRNA and tRNA mapped small RNAs in IR library.

Currently, nothing is known about the possible influences of adverse environmental factors on germ line specific small RNAs. Here, we show that ionizing radiation has the potential to change the small RNAome in testes and that this effect could still be detected in the adult male germ line of an F1 generation derived from fertilization with irradiated epididymal spermatozoa. Therefore, ionizing radiation affects germ line small RNAs in a transgenerational manner. On the level of general composition, both direct and transgenerational effects were expressed as a subtle decline in repeat derived piwi-like RNAs and almost a doubling of the reads mapping to known miRNAs.

A number of studies showed that piRNAs are detected only in the germ line and that they are involved in the silencing of transposable elements in Drosophila, mice, and zebrafish (Klattenhoff and Theurkauf 2008). Mutations of proteins involved in piRNA production in Drosophila lead to dramatic up-regulation of transposable elements, accumulation of double-strand breaks, and failure of meiosis (Siomi, Miyoshi et al. 2010), while mutations of Piwi proteins in mice cause sterility and failure of

spermatogenesis (Carmell, Girard et al. 2007). Among the known Piwi proteins, only Mili and Miwi are expressed in adult testes (Aravin, Gaidatzis et al. 2006; Grivna, Pyhtila et al. 2006); the third piwi protein Miwi2 is detected only in fetal testes and up to 4 days post partum (Aravin, Sachidanandam et al. 2008). Mutations in Mili in Miwi2 proteins lead to derepression and DNA hypomethylation of retro-transposable elements in fetal testes (Carmell, Girard et al. 2007; Aravin, Sachidanandam et al. 2008; Kuramochi-Miyagawa, Watanabe et al. 2008). In addition, male germ line Miwi2 knockouts exhibit increased levels of double-strand breaks (Carmell, Girard et al. 2007).

Recent work showed that control over the activity of transposable elements in the Drosophila germ line is achieved by heterochromatin formation directed in a sequence-specific manner by piRNAs (Todeschini, Teysset et al. 2010). Unlike those in Drosophila, the mammalian TEs require both methylation of cytosine in DNA and heterochromatin formation for silencing (Bernstein and Allis 2005; Cheung and Lau 2005). It is still unclear how transposon sequences are specifically recognized to receive these types of modifications. It is extremely tempting to hypothesize that similar mechanisms operate in the Drosophila and mouse germ line, and that small RNAs act in a sequence-specific manner to attract chromatin modification machinery to the developing germ cells undergoing rapid epigenetic re-modeling (Aravin, Hannon et al. 2007; Kuramochi-Miyagawa, Watanabe et al. 2008).

5.4.3. Detailed analysis of repeat derived piwi-like RNAs

The overwhelming majority of small RNA pool in adult testes was derived from repeats (Figure 1. A and B). Since both direct and paternal X-Ray irradiations induced a decrease in the cloning frequency of repeat derived piwi-like RNAs, we decided to

proceed with a detailed analysis of repeat associated sequencing reads.

Repeat derived RNAs recovered from our dataset revealed a complex picture, wherein small non-coding RNAs map to diverse classes of mobile elements including retroviral-like elements, DNA transposons, and simple and low complexity repeats. All of these were derived from 314 repeats, which accounted for around one third of all repeat elements shared ancestrally or specific to the genus *Mus* (Jurka 2000). The vast majority of small RNA reads mapped to long tandem repeat (LTR) retrotransposons (8%), long interspersed nucleotide elements (LINEs) (5%), and short interspersed nucleotide elements (SINEs). Other classes of repeat elements were present at lower frequencies: simple repeats occupied 2%, DNA transposons1.3% and low complexity repeats 0.6% from total reads mapped. The discovery of piwi-like repeat derived RNAs associated with simple and low complexity repeats was a novel finding.

The relative abundance of repeat derived small RNAs showed no direct dependence on copy number of the corresponding class of repeat element in the genome. For instance, simple and low complexity repeats with more than $1.38*10^6$ copies in the genome generated a number of small RNA reads comparable to those of DNA transposons with approximately 155.000 (Figure 5.3.). Small RNA drived from SINEs appeared to be under-represented in comparison to LINEs, LTRs, and DNA transposons (Figure 5.3.).

Direct exposure to X-rays led to a subtle down-regulation of repeat derived small RNAs that mapped to all of the repeat classes, with the exception of low complexity repeats (Figure 5.4. A). Analysis of repeat small RNA pools in the progeny of irradiated mice, in comparison to those of the progeny of control mice, revealed a more complex

picture, wherein most of the repeat classes showed no change between treatment groups, with the exception of the down-regulation of piwi-like RNAs derived from SINEs and low complexity repeats and of the up-regulation of small RNA reads that mapped to LINEs (Figure 5.4. B). A slight decline in the overall pool of repeat derived small RNAs in the progeny of irradiated mice represented a net effect of these processes.

In order to increase the resolution of the analysis of radiation changes in the repeat derived small RNAs, we compared read frequencies at the level of individual repeat elements. Following direct exposure, we observed a bidirectional effect on the expression of repeat derived small RNAs, with about two thirds of the detected repeats displaying a trend towards down-regulation. The list of individual repeat elements that showed more than a 2-fold change in the number of corresponding reads, depending on direct or paternal exposure, can be found in tables 4 and 5, respectively.

The differences in piwi-like RNA expression on the level of individual repeats were more pronounced in direct exposure libraries. Overall, 40 repeat elements showed more than 2-fold changes, 21 were up- and 19 down-regulated. The extent of change was higher in the case of down-regulated repeats; these also had a higher number of reads mapped, with 5 highly expressed elements exceeding 1000 RPM (Table 5.4.). Small RNA changes detected in the unexposed progeny were milder in comparison, as only 24 repeat elements showed more than 2-fold difference; most of these (16) were up-regulated. With the exception of MER110 (LTR), Murvy-int (LTR), and $(TTCG)_n$ (simple repeat) that were found in both lists, no overlap occurred.

Differentially expressed repeat derived piwi-like RNAs covered a wide spectrum of known repeat sequences in the genome, including LTRs, LINEs, DNA transposons,

and simple and low complexity repeats. The only commonality we were able to determine between these was a significantly higher strand bias found among altered repeat derived RNAs as compared to all repeats detected in, both direct and paternal exposure libraries: a) strand bias in the case of repeats that were altered after direct exposure was 5.87±2.83 as compared to 3.78±3.00 in all of the repeats detected, $P<10*10^{5}$; b) strand bias for repeats that were changed after paternal exposure was 5.1±2.93 as compared to 3.6±2.9 in all of the detected repeats, P=0.013 (unpaired, 2-tailed t-test). The strand bias was calculated as the absolute of $\log_2$ ratio of reads that mapped to opposite strands for the specific repeat element. This indicates that repeats affected by radiation tended to be expressed from single-stranded precursors.

Although the net effects of direct and paternal radiation exposure on piwi-like RNAs in testes was expressed as a decrease in the population of repeat derived sequences, the detailed analysis revealed a more complex picture. Repeat derived RNAs that mapped to specific repeat elements could be both up- or down-regulated in response to direct or paternal exposure. Changes induced by direct exposure were, in general, more pronounced, but they were still detectable in the germ line of un-exposed progeny. Radiation responsive piwi-like RNAs mapped to diverse repeat sequence classes and the only detectable common feature was the high strand bias.

The mechanism by which IR exposure can induce changes in piwi-like RNA expression is unknown. The simplest explanation of piRNA population changes after direct irradiation involves radiation-induced cell killing. The spermatogonial cell type is, in general, considered to be the most radiation-sensitive spermatogenic cell, as a massive depletion of the spermatogonia A population is observed 4 days after irradiation and its

levels only start to return to normal at day 40 (Oakberg and Clark 1961; Cordelli, Fresegna et al. 2003). A decline in subsequent stages is detectable later in time and depends mainly on depletion of type A spermatogonia (Hasegawa, Wilson et al. 1997). Apoptosis was rarely detected in spermatocytes, the spermatogenic cells that serve as the main contributors of abundant piRNA expression in adult testes (Hasegawa, Wilson et al. 1997). The results of our experiment suggested that IR exposure causes diminished expression of piwi-like RNAs derived from repeats, while piRNAs derived from sense transcripts and intergenic regions remained unaffected. This suggests that the effect observed here was not a passive consequence of cell killing, but rather was the result of IR influence on the piRNA processing machinery.

Recent findings from our laboratory suggested that the expression of Mili and Mael were reproducibly down-regulated at the same experimental conditions, while the expression of Miwi appeared to increase (Matt Merifield, personal communication). Mael maybe the main target for this decrease, since processing of the abundant fraction of the 28-30 nt piRNAs disappeared in Mael mutants. In addition, changes in the piwi-like RNA transcriptome were still detected in the germ line of F1 progeny conceived by IR damaged spermatozoa, suggesting that aberrant cellular signaling induced by irradiated sperm persists across multiple cell divisions and can survive epigenetic reprogramming in primordial germ cells (Hajkova, Erhardt et al. 2002)

5.4.4. Radiation-induced changes of the microRNA in the male germ line after direct and paternal exposure

We applied Illumina sequencing to create comprehensive profiles of small RNA transcriptomes in the mouse male germ line treated with an acute dose of 2.5 Gy of X-

rays and in the male germ line of F1 progeny paternally irradiated at the spermatozoa stage and we compared these to matched controls. Our experiment identified a substantial fraction of small RNA reads that mapped to known miRNA loci. The relative frequency of reads mapping to known miRNA loci increased from 3.4% in CT to 6.46% in the IR library. A similar increase from 4.6 to 8.3% was observed as a result of paternal irradiation (Figure 5.1, A). As expected, the miRNA fraction in testes was much lower than the fraction of piwi-like RNAs. The length distribution analysis demonstrated an expected peak of 18-23 nucleotides, compatible with DICER-dependent processing.

The precise genomic mapping and calculation of relative read frequencies allowed us to perform global profiling of miRNA expression in normal mouse testes and to determine the exact nature of miRNA transcriptome changes after direct and paternal radiation exposures. We obtained reads that mapped to 360 out of 672 reads annotated in the miRBase (Griffiths-Jones, Saini et al. 2008); inclusion of candidate novel and homologous miRNAs resulted in 513 miRNAs detected in the control mouse germ line. In most cases, we obtained reads from both the 5' and 3' arms of the hairpin precursor and, in our analysis, these were treated as separate entities. Although the arms with the lowest read frequency are likely to represent the passenger strands bound for degradation, these can still down-regulate protein targets and their expression was shown to depend on tissue and developmental stage (Ro, Park et al. 2007; Okamura, Phillips et al. 2008).

Known and novel miRNAs detected in the CT library were classified according to their scaled cloning frequency as those with high (>1%), moderate (0.1-1%) and low (<0.1% from total miRNA pool) expression levels. Only 17 out of 513 (3.3%) of the detected miRNAs were highly expressed in control testes; 43 (8.4%) were found in the

moderate category and the largest list – 454 (88.5%) was composed of those with low expression (Figure 5.6. B). Candidate novel miRNAs were found only among the low expression category. The list of miRNAs with high expression in the male germ line was enriched in members of let-7 family (let-7g, let-7f, let-7c, let-7a, let-7b, let-7i, i.e. 6 out of 13). Let-7g and let-7f were 2 miRNAs with the highest frequency of sequencing reads that occupied 31% of the CT library. Highly expressed miRNAs that did not belong to the let-7 family included mmu-184, mir-34c, mir-871, mir-470, mir-423, mir-140, mir-375, mir-741 and mir-103. These data were in good agreement with previous low scale sequencing efforts. High expression of the let-7 family miRNAs, mir-34c, mir-143, and mir-741 was previously detected in testes by Landgraf and colleagues (2007); mir-184 was detected as highly expressed in testes by Takada et al (2006). However, the rest of the miRNAs detected at high frequency in our control library, such as mir-871, mir-470, mir-423, mir-375, mir-103 were previously detected only at intermediate frequencies by Landgraf et al (2007).

Next, we compared relative cloning frequencies of miRNA between the CT and IR libraries. Fold changes were calculated as $\log_2$(IR/CT) for each of the known and candidate miRNAs. The data for miRNAs with low expression were excluded from this analysis as un-reliable. Considering the absence of replicas, we applied a strict log2 ratio with a minimum “3” as the criterion to consider miRNA as differentially expressed. In addition, we filtered out all of the miRNAs with less than 100 RPM in both libraries in order to create a focused list of differentially expressed miRNAs with relatively high transcript levels (Table 5.5. A). The vast majority (12 out of 15) of miRNAs altered after direct irradiation were up-regulated, while only 3 miRNA species were down-regulated.

Most of the miRNAs found in the list showed a high degree of conservation.

A literature search revealed that many of these miRNAs have experimentally indentified protein targets and cellular functions. The common functional theme was promotion of cell proliferation and inhibition of apoptosis; not surprisingly, many were found in cancers, including hematopoietic malignancies, commonly induced by radiation. For instance, miR-93 was shown to be up-regulated in T-cell leukemia, it was shown to target tumor protein 53-induced protein 1 (TP53INP1) and cyclin dependent kinase inhibitor 1(p21/Waf1) (Yeung, Yasunaga et al. 2008; Kim, Yu et al. 2009). Ectopic expression of this miRNA facilitated G1 to S phase progression (Kim, Yu et al. 2009).

MiR-30 was shown to down-regulate pro-apoptotic protein Bcl6 and to promote lymphomagenesis (Lin, Lwin et al. 2010). In addition, miR-30 suppressed apoptotic responses by targeting p53 and its downstream effector Drp1 (Li, Donath et al. 2010). Another miRNA heavily involved in tumorigenesis was miR-181b; it was shown to promote cellular proliferation and to inhibit apoptosis, at least in part by down-regulating a Pten oncosupressor (Iliopoulos, Jaeger et al. 2010). MiR-222 was shown to promote cell survival by targeting cyclin dependent kinase inhibitor 1b (Ckn1b) and pro-apoptotic transcription factor Puma (Zhang, Zhang et al. 2010; Zhang, Zhang et al. 2010). MiR-7 was previously detected as down-regulated in irradiated thymus (Ilnytskyy, Zemp et al. 2008); in contrast, in this study, it appears to be up-regulated in testes. Up-regulation of miR-7 can suppress Lsh, a SWI/SNF family helicase-like protein capable of targeting methylation machinery to repeat elements (Ilnytskyy, Zemp et al. 2008). Overall, miRNA expression changes in directly irradiated testes appear to be favorable for cell survival and for inhibition of apoptosis, at least the at 96 hr time-point after treatment. A number

of miRNAs (miR-34a/b; miR-93; miR-7; miR-181b) detected in this study as changed under the influence of radiation were previously shown to be radio-responsive (Ilnytskyy, Zemp et al. 2008). Interestingly, a MicroCosm search (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) revealed that Mael is one of the putative targets for miR-93, while Mov10l is targeted by miR-34a/c and miR-181b. Thus, a putative connection is apparent between up-regulated miRNAs and diminished piwi like RNA pool in irradiated testes.

The highest fold change in miRNA cloning frequency between IR and the CT library was detected in case of miR-143* (Table 6, A), which increased from 1 RPM in CT to 158 RPM in IR, while miR-143 was unchanged. This miRNA is processed from the passenger strand miR-143 hairpin and normally is bound for degradation; thus, this finding is novel and serves as evidence that a stress response can alter not only level of mature RNA, but also specifically affect degradation of the passenger strand. Passenger miRNAs were shown to have their own targets and their expression can depend on tissue and developmental stage (Ro, Park et al. 2007; Okamura, Phillips et al. 2008).

The anti-apoptotic theme was counterbalanced by 2 members of miR-34 family present in the list; both were shown to be directly induced by p53 tumor suppressor transcription factor (Corney, Flesken-Nikitin et al. 2007) and are capable of down-regulating the cellular proliferation driver – Notch1 (Ji, Hao et al. 2009).

A dramatic differential expression of miRNA was detected not only after direct, but also after paternal irradiation. We applied the same strict criteria in order to compile a list of miRNAs altered in the mouse germ line after paternal irradiation, focusing on sequences with high cloning frequency in at least one of the libraries (more than 100

RPM) and a fold change with corresponding log2 ratio higher than “3”. We assembled a list of 29 highly up-regulated miRNAs; none was down-regulated under given selection criteria. All except miR-741 were conserved in at least 2 species; miR-741 appears to be rodent-specific. Highly conserved let-7 family miRNAs were enriched, with 6 out of 12 members appearing in this list. A literature search revealed that a number of differentially expressed miRNAs have defined protein targets and cellular functions.

The most dramatic increase was the case of miR-29b, which changed from 5 RPM in progeny of controls to 905 RPM in the progeny of irradiated mice (Table 6, B). Confirmed targets of miR-29b include the de novo methyltranferases DNMT3a/b, which possibly prevent aberrant de novo methylation (Fabbri, Garzon et al. 2007; Takada, Berezikov et al. 2009). Mir-29b was previously induced by radiation in mouse testes (Tamminga, Kathiria et al. 2008; Filkowski, Ilnytskyy et al. 2010). In addition, it was shown to have tumor-suppressive and pro-apoptotic activity (Garzon, Heaphy et al. 2009; Park, Lee et al. 2009; Urdinguio, Fernandez et al. 2010). All members of miR-15 family (miR-15a/b and miR-16-1/2) appear on the list (Table 6, B); they were known to be expressed from two different clusters (mirbase.org), which implied similarity of regulation. The MiR-15 family is frequently down-regulated in hematopoietic malignancies and exhibits strong pro-apoptotic activity that is mediated by direct suppression of Bcl2 protein (Cimmino, Calin et al. 2005). Clustered miR-143 and miR-145 were highly up-regulated in the germ line of PPI mice (Table 6, B); both are known tumor suppressors, inducers of apoptosis, and are consistently suppressed in various cancers (Wang and Lee 2009). Another miRNA with described cellular function was miR-22 – a tumor suppressor and an antagonist of cMyc oncogene, which acts as

negative regulator of cell migration and metastatic invasion (Xiong, Du et al. 2010). Only miRNAs with well described anti-apoptotic activity were the members of miR-30 family – miR-30c and miR-30b – that were shown to suppress p53 and the down-stream initiator of mitochondrial fission Drp1 (Li, Donath et al. 2010). MiR-30c was the only miRNA detected in as being up-regulated after both direct and pre-conceptual irradiation (Table 5.6. A, B).

Six out of 12 members of the highly conserved let-7 family were detected among those up-regulated by pre-conceptual irradiation; the let-7 family is involved in the regulation of cell cycle effectors including Ras, cMyc, Hmga2, and Cdc25a (Johnson, Grosshans et al. 2005; Johnson, Esquela-Kerscher et al. 2007; Rahman, Qian et al. 2009; Viswanathan, Powers et al. 2009). The let-7 family suppresses factors responsible for self-renewal and pluripotency and is down-regulated in a variety of cancers (Boyerinas, Park et al. 2010; He, Chen et al. 2010). The listed miRNAs with poorly defined targets and functions included miR-32, miR-871, miR-136, miR-425, miR-741, miR-322, and miR-24 (Table 6, B).

Overall, miRNAs altered in the germ line of F1 progeny of irradiated males appear to be involved in positive regulation of apoptosis, growth arrest, and suppression of tumorigenesis and therefore may represent a protective mechanism in response to paternal exposure.

5.4.5. Aberrant methylation of repeat elements in the germ line of F1 progeny of irradiated mice

Male mice exposed to 2.5 Gy of X-Rays were mated to control females in order to obtain PPI progeny. Mating was performed 7 days, and 3 and 6 weeks after exposure to

ensure fertilization by spermatozoa irradiated at epididymal spermatozoa, spermatid, and spermatogonial stages, respectively. Spermatid stage offspring were interbred in order to produce an F2 generation. Progeny of concurrent controls was available for each of the treatment groups.

Testes were sampled only at the spermatozoal stage of irradiation and were used to design small RNA deep sequencing libraries for F1 PPI mice.

Since only SINEs and LINEs were previously shown to change under the influence of direct and paternal irradiation (Filkowski, Ilnytskyy et al. 2010) and their repeat associated RNAs were altered simultaneously after direct and pre-conceptual irradiation (Figure 5.4. A and B), we first focused on DNA methylation analysis of these repeat elements in the germ line of F1 progeny. The goal was to establish a possible association between PPI-induced dysregulation of repeat derived piwi-like RNAs and DNA methylation at corresponding repeats. In order to measure DNA methylation levels at these repeat elements, we used McrBC digestion, followed by semi-quantitative PCR (see materials and methods for details). A statistically significant decrease in methylation level was detected in the case of SINE B1 ($p<0.05$, 2-tailed un-paired t-test), but not for LINE ORF2 (Figure 5.8. A, B).

Next, we used genomic bisulfite sequencing in order to confirm the observed methylation change and to explore it in more detail. Four individual repeat elements were selected; primers were designed to the flanking sequences to ensure amplification from the particular region rather than from the range of loci. Bisulfite sequencing was performed using DNA pools from the progeny of irradiated mice and compared to the progeny of controls. Ten to 20 clones were analyzed in each case. The difference in CpG

content approached statistical significance (p=0.08, Fisher exact test) only in case of B3A, SINE (chr11: 101,472,302-101,472,499). The methylation level in this region decreased from 40% in the progeny of controls to 25% in the progeny of exposed mice (Figure 5.7. 3). The rest of the regions analyzed were heavily methylated (83-95%) and unchanged (Figure 5.7. 1, 2, 4). Detailed examination of small RNA reads, which were mapped using the UCSC genomic browser custom tracks assembled with sequencing data from the progeny libraries, revealed that piwi-like repeat derived small RNAs map to the exact sequenced locations; however, the read counts were un-changed at these specific loci (data not shown) and all of the small RNA reads were in the sense orientation. In addition, we noticed that heavily methylated SINEs (Figure 5.7. 1, 2, 4) were located in intergenic regions; in contrast, moderately methylated SINEs, altered in PPI mice, were located in the second exon of a long coding RNA transcript (GenBank accession BC048585) – the mouse specific non-coding RNA was expressed at high levels in the embryo, ovary, and testes (USCS, expression data).

Hypomethylation of LINEs and SINEs was previously detected in our previous work on thymus tissue of 6-month-old male mice subjected to paternal irradiation at the spermatozoal stage (Filkowski, Ilnytskyy et al. 2010). Here, we report a significant decrease in DNA methylation at the SINE B1 repeat element, but not at LINE1. This discrepancy with the previous work may be explained by the use of semi-quantitative instead of real time PCR, the different tissue, and the ages of the animals. Bisulfite sequencing analysis of 4 individual SINEs failed to confirm the results of the McrBC-PCR assay. Bisulfite sequenced SINEs appeared to be heavily methylated, with the exception of B3A SINE (Figure 5.7, 3) which had overall lower methylation and was

localized to the exon of non-coding RNA transcript expressed in the germ line. Coincidentally, this was the only repeat showing methylation change, albeit not reaching significance. We suggest that DNA hypomethylation at SINEs in the progeny of exposed fathers occurs at specific loci, rather than sporadically across multiple regions. Repeat elements with overall lower methylation may be a target for this effect. We also noted a substantial increase in miR-29b in testes tissue of PPI mice. MiR-29b was shown to target de novo Dnmts and perhaps caused down-stream changes in the methylation level of this repeat (Fabbri, Garzon et al. 2007; Takada, Berezikov et al. 2009).

Previously, cooperation Dnmt3a/b was shown to be required for the maintenance of DNA methylation at repeat elements (Liang, Chan et al. 2002). Down-regulation of de novo Dnmts leads to the loss of methylation at SINE B1, but not at LINE1, IAPs, or minor satellites in spermatogonial stem cells (Takashima, Takehashi et al. 2009). Perhaps the observed SINE B1 hypomethylation is mediated by increased expression of miR-29b, while the same increase would not affect LINE1 elements (Takashima, Takehashi et al. 2009).

Induction of these epigenetic changes at the spermatozoal stage of irradiation indicated that these changes could not be established in a paternal germ line due to absence of noticeable metabolic activity in mature spermatozoa (Lalancette, Miller et al. 2008). Therefore, they had to be induced upon fertilization with spermatozoa carrying unrepaired DNA damage, which was then maintained and propagated in adult tissue.

To date, few studies have shown that fertilization with mature spermatozoa subjected to genotoxic agent leads to genomic instability, altered intracellular signaling in the progeny, and changes in chromatin packaging (Shimura, Inoue et al. 2002; Barton,

Robaire et al. 2005; Hales, Barton et al. 2005; Tateno, Kusakabe et al. 2010). Recent work showed that F1 offspring derived from neutron irradiated spermatozoa produce abnormal sperm, have increased embryonic lethality in F2, and tended to develop liver tumors (Watanabe, Toyoshima et al. 2010)

5.4.6. Aberrant methylation of repeat elements in somatic tissues of the progeny of irradiated fathers

Next, we tested whether pre-conceptual irradiation can induce aberrant DNA methylation in somatic tissue of the progeny. Samples of bone marrow and spleen were available for all stages of spermatogenesis that received radiation treatment; i.e., the spermatozoal (7 days), spermatid (3 weeks), and spermatogonial stages (6 weeks).

We first compared DNA methylation levels of SINE B1 and LINE1 ORF2 in the spleen tissue of the progeny of mice that were paternally irradiated at spermatogonial stage (Figure 5.8. A, B). The results were consistent with those obtained for the germ line, with statistically significant decreases in methylation levels noted in case of SINE B1, but not LINE repeat elements.

We then used bisulfite sequencing to analyze DNA methylation at selected repeats. The selection of repeat elements for this analysis was based on the number of repeat derived small RNAs mapping to them directly or within their immediate vicinity, and possibly guiding DNA methylation between spermatogonial and round spermatid stages, when piRNAs are known to be expressed. In order to define these types of regions, the mouse reference genome was divided into 5 kb bins and the number of sequencing reads was counted within each bin for direct irradiation and control libraries.

Bins with more than 2-fold differences in small RNA counts were considered significantly changed. Overall, 415 5kb intervals in the genome had more than 2-fold differences in repeat derived small RNA mapping.

Next, bins were visually examined using the UCSC genomic browser. In many cases, it was possible to define the exact repeat element that contributed to the altered read counts; this way, we were able to narrow down piwi-like small RNA expression changes to a specific locus, feasible for bisulfite sequencing. Methylation analysis was performed for 6 such repeat elements in a pooled DNA sample from each of the PPI groups. The F2 was obtained by interbreeding animals from the spermatid stage of irradiation and comparing them to the progeny of controls (Figure 5.9.). Statistically significant changes were observed in 3 out 6 repeats tested. A particularly interesting difference was seen in case of 2 LTR, ERVK family retroviral elements that showed significant DNA hypermethylation at spermatid and spermatogonial PPIs. In the case of RLTR9B2, this change was transmitted to the F2 generation (Figure 5.9.).

Bisulfite sequencing of select repeat elements showed that paternal exposure can induce aberrant DNA methylation in the somatic tissue of the progeny and that these effects may stem from the radiation-induced changes at the level of repeat derived piwi-like RNAs. Hypermethylation of both LTR elements tested was detected only at spermatid and spermatogonial stages of irradiation, within the timeframe of active piRNA expression. It is tempting to suggest that piRNAs are capable of directing DNA methylation and chromatin modifications to the genome. Changes in the levels of specific piRNAs induced by environmental influences could therefore lead to locus-specific chromatin remodeling retained in mature sperm and transferred to developing embryo.

Should such events survive epigenetic reprogramming in pre-implantation embryo (Oswald, Engemann et al. 2000; Lane, Dean et al. 2003), they could be propagated during embryonic development and retained in adult tissue. Interestingly, both of the altered LTRs belonged to the ERVK family, recently shown to be resistant to DNA methylation reprogramming (Popp, Dean et al. 2010). Although both of the changed hypermethylated repeats were intergenic, the possibility exists that these types of events are quite frequent as a result of paternal exposure and that they affect multiple repeated loci. Hypermethylation of repeat elements localized at the 5'ends of coding transcripts may lead to decreased transcription rates of the corresponding genes and persistent gene expression changes (Cho, Kim et al. 2007). Alternatively, repeat hypermethylation may be a protective reaction in response to the detection of DNA damage and disturbed epigenetic patterns in pro-nuclei derived from irradiated spermatozoa.

5.4.7. CONCLUSIONS

This work was aimed at analyzing the influence of IR exposure on small RNAs in the germ line and possibly to link the observed changes to aberrant epigenetic markers, which were previously shown to be introduced by direct and paternal radiation (Koturbash, Baker et al. 2006; Tamminga, Koturbash et al. 2008; Filkowski, Ilnytskyy et al. 2010). In the process, we also assembled a comprehensive small RNA library for the adult mouse testes, which had much higher coverage than previous sequencing efforts (Aravin, Gaidatzis et al. 2006; Grivna, Pyhtila et al. 2006; Ro, Park et al. 2007). Here we report following findings:

1. A majority of small RNAs in adult testes are generated from repeats most

probably in pachytene spermatocytes; this contradicts the results of earlier sequencing efforts showing that piRNA in testes are depleted in repeats;

2. Repeat derived small RNAs map not only to mobile elements, but also to simple, low complexity repeats and satellites that are not transposable elements per se; this was never shown previously;
3. Both direct and paternal exposure leads to decreased pools of repeat derived small RNAs. Following direct exposure, this decrease affects almost all of the repeat classes; however, analysis at the level of individual repeats showed that some of them became up-regulated. Only SINEs were down-regulated in the paternal exposure library.
4. Direct and paternal irradiations had profound effects on miRNA abundance. Among the miRNAs significantly changed, the vast majority were up-regulated. Altered miRNAs in both cases were involved primarily in DNA damage responses, apoptosis, and the cell cycle; however, they appeared to have opposite functional themes: a) in the case of direct exposure, they were geared towards inhibition of apoptosis; b) in the case of paternal exposure, they promoted induction of apoptosis and inhibition of cellular proliferation;
5. We detected a very dramatic radiation-induced up-regulation of miR-143*, which was independent of miR-143. If confirmed, this shows that environmental exposure can specifically affect the stability of miRNA derived from the passenger strand, which provides a novel mechanism of the stress response at the miRNA level;
6. MiRNAs might be central for the regulation of radiation responses in the male

germ line, and they provided a mechanistic clue as to how the piwi-like RNA transcriptome can be down-regulated. Four miRNAs dramatically up-regulated after direct exposure have high scoring seeding sites in the 3'-UTR regions of Mov10l and Mael – proteins, whose knockout leads to a virtually identical phenotype of increased TE activity, meiotic arrest, disappearance of pachytene piRNAs, and failure of spermatogenesis. If confirmed, this will provide a new model for the induction of genomic instability in spermatocytes, where radiation exposure induces expression miR-34a/c, miR-93 and miR-181b, all of which were previously shown to be radioresponsive (Ilnytskyy, Zemp et al. 2008). This, in turn, causes down-regulation of Mael and Mov10l, decreases in Piwi proteins and piRNAs, and enhanced activity of TEs. Increased TE expression leads to induction of double strand breaks due to activity of TE encoded proteins, insertinal mutagenesis, and changes in chromatin structure and these, upon transmission to the zygote, cause transgenerational genomic instability and abnormal development. This would also suggest that the pachytene spermatocyte is a sensitive target for induction of radiation-induced transgenerational effects.

7. We detected hypomethylation of SINE B1 TE in the germ line and somatic tissue of paternally-exposed progeny. The presence of this change in the germ line and soma suggests that it was induced early in development, due to fertilization with irradiated spermatozoa and maintained over multiple cells divisions. Hypomethylation of SINE B1 may be explained by up-regulation of miR-29b in the progeny. MiR-29b was shown to target de novo DNA

methyltrasferases responsible for the maintenance of SINE B1 methylation in spermatogonial stem cells (Takada, Berezikov et al. 2009; Takashima, Takehashi et al. 2009).

8. Bisulfite sequencing of select TEs identified 2 regions hypermethylated in the progeny upon spermatid and spermatogonial exposure. In one case, this hypermethylated status was transmitted to the F2 generation. Both elements were ERVK family LTRs, which were shown to be resistant to epigenetic reprogramming in the pre-implantation embryo (Popp, Dean et al. 2010). If confirmed, this would be a case of transgenerational epigenetic inheritance in mammals. To date, only 2 such cases have been identified in mammals: maternal inheritance of the methylation status of the Agouti viable allele and paternal/maternal inheritance of the Axin fused allele (Rakyan, Chong et al. 2003; Dolinoy, Weidman et al. 2006). In both cases, heritable methylation changes occurred at LTR insertion. Our find suggests that altered methylation status might be inherited for a number of LTRs, but only a few have an easily detectable phenotype.
9. Potential for sequencing based discovery of novel miRNAs is not exhausted since we were able to detect a number of confident novel miRNAs in our datasets.

We have to point out that caution needs to be taken at interpreting these results, since the study had several important limitations. First of all, due to high cost of the sequencing, only one library was prepared per treatment from pooled sample of treated individuals. This eliminated the possibility of assessing inter-individual variation and

detection of biological outliers. Secondly, there was a very dramatic difference in the number of perfectly matching reads between direct exposure and paternal exposure libraries, introduced by un-identified technical variability (Table 2). These 2 sets of libraries were sequenced at different times, and controls from direct exposure cannot serve as technical replicas for control in paternal exposure, although the animals were of the same age.

The presence of one library per treatment eliminated the statistical power and disabled assessment of experimental variation. Therefore, we used stringent fold change criteria when analyzing miRNA expression. Bisulfite sequencing was also performed on pooled samples leading to a similar problem; in some cases, we performed assembly and comparison of 2 alternative pools from the same treatment group and did not see significant differences (data not shown).

We have yet to provide downstream experimental confirmation for changed miRNAs using real time PCR and to show that these indeed can down-regulate putative targets involved in piRNA processing.

Table 5.1. Parameters of analysis applied in Mir-Intess^{TM} bioinformatics pipeline.

Reference organism: mouse	**Assembly: ncbim37**	
Ensemble database: mus_musculus_core_54_37g	UCSC database: mm9	Mirbase version: 13.0
Min read length: 18	Max read length: 36	Min mapped read length: 18
READ trimming: THREE_PRIME_ALL_TRIM	Editing analysis: 0	Max distance between mature and star: 50
Hairpin classification:		
	Distance from loop: 0	Relative read abundance for signature calculation: 0.3
Distance from stem base: 5	Max mature length: 24	Max Dicer/Drosha overhang: 3
Min mature length: 20	Min Dicer/Drosha overhang:1	Lib/read overlap for confident novel miRNAs: AND
Max mature GC: 0.9	Min required libs for novel confident miRNA: 1	RNA forester threshold for homology: 0.3
Min required reads for novel confident miRNA: 10	Species for conservation analysis: rat, human, zebrafish, chicken	
Randfold threshold for confident novel miRNAs: 0.01		

Table 5.2. Library information, quality and adapter processing. This table presents information about the total number of sequencing reads that passed internal quality control analysis, number of reads that could not be aligned to the reference genome and were removed from the analysis, number of reads that were aligned perfectly, number of reads that could be aligned after 3'adapter trimming, total number and the number of unique sequences mapped. Only perfect and 3' trimmed matches with at least 18 bases perfectly matching were mapped to the genome.

Lib ID	**Description**	**Accepted**	**Nonmatching**	**Perfect**	**Trimmed 3 only**	**Mapped to genome**	**Mapped non redundant reads**	**Mapped loci**
CT	control	4573953	364524	824	4208605	4209429	547800	654300
IR	2.5 Gy	4786092	774252	3770	4008070	401840	638478	703524
PROG CT	Progeny of control	2887826	468099	195690	2224037	2419727	523754	661643
PROG IR	Progeny of irradiated	4070047	769868	254474	3045705	3300179	787750	960647

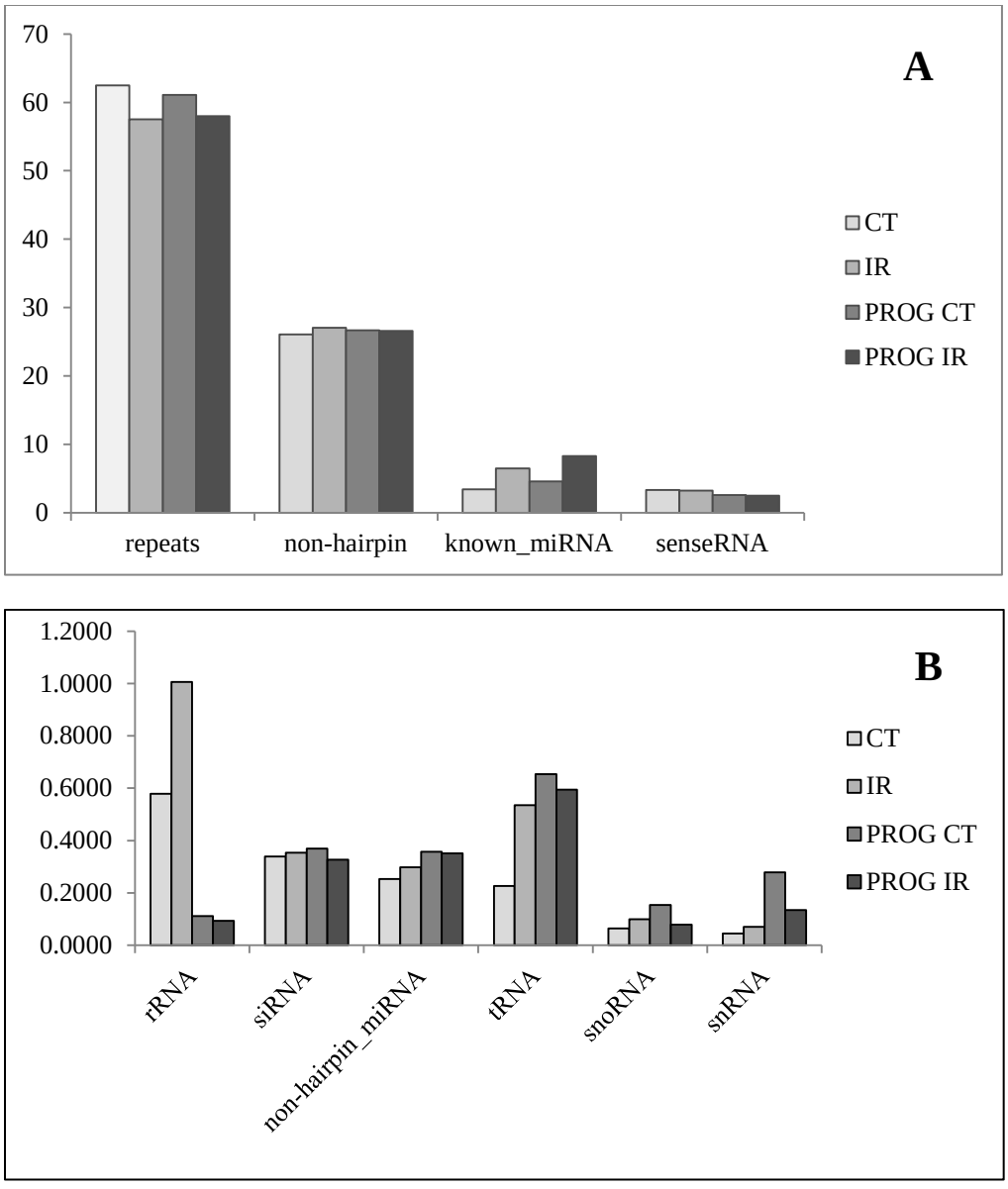


Figure 5.1. General compositional analysis of small RNA libraries sequenced from total RNA pools of adult mouse testes subjected to direct or paternal irradiation, where CT, IR, PROG CT, PROG IR represent sham treatment, 2.5 Gy direct X-Ray exposure, progeny of sham treated animals and irradiated animals respectively. Height of the bars represents the percent of small RNA pool occupied by certain sequence type in the corresponding library. A. sequence categories with more than 1% representation; B. sequence categories with less than 1% representation.

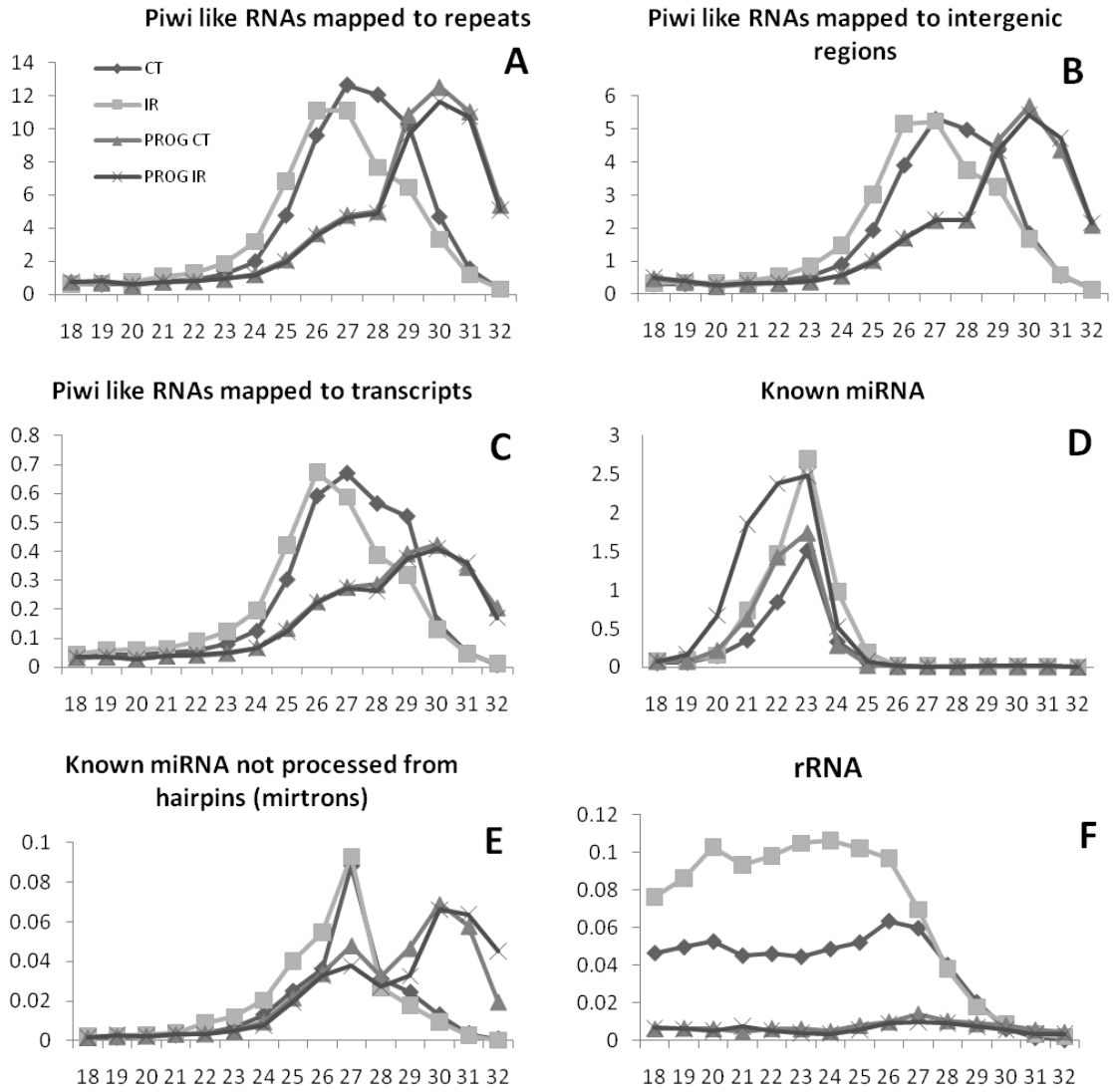


Figure 5.2. Examples of length distribution of sequencing reads derived from distinct classes of genomic sequences: Y-axis represents percent from the total reads mapped; X-axis – length in nucleotides. Assumption as to the origins and nature of this piwi like small RNAs were based solely on the genomic regions they were mapped to and length distributions reported in the literature (Aravin, Gaidatzis et al. 2006; Girard, Sachidanandam et al. 2006; Grivna, Pyhtila et al. 2006).

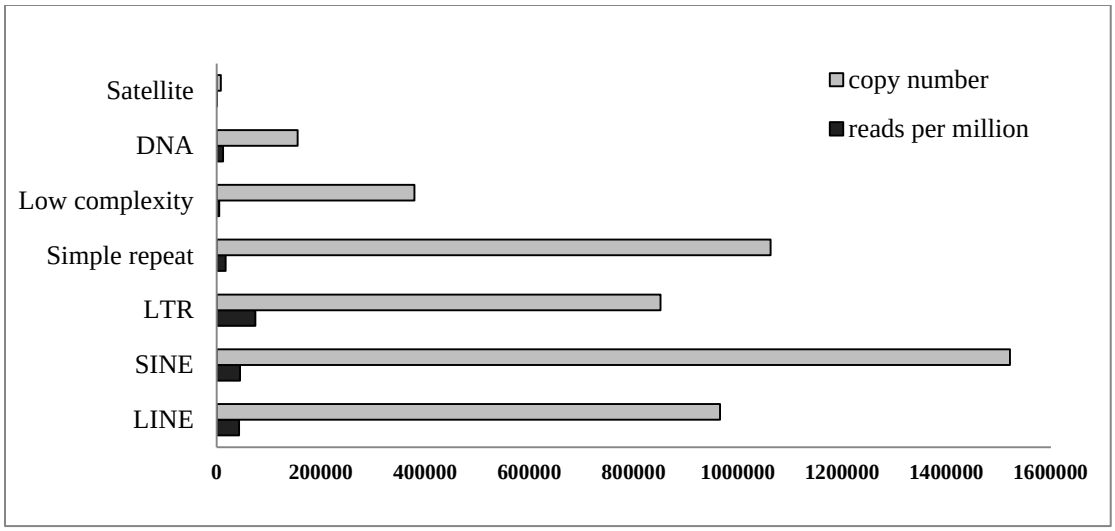
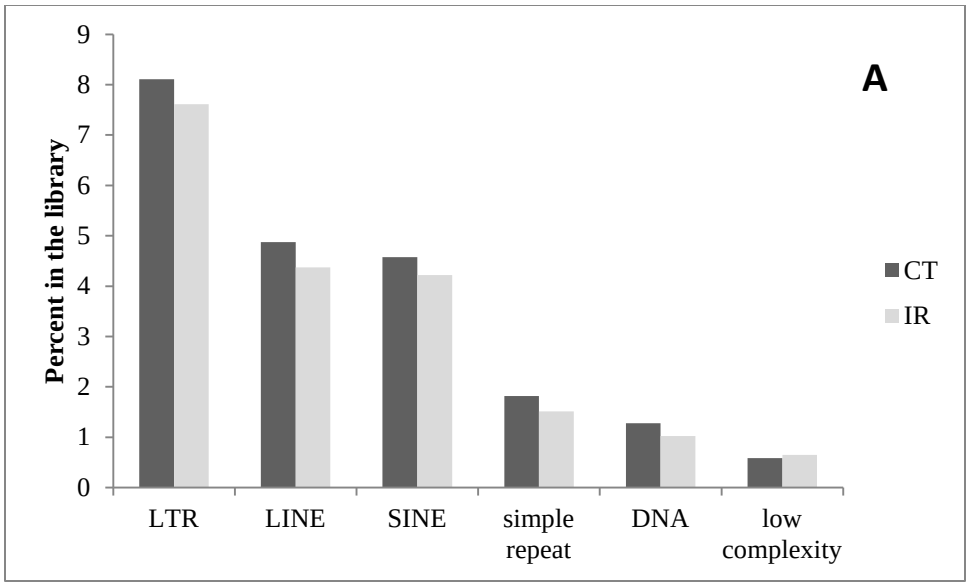


Figure 5.3. Comparison of the relative abundance of repeat derived short RNA in CT library to the copy number of corresponding repeat classes in the mouse genome uploaded using UCSC table browser.

Table 5.3. List of repeat elements identified as over-represented in CT sequencing library (log10 **(**Reads/Copy number**)** > 0).

#	Element	Repeat family	Reads per million	Copy number	LOG10(Reads/Counts)
1	MLT1I-int_(class=LTR)	MALR	408.31	62	0.81
2	CR1_Mam_(class=LINE)	CR1	832.38	267	0.49
3	LTR68_(class=LTR)	ERV1	296.49	112	0.42
4	Ricksha_(class=DNA/MuDR)	MuDR	531.94	232	0.36
5	MLT2F_(class=LTR)	ERVL	1289.71	569	0.35
6	MER68-int_(class=LTR)	ERVL	170.67	78	0.34
7	MER52-int_(class=LTR)	ERV1	413.34	199	0.31
8	Charlie9_(class=DNA/MER1_type)	MER1-type	401.75	226	0.24
9	Charlie18a_(class=DNA)	MER1-type	381.61	243	0.19
10	MER58D_(class=DNA)	MER1-type	374.39	261	0.15
11	RLTR24_(class=LTR)	ERV1	1953.82	1442	0.13
12	(TTCG)n_(class=Simple_repeat)	Simple repeat	64.77	51	0.10
13	MER74B_(class=LTR)	ERVL	397.15	326	0.08
14	(CAG)n_(class=Simple_repeat)	Simple repeat	2685.99	2224	0.08
15	MLT-int_(class=LTR)	MALR	986.65	849	0.06
16	RLTR14-int_(class=LTR)	ERV1	1992.12	1827	0.03
17	LSU-rRNA_Hsa_(class=rRNA)	rRNA	529.75	491	0.03
18	MER112_(class=DNA)	MER1-type	394.31	366	0.03
19	LTRIS4_(class=LTR)	ERV1	331.94	323	0.01
20	ORR1E-int_(class=LTR)	MALR	1614.66	1,588	0.01



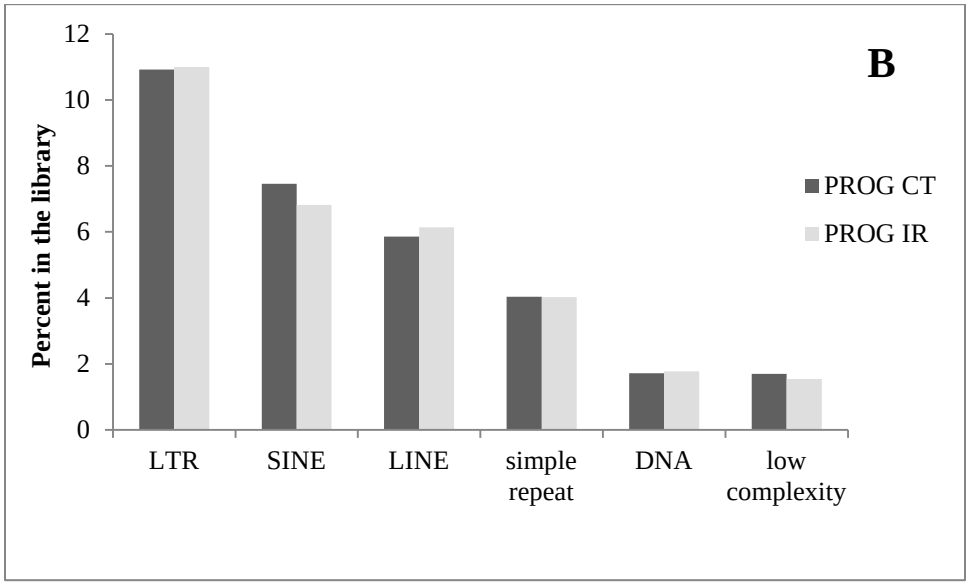


Figure 5.4. Influence of ionizing radiation on repeat derived piwi-like RNAs in adult mouse testes shown at the level of repeat element classes: A. Direct whole body exposure, B. Paternal exposure. Number of short sequencing reads mapped to repeat class was normalized to the size of the library as percent from total number of reads in the library.

Table 5.4. List of repeat elements with altered expression of corresponding small RNAs log_2(IR/CT) in directly exposed testes as compared to controls. Repeats with more than 1000 RPM in at least one of the libraries are shown in bold; asterisk indicates repeats that were also detected as changed in the progeny.

Repeat element	**CT (RPM)**	**IR (RPM)**	**log_2 (IR/CT)**
Up-regulated upon direct irradiation			
RLTR19-int_(class=LTR/ERVK)	25.9	178.2	2.8
(GGGA)n_(class=Simple_repeat)	26.6	154.3	2.5
(GGGTG)n_(class=Simple_repeat)	31.4	122.4	2.0
IAPLTR2a_(class=LTR)	73.9	278.9	1.9
LTR28_(class=LTR)	168.0	480.3	1.5
MLT1J1_(class=LTR)	178.4	498.0	1.5
MurSatRep1_(class=Unknown)	236.6	651.3	1.5
MER3_(class=DNA)	114.5	305.6	1.4
L1M1_(class=LINE)	224.5	557.6	1.3
(TTCG)n_(class=Simple_repeat)*****	70.3	169.2	1.3
MLT1J-int_(class=LTR)	406.5	967.6	1.3
L4_(class=LINE/RTE)	55.6	131.6	1.2
(C)n_(class=Simple_repeat)	37.1	85.5	1.2
RMER6A_(class=LTR)	67.2	146.6	1.1
GC_rich_(class=Low_complexity)	78.4	170.0	1.1
IAPLTR2_Mm_(class=LTR)	244.5	522.0	1.1
IAPLTR3_(class=LTR)	43.2	92.0	1.1
(TTCC)n_(class=Simple_repeat)	34.7	73.3	1.1
ORR1A4-int_(class=LTR)	91.9	192.4	1.1
IAPLTR2b_(class=LTR)	131.1	269.7	1.0
MER110_(class=LTR)*	36.8	74.3	1.0
Down-regulated upon direct irradiation			
(TTTG)n_(class=Simple_repeat)	89.1	42.6	-1.1
Charlie1a_(class=DNA)	**2283.9**	**1084.8**	**-1.1**
(AGGGGG)n_(class=Simple_repeat)	281.3	129.1	-1.1
L1MC4_(class=LINE)	448.5	190.4	-1.2
RNLTR23_(class=LTR)	498.6	211.1	-1.2
RLTR27-int_(class=LTR/ERVK)	114.5	47.4	-1.3
RLTR43A_(class=LTR/ERVK)	97.9	39.6	-1.3
L1MD2_(class=LINE)	**1600.0**	**575.3**	**-1.5**
MER5A1_(class=DNA)	**1133.2**	**376.9**	**-1.6**
MurERV4_19_(class=LTR/ERVK)	**7211.0**	**2132.2**	**-1.8**
MURVY-int_(class=LTR)*****	341.1	100.7	-1.8
(GAAAA)n_(class=Simple_repeat)	198.8	56.6	-1.8
ZP3AR_(class=Satellite)	86.2	23.7	-1.9
(CAG)n_(class=Simple_repeat)	**2916.1**	**777.9**	**-1.9**
(TAAAAA)n_(class=Simple_repeat)	363.2	96.5	-1.9
L1ME3_(class=LINE)	378.7	92.0	-2.0
RLTR15_(class=LTR)	323.3	72.8	-2.2
L1MD3_(class=LINE)	184.3	30.7	-2.6
L1_Mur2_(class=LINE/L1)	400.3	47.1	-3.1

Table 5.5. List of repeat elements with altered expression of corresponding small RNAs log_2(IR/CT) in the male germ line of paternally exposed mice as compared to controls. Repeats with more than 1000 RPM in at least one of the libraries are shown in bold; asterisk indicates repeats that were also detected as changed upon direct exposure.

Repeat element	**CT (RPM)**	**IR (RPM)**	**log_2 (CT/IR)**
Up-regulated after paternal irradiation			
(TCC)n_(class=Simple_repeat)	227.7	797.5	1.8
MER110_(class=LTR)*****	31.4	107.0	1.8
(TTCG)n_(class=Simple_repeat)*	113.2	339.7	1.6
L1MA4_(class=LINE)	114.9	317.0	1.5
L1ME3A_(class=LINE/L1)	109.1	294.8	1.4
RLTR10C_(class=LTR)	103.3	270.0	1.4
(TAGA)n_(class=Simple_repeat)	104.6	264.8	1.3
RMER30_(class=DNA)	204.6	480.9	1.2
L1_Mus3_(class=LINE)	**1522.1**	**3515.3**	**1.2**
MLT1E2_(class=LTR)	53.7	118.5	1.1
L1MA6_(class=LINE)	385.2	846.9	1.1
MLT1I-int_(class=LTR)	176.1	386.3	1.1
MurERV4_19-int_(class=LTR)	429.0	937.5	1.1
MURVY-int_(class=LTR)*	117.0	253.9	1.1
MLT2C1_(class=LTR/ERVL)	62.8	132.1	1.1
(GAA)n_(class=Simple_repeat)	123.6	252.7	1.0
Down-regulated after paternal irradiation			
MER115_(class=DNA)	412.4	203.0	-1.0
Charlie9_(class=DNA/MER1_type)	469.9	223.6	-1.1
tRNA-Ala-GCY__(class=tRNA)	102.1	46.7	-1.1
(CG)n_(class=Simple_repeat)	480.2	217.3	-1.1
RLTR13C1_(class=LTR)	124.8	48.2	-1.4
C-rich_(class=Low_complexity)	**1589.8**	**494.5**	**-1.7**
MER45A_(class=DNA)	150.8	45.1	-1.7
MLT1A1_(class=LTR/MaLR)	192.2	49.1	-2.0

Table 5.6. MiRNAs altered by direct and paternal radiation exposure in male mouse germ line. The list contains miRNA with more than 100 RPM in both libraries and a log2 ratio

of the fold change exceeding 3. Read counts were scaled to reads per million. Conservation data was retrieved using miRIntess pipeline. A. Direct exposure to 2.5 Gy of X-Ray, 96 hours time-point; B. Paternal exposure to the same dose.

A

miRBase ID	conserved species	CT (RPM)	IR (RPM)	Log2ratio (IR/CT)
up-regulated 96 hours post irradiation				
mmu-mir-143*	chicken, human, rat, zebrafish	1	158	7
mmu-mir-93	human, rat, zebrafish	15	952	6
mmu-mir-30c	chicken, human, rat, zebrafish	27	370	4
mmu-mir-7a-1	chicken, human, rat, zebrafish	208	2663	4
mmu-mir-451	unknown	25	294	4
mmu-mir-181b-2	chicken, human, rat, zebrafish	33	376	4
mmu-mir-541	rat	38	430	3
mmu-mir-34b	chicken, human, rat	70	777	3
mmu-mir-883b	chicken, rat	16	177	3
mmu-mir-34a	chicken, human, rat, zebrafish	19	179	3
mmu-mir-222	chicken, human, rat, zebrafish	20	181	3
mmu-mir-7a-2	chicken, human, rat, zebrafish	29	256	3
down-regulated 96 hours post irradiation				
mmu-mir-98	chicken, human, rat, zebrafish	562	69	-3
mmu-let-7i	chicken, human, rat, zebrafish	5186	605	-3
mmu-mir-741	rat	1738	79	-4

Table 5.6. Continued.

B

miRBase ID	conserved species	CT(RPM)	EXP(RPM)	Log2ratio (EXP/CT)
up-regulated after paternal exposure				
mmu-mir-29b-2	chicken, human, rat, zebrafish	5	905	8
mmu-mir-29b-1	chicken, human, rat, zebrafish	5	902	8
mmu-let-7f-1	chicken, human, rat, zebrafish	11	1792	7
mmu-let-7f-2	chicken, human, rat, zebrafish	13	1866	7
mmu-let-7a-1	chicken, human, rat, zebrafish	14	870	6
mmu-let-7a-2	chicken, human, rat, zebrafish	15	914	6
mmu-let-7c-2	chicken, human, rat, zebrafish	21	1241	6
mmu-mir-32	chicken, human, rat	9	419	6
mmu-let-7c-1	chicken, human, rat, zebrafish	28	1309	6
mmu-let-7b	chicken, human, rat, zebrafish	21	543	5
mmu-mir-871	human, rat	16	404	5
mmu-mir-16-1	chicken, human, rat, zebrafish	18	396	4
mmu-mir-136	human, rat, zebrafish	21	456	4
mmu-mir-16-2	chicken, human, rat, zebrafish	22	465	4
mmu-mir-15a	chicken, human, rat, zebrafish	19	406	4
mmu-mir-18a	chicken, human, rat, zebrafish	7	142	4
mmu-mir-15b	chicken, human, rat, zebrafish	6	100	4
mmu-mir-425	human, rat	57	869	4
mmu-mir-30c-2	chicken, human, rat, zebrafish	24	361	4
mmu-mir-30c-1	chicken, human, rat, zebrafish	24	361	4
mmu-let-7g	chicken, human, rat, zebrafish	82	1192	4
mmu-mir-741	rat	271	3185	4
mmu-mir-322	human, rat	36	378	3
mmu-mir-30b	chicken, human, rat, zebrafish	18	180	3
mmu-mir-22	chicken, human, rat, zebrafish	76	701	3
mmu-mir-145	chicken, human, rat, zebrafish	22	194	3
mmu-mir-206	chicken, human, rat, zebrafish	67	572	3
mmu-mir-143	chicken, human, rat, zebrafish	1779	15136	3
mmu-mir-24-2	rat, zebrafish	55	453	3
mmu-mir-24-1	human, rat, zebrafish	55	453	3

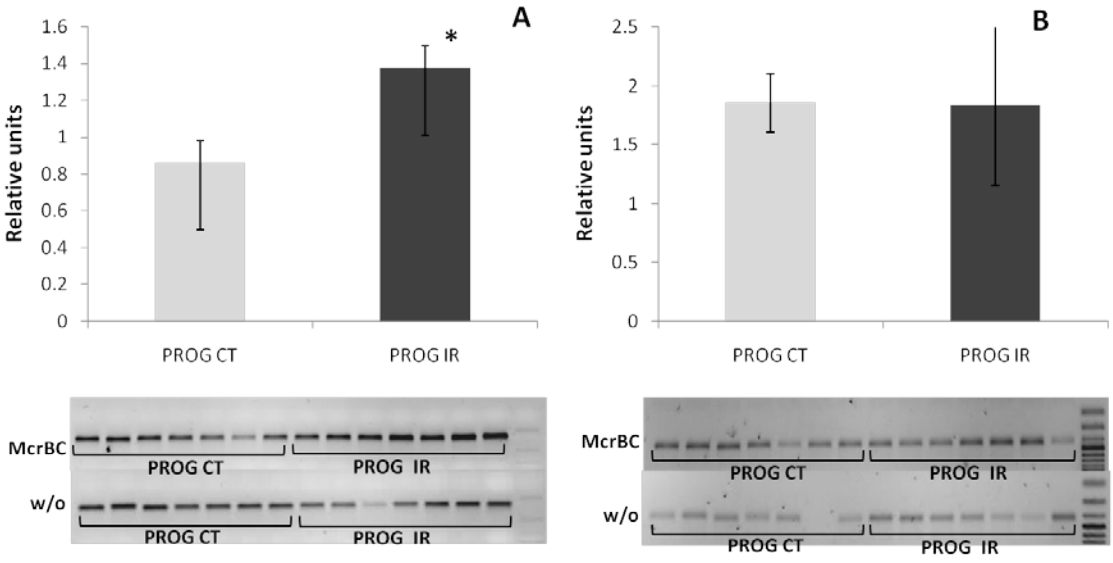


Figure 5.5. DNA methylation levels at SINE B1 (A) and LINE1 ORF2 (B) in testes of male mice paternally exposed to acute dose of X-Ray as compared to the progeny of controls. Methylation levels were measured using McrBC digestion followed by semi-quantitative PCR as described in Materials and Methods. Relative units represent ratios of band intensities of PCR product amplified after McrBC digestion to those of un-digested controls. The data points represent mean values from 2 technical repeats. Asterisk indicates statistical significance $p<0.05$ in 2-tailed un-paired t-test.

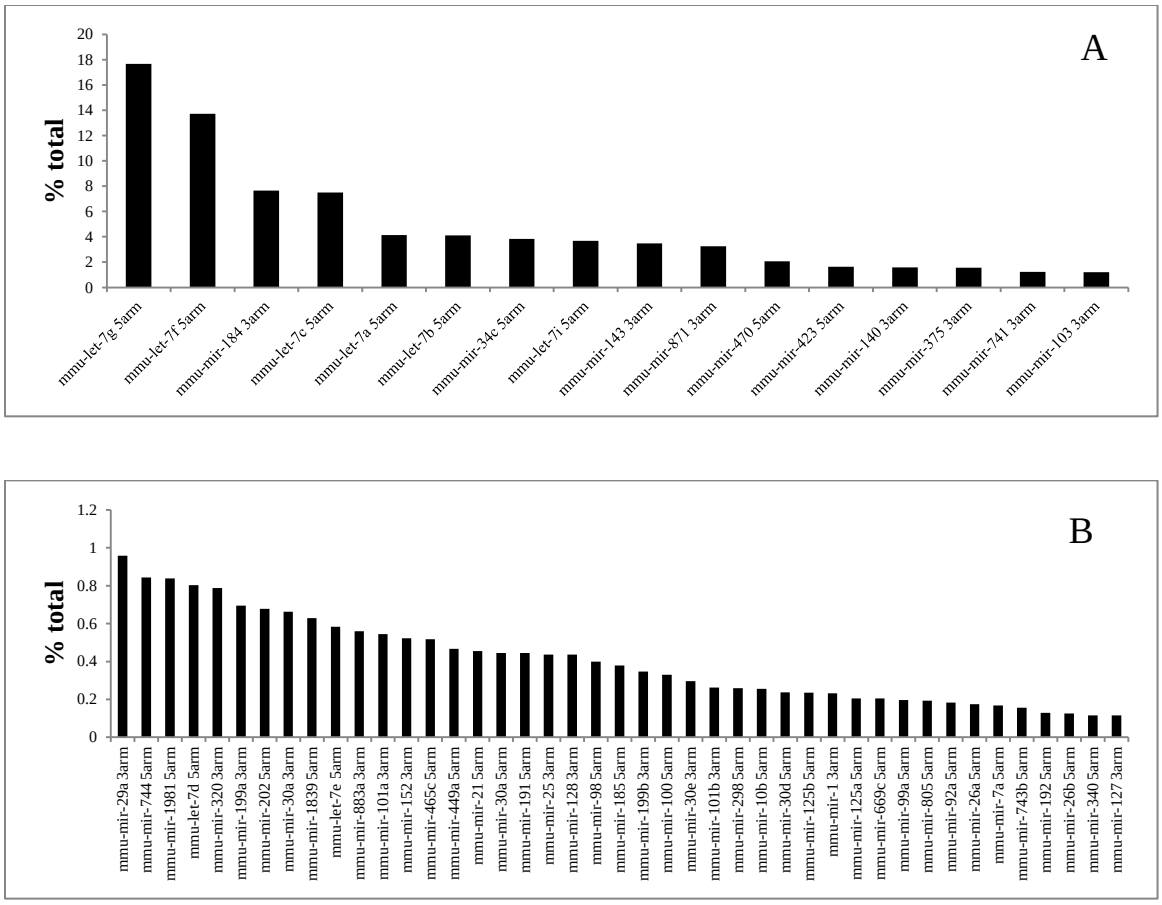


Figure 5.6. Known and candidate novel miRNAs ranked according to their expression in control mouse testes. Percent of sequencing reads mapped to specific miRNA in total pool of miRNA mapped reads was used as a surrogate for expression. A. High expression category (> 1% of total miRNA reads); B. Moderate expression (0.1-1%).

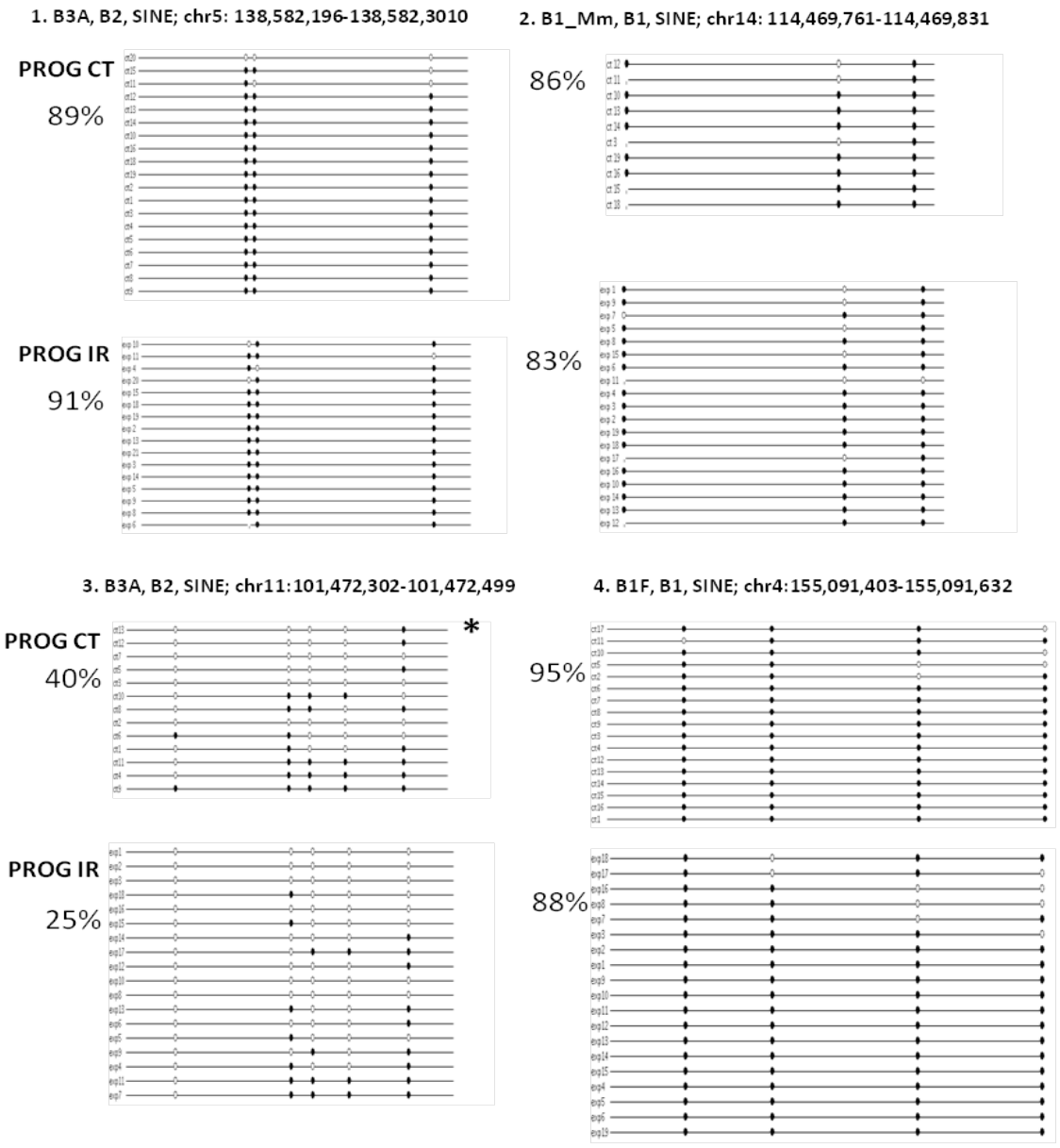


Figure 5.7. Bisulfite sequencing of selected individual short interspersed nuclear elements (SINEs) using pooled testes gDNA sample from 7 progeny of irradiated fathers as compared to the pooled sample of 7 progeny of controls. In both cases the progeny represent 3 different litters. Black circles represent methylated CpGs, clear circles – unmethylated CpGs. Distance between circles to proportional to the distance between corresponding CpGs in the genome. Repeat element's classification and genomic position

is indicated above the diagram. Overall methylation level is calculated as a percent of un-methylated CpGs in the region. Asterisk indicates SINE element that showed nearly significant change between treatment groups (p=0.08, Fisher exact test).

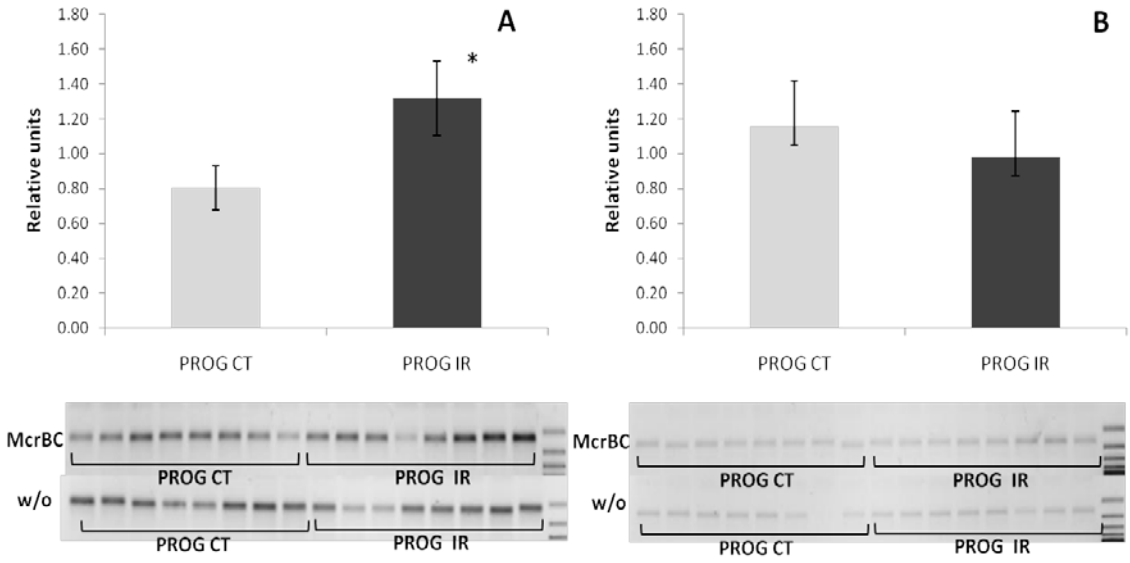


Figure 5.8. DNA methylation levels at SINE B1 (A) and LINE1 ORF2 (B) in spleen of the male mice paternally exposed to acute dose of X-Ray as compared to the progeny of controls. Methylation levels were measured using McrBC digestion followed by semi-quantitative PCR as described in Materials and Methods. Relative units represent ratios of band intensities of PCR product amplified after McrBC digestion to those of un-digested controls. The data points represent mean values from 2 technical repeats. Asterisk indicates statistical significance $p<0.05$ in 2-tailed non-paired t-test.

			Methylation level					Read counts	
Region	Repeat	Location	CT	SZ	SP	SG	F2	CT	IR
SI001	**RMER17B, LTR**	**chr7:120,071,640-120,072,576**	**53.6**	**58.9**	**73.3***	**75.3***	**66.7**	**939**	**31**
SI005	(TGAA)n, simple	chr18:35,903,253-35,903,361	92.7	89.5	78.3	94.8	85.7	213	39
SI009	**B1_Mm, SINE**	**chr14:114,468,640-114,470,859**	**42.6**	**61.4***	**33.3**	**47.9**	**39.4**	**1003**	**168**
SI025	B3A, SINE	chr11:101,472,104-101,472,697	65.8	71.7	58.8	68	67.3	82	0
SI043	**RLTR9B2, LTR**	**chr16:12,744,286-12,764,085**	**60.2**	**60.6**	**84.2***	**84.8***	**84.6***	**169**	**17**
SI049	B1_Mur1, SINE	chr4:155,091,405-155,091,631	79.8	96.4	81.9	83.3	97.6	101	10

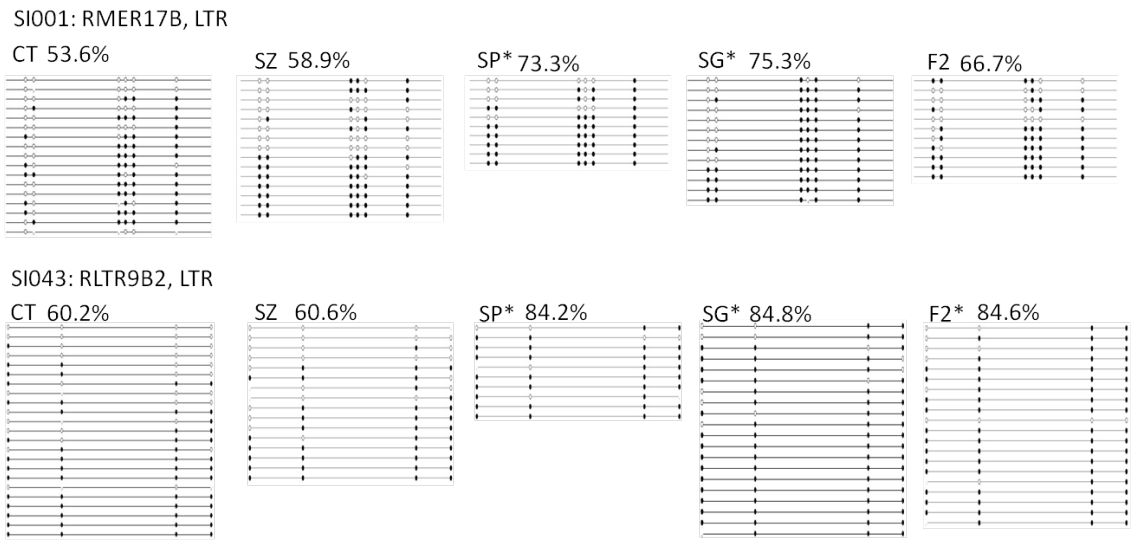


Figure 5.9. Bisulfite sequencing of selected repeat elements in the bone marrow of the PPI progeny mated to un-treated females at various time-points post irradiation. Black circles on the diagram represent methylated CpGs, clear circles – unmethylated CpGs. Distance between circles is proportional to the distance between corresponding CpGs in the genome. Bisulfite sequencing was performed using pooled DNA sample from 6 mice from 3 separate litters, 10 – 20 clones was obtained in each case. DNA methylation levels were calculated as percent of methylated CpGs within the sequenced region. Table holds information with regard to repeat classification, genomic location (UCSC, mm9), methylation level (CT – control; SZ – spermatozoal; SP – spermatid; SG – spermatogonial stages; F2 – obtained by interbreeding progeny from spermatogonial stage) and repeat derived small RNA mapping in direct exposure libraries. Asterisks indicate statistical significance at $p<0.05$ in Fisher exact test.

5. FUTURE WORK

Experimental results presented in this thesis suggest that ionizing radiation acts as a potent epigenetic modifier in hematopoietic tissues and in the germ line. The epigenetic effects were persistent and detectable both on the level of DNA methylation and expression of regulatory non-coding RNAs, furthermore epigenetic changes were introduced by paternal irradiation in un-exposed progeny. The next step after detection of radiation-induced epigenetic changes has be aimed at better understanding of the molecular mechanisms of their induction and identification of genomic locations susceptible to this effect.

To date large number of environmental factors, including ionizing radiation, chemotherapeutic drugs, metals, arsenic and organic synthetic compounds were shown to modify epigenetic patterns in mammalian organisms (Bollati and Baccarelli 2010). Heritable nature of epigenetic changes raises interesting possibility of transgenerational transmission of epigenetic errors to subsequent generations with the possibility of impact on developmental outcomes and adult onset disease (Jirtle and Skinner 2007). At this point it is unclear whether epigenetic transgenerational effects are widespread and whether they pose significant threat to human health. Further research is required to gain better understanding of how epigenetic marks are set in the germ line and how environmental factors can modify these processes. On the other hand it is important indentify areas in the genome susceptible to epigenetic modifications and resistant to epigenetic reprogramming that occurs in pre-implantation embryo and in primordial germ cells.

Recently, non-coding RNAs (piRNAs and microRNAs) have emerged as a potent regulator of epigenetic programming in mammalian germ line, yet influence of environmental factors on non-coding RNAs in the germ line and on possible down-stream outcomes (such as DNA methylation and histone modifications) is practically un-explored. Here we suggest a number of experiments that in our opinion would facilitate the study epigenetic programming in the future.

6.1. CHARACTERIZATION OF CHROMATIN BOUND FRACTION OF SMALL NON-CODING RNAS AT DIFFERENT STAGES OF SPERMATOGENSIS

Spermatogenesis is a very complex and finely regulated process aimed at the production of a narrowly specialized cell with a densely packaged haploid genome and a capability for locomotion. One of the most challenging parts of this process is rapid and precise regulation of chromatin packaging and accompanying gene expression. Adverse environmental factors, including genotoxic chemicals and ionizing radiation, can influence this process and introduce chromatin abnormalities that may interfere with the unfolding of developmental programs in the early embryo. Ultimately, these chromatin abnormalities could lead to the emergence of persistent changes in gene expression, cellular signaling, and genomic stability that manifests as a pathological state. Therefore, one of the important areas in the field of transgenerational effects and gene-environment interaction is the study of mechanisms of epigenetic reprogramming in the male germ line.

Non-coding RNAs have been recently recognized as a crucial factor involved in the direction of chromatin packaging, DNA methylation, and gene expression (Costa 2008; van Wolfswinkel and Ketting 2010). Long non-coding RNAs were shown to bind

directly to chromatin and to regulate parental imprinting and X-chromosome inactivation (Murakami, Ohhira et al. 2009; Gupta, Shah et al. 2010; Tsai, Manor et al. 2010). It is not yet clear how universal and widespread these mechanisms are in mammalian cells. Can abundant small RNAs direct chromatin modifying machinery to genetic material in a way similar to RNA-induced transcriptional silencing in yeast (Verdel, Jia et al. 2004)? Do piRNAs direct epigenetic patterning during sperm cell differentiation? One of the important steps towards answering these questions would be to characterize a small RNA population bound to chromatin at different stages of spermatogenesis.

In order to achieve this goal, we suggest the following workflow:

1. First, we will isolate germ cells at various stages of spermatogenesis by centrifugal elutriation and confirm the results by DNA content analysis and stage specific markers as described in Barchi et al. (2009).

2. Next, we will isolate soluble chromatin from each of the fraction, isolate, and precipitate RNA and finalize the process with extensive DNAse treatment (Mondal, Rasmussen et al. 2010).

3. RNA will be resolved on 20% polyacrylamide gel and examined for the presence of 18-32 nucleotide bands, which will be excised and purified. In order to determine that this fraction is indeed enriched in chromatin bound small RNAs, we will conduct real time PCR analysis of one the housekeeping miRNA and 2 small nuclear RNAs, 7SK and U1, known to be enriched in chromatin fraction.

4. Part of this fraction will be treated with T4 ligase and run on the gel to check for concatamerization. PiRNAs were shown to have 5’phosphate

group and 3'hydroxyl group and, therefore, can be concatamerized by T4 ligase (Watanabe, Takeda et al. 2006)

5. Next, we will perform next generation sequencing of small chromatin associated RNAs according to standard Illumina protocols.

6. Small RNA reads will be mapped to the repeat unmasked genome and counted. We will select several highly expressed transcripts for the given cell type and assess their presence in the library as a measure of contamination with nascent transcripts. This will also allow us to establish enrichment cut-offs;

7. Next, we will establish the regions of preferential small RNA binding, their dynamics between developmental stages displayed by maturing germ cells and level of evolutionary conservation;

8. Subsequent stages of analysis: down-stream confirmation and functional analysis would depend on the actual results.

6.2. X-RAY EXPOSURE SUPPRESSES PIRNA PROCESSING BY MIRNA MEDIATED MECHANISM

In Chapter V of this work, we found evidence that IR can induce the expression of miR-93, miR-181b, miR-34c, and miR-34a. A strong binding site for miR-93 was predicted by MicroCosm search (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) within 3'-UTR of the Mael transcript, and binding sites for miR-181b, miR-34a, and miR-34c were predicted for Mov10l. Both proteins are involved in piRNA processing, and their loss of function mutation produces remarkably similar phenotypes, expressed as dramatic up-regulation of transposable elements activity, meiotic arrest, and disappearance of the pachytene fraction of piRNAs (Soper,

van der Heijden et al. 2008; Frost, Hamra et al. 2010). It was suggested that the main function of pachytene piRNA is the suppression of transposable elements that undergo transient relaxation at the onset of meiosis (van der Heijden and Bortvin 2009).

Radiation-induced up-regulation of miRNAs that target the Mael and Mov10l transcripts can cause suppression of piRNAs and activation of transposable elements thus causing loss of genomic stability in irradiated male germ line. All of the candidate miRNAs were previously reported as radiation responsive (Ilnytskyy, Zemp et al. 2008). In addition, miR-34a/c are directly activated by p53, which acts as the primary radiation sensing transcription factor and inducer of apoptosis (Corney, Flesken-Nikitin et al. 2007). It would be interesting to establish whether radiation exposure can indeed down-regulate piRNA processing and induce activation of transposable elements through an miRNA mediated pathway.

During the first stage of suggested research, we will have to show that given miRNAs are indeed up-regulated in testes tissue as a response to IR exposure and that this up-regulation takes place in spermatocytes. There are a few ways to determine this; for instance, we could irradiate testes of 14-day old mice and measure the expression of miRNAs of interest at several time-points post irradiation using real-time PCR. At 14 days of age, mouse testes comprise only pre-meiotic cells and spermatocytes entering meiosis. Radiation-induced over-expression of these miRNAs can be confirmed by *in situ* detection by a complementary DNA probe. This procedure would allow us to localize the expression change to a particular type of cells.

Next, we will have to establish that given miRNAs are indeed able to down-regulate the expression of target proteins. This can be achieved by the use of the well-

tested luciferase assay, which relies on the measurement of activity of the reporter gene fused to the 3'-UTR of the target upon application of various concentrations of miRNA. After that, we will have to show that ectopic application of given miRNAs can down-regulate endogenous expression of the target protein. Since our target proteins are expressed strictly in the germ line and specifically in spermatocytes, this could be technically challenging. A recent paper reported an experimental procedure for short-term culture of spermatocytes and pharmacological induction for up to prophase-to-division phase transition (La Salle, Sun et al. 2009). Potentially, we could use this system for miRNA transfection, followed by quantification of target protein expression by Western blot.

Upon completing that step, we will then have to demonstrate that miRNA transfection causes transposon activation and increased DNA damage in a spermatocyte culture. Activation of transposons can be detected on the transcriptional level by real-time PCR, as well as by antibodies raised to the proteins translated from full length LINE1 and intracisternal A particle (IAP) transcripts. DNA damage can be evaluated by γH2AX staining and Comet assay. The same end-points have to be assessed *in vivo* after exposure to a relevant dose of IR.

6.3. USE OF AXIN(FU) MICE AS A MODEL FOR THE STUDY OF MALE MEDIATED TRANSGENERATIONAL EFFECTS

Number of genotoxic agents, including chemicals and ionizing radiation, are suggested to cause epigenetic reprogramming in the offspring of affected fathers (Vorobtsova, Aliyakparova et al. 1993; Barber, Plumb et al. 2002; Barton, Robaire et al. 2005; Nomura 2008). The exact mechanism of this reprogramming is unknown, but it

may involve changes of DNA methylation in a number of repeat elements resistant to erasure of DNA methylation marks in pre-implantation embryos (Lane, Dean et al. 2003; Popp, Dean et al. 2010). One of the potential outcomes of aberrant methylation of repeat elements in the progeny is altered gene expression, as many repeat elements are localized in the vicinity of functional transcripts and carry cryptic promoters normally silenced by DNA methylation. To date, only two such cases have been discovered in the mouse model, and both were shown to depend on IAP insertion in genomic loci with a clearly detectable phenotype (Rakyan, Chong et al. 2003; Dolinoy, Weidman et al. 2006). We refer to Agouti viable (A(vy)) and Axin1 Fused (Axin(Fu)) alleles.

An A(vy) allele arises as a result of IAP insertion in one of the exons of the gene coding for the coat coloration. The gene expression in A(vy) is driven by a cryptic promoter located within the IAP insert and depends on its methylation status. Low methylation level results in active ectopic transcription from the IAP promoter and yellow coloration in the mouse. On the other hand, hypermethylation results in silencing and brown coloration, while intermediate methylation results in "mottled" coloration of various penetrance (Morgan, Sutherland et al. 1999; Dolinoy, Weidman et al. 2006). Methylation status of dominant A(vy) is inherited through the maternal but not paternal germ-line and can be affected by dietary exposure to methyl-group donors, bisphenol A, and genistein (Dolinoy, Weidman et al. 2006; Dolinoy, Huang et al. 2007). Unfortunately, since A(vy) is maternally inherited, it cannot serve as a model for male-mediated epigenetic reprogramming.

A similar mechanism determining epigenetic inheritance was discovered in the case of Axin(Fu) alleles in a C57Bl background. Axin(Fu) codes for a multifunctional

protein that regulates a number of signaling pathways. An abnormal expression of this gene is caused by transcription from the cryptic promoter located within the recently inserted IAP element. Presence of retroviral particle results in various alternative transcripts. Mice expressing Axin(Fu) demonstrate a “kinked tail” phenotype attributed to axial duplication during embryogenesis (Flood and Ruvinsky 2001; Rakyan, Chong et al. 2003). The activity of IAP insertion is strongly correlated with methylation status. Moreover, the methylation status of inserted IAP was transmitted to the progeny from the paternal and maternal germ lines (Rakyan, Chong et al. 2003). Maternal exposure to methyl donors was shown to increase methylation at Axin(Fu) (Waterland, Dolinoy et al. 2006).

Expression of Axin(Fu) phenotypes strongly depends on methylation status, it is paternally heritable and can be readily detected; these features make Axin(Fu) a suitable model for the study of epigenetically transmitted male-mediated toxicity. We suggest a simple experiment that will demonstrate whether radiation exposure can alter the epigenetic status of IAP elements in a transgenerational manner.

Axin(Fu) male mice will be sorted according to the penetrance of the “kinked tale” phenotype. Mice that belong to opposite ends of the spectrum of “kinked tale” expression will be selected for further experiments. Methylation status of the Axin(Fu) embedded IAP element will be determined in each of the males by bisulfite sequencing performed on the DNA from blood lymphocytes. Selected males will be randomly distributed to experimental groups and mated to control wild-type females at various time-points after irradiation. Penetrance of the “kinked tail” phenotype will be analyzed in the F1 progeny and compared between experimental groups. Bisulfite sequencing will

be performed in order to confirm methylation status. IR-induced hypermethylation at Axin(Fu) will result in decreased “kinked tail” penetrance in the progeny of irradiated low penetrance males, and there will be no phenotypic shift for irradiated high penetrance group. IR-induced hypomethylation will produce the opposite effect. Mating at different time-points after irradiation is necessary to determine stages of spermatogenesis susceptible to this effect.

If successful, this model can be used for relatively quick and inexpensive screening for transgenerational epigenetic effects of suspected epigenetic modifiers, which include different types of ionizing radiation, genotoxic drugs used in chemotherapy, and drugs specifically designed to target components of DNA methylation and histone modification machinery.

7. BIBLIOGRAPHY

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