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Hypomethylation and genome instability in the germline of exposed parents and their progeny is associated with altered miRNA expression

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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.

Introduction

Paternal exposure to genotoxic agents such as ionizing radiation (1–5), environmental toxicants (6,7) and chemotherapeutic drugs (8,9) 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 (2,10–12). Epigenetic changes are alterations in gene expression that include DNA methylation, histone modification and RNA-associated silencing (13–15). 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 (15). 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 (16,17).

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 (18,19). We found that it also influences global DNA methylation in bone marrow, thymus and thymus in unexposed offspring (19,20).

Abbreviations: DNMT, DNA methyltransferase; LINE1, long interspersed nuclear elements 1; LSH, lymphoid-specific helicase; miRNA, microRNA; PCR, polymerase chain reaction; SINE B2, short interspersed nuclear elements B2; UTR, untranslated region.

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Materials and methods

Model

In this study, we utilized an *in vivo* murine model to analyze the role of epigenetic alterations in transgenerational radiation effects. The murine model is widely used, well characterized and generally accepted for studies of radiation-induced changes and transgenerational effects (21–24). 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 (20). For the irradiation procedure, animals were placed in small (10 × 5 × 6 cm) 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 −80°C until the analysis.

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 (25,26). Quantitative real-time-PCR was conducted using tissues of six animals per group (paternal germline analysis) or eight animals per group (progeny analysis).

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).

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 1× 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 (28,29).

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.

Western immunoblotting

Western immunoblotting was conducted as described previously, using antibodies against LSH (1:500; Abcam, Cambridge, MA), DNA methyltransferase (DNMT)3a (1:500; Santa Cruz Biotechnology) and actin (loading control) (1:2000; Abcam).

DNA extraction and DNA methylation analysis

DNA was extracted from thymus using a QIAGEN (Valencia, CA) 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 (28). Genomic DNA (1 μg) 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.* (28). Two-step quantitative PCR was performed using a SYBR® GreenER™ SuperMix (Invitrogen, Carlsbad, CA) for iCycler (Bio-Rad, Hercules, CA) with 40 cycles of 45 s at 95°C and 90 s at 58°C. After the final cycle, melting curve analysis of all samples was conducted within the range of 55–95°C. All reactions were run in triplicate. The threshold cycle (C_t) is defined as the fractional cycle number that passes the fixed threshold. C_t 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 (19,20).

Statistical analysis

Statistical analysis was performed using MS Excel 2007 and JMP5 software packages.

Results

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 methylation status 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 (18), particularly in a significant upregulation of miR-29a and 29b (Figure 1A). These changes were confirmed by real-time PCR. The miR-29 family influences the *de novo* DNMTs, DNMT3a and DNMT3b. Fabbri *et al.* (31) 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 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 (18). 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 DNA methylation (supplementary Figure 1 is available at *Carcinogenesis* Online). Thus, the main changes were seen only in the levels of *de novo* methyltransferases.

A

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

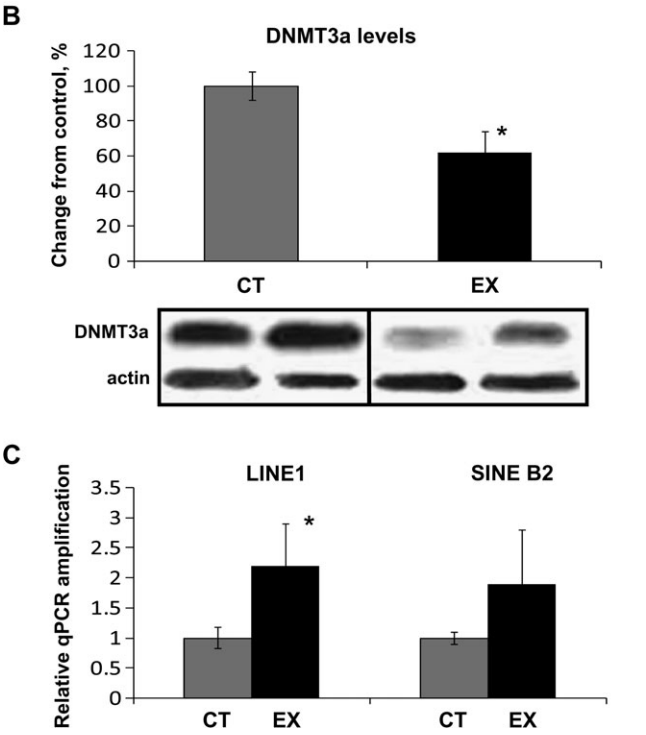

Fig. 1. Radiation exposure alters miRNA expression and methylation of LINE1 and SINE B2 retrotransposons in male germline. We utilized an *in vivo* murine model to analyze the role of epigenetic alterations in transgenerational radiation effects. The murine model is widely used, well characterized and generally accepted for studies of radiation-induced changes and transgenerational effects (21–24). 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 (20). 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 (18). Levels of miR-29a and miR-29b were confirmed by quantitative real-time PCR using specific primers (Ambion) as described (26). (**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. The methylation-sensitive McrBC endonuclease is a restriction enzyme that digests only methylated DNA sequences (30), $n = 10$; *$P < 0.05$, Student's *t*-test. CT, control animals; EX, exposed animals, $n = 10$; *$P < 0.05$, Student's *t*-test.

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 (32). The status of LINE1 and SINE B2 methylation was determined by methylation-sensitive McrBC quantitative PCR analysis (30). The methylation-sensitive McrBC endonuclease is a restriction enzyme that digests only methylated DNA sequences but does not cleave unmethylated DNA (33). Importantly, we found a significant loss of LINE1 methylation and a tendency of SINE B2 hypomethylation upon radiation exposure in the male germline (Figure 1C). This is a very interesting finding because hypomethylation of certain repetitive elements, including LINE1, is considered to be a hallmark of genomic instability (34,35).

Paternal exposed 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 (36). 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 (20). One of the main functions of DNA methylation in normal mammalian somatic tissues is the suppression of transposable repetitive elements (37,38). 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 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 (34,35).

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 2B). LSH, a member of the SNF2 family of chromatin remodeling proteins, is thought to be crucial for the maintenance of genome-wide CpG methylation (39), especially for methylation and silencing of repetitive elements such as LINEs and SINEs (40,41). Recently, it has been demonstrated that LSH is directly involved in the control of *de novo* methylation of DNA (42), and LSH deficiency leads to aberrant upregulation of retroviral repetitive elements in the genome, abnormal mitosis with amplified centrosomes and genomic instability (43). 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.

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 (31,44–46). In total, miRNAs

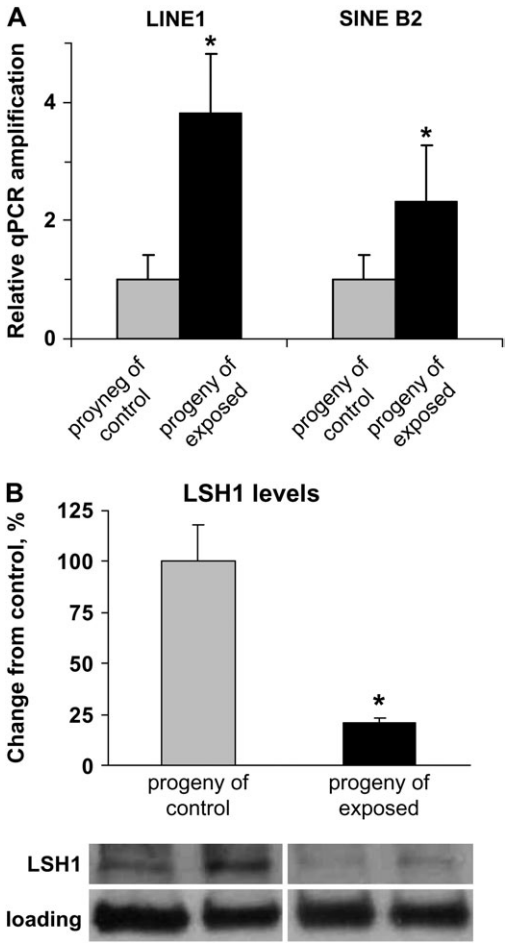

Fig. 2. Paternal radiation exposure causes hypomethylation of LINE1 and SINE B2 retrotransposons and alters cellular levels of the methylation regulator LSH. To analyze the effects of exposure on the progeny, 4 days after irradiation control and exposed animals were mated with unexposed females. Both sets of progeny were killed 6 months after birth. The thymus tissues were extracted, immediately frozen and stored at −80°C 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 analysis. The methylation-sensitive McrBC endonuclease is a restriction enzyme that digests only methylated DNA sequences (30), $n = 10$; $^*P < 0.05$, 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 from four independent experiments are shown; each experiment included pooled lysates from four animals for each exposure condition with equal representation of each animal.

control the activity of ∼30% of all protein-coding genes (47), 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 (48). Indeed, Figure 3A 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 exposed parents. Cluster analysis revealed that preconceptional 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 3B). Interestingly, we did not detect changes in the expression of two miRNA families, miR-29 and miR-290, that regulate levels of DNMTs (31,48,49). However, computational analysis revealed that miR-468, which was upregulated in the progeny of exposed parents, targets

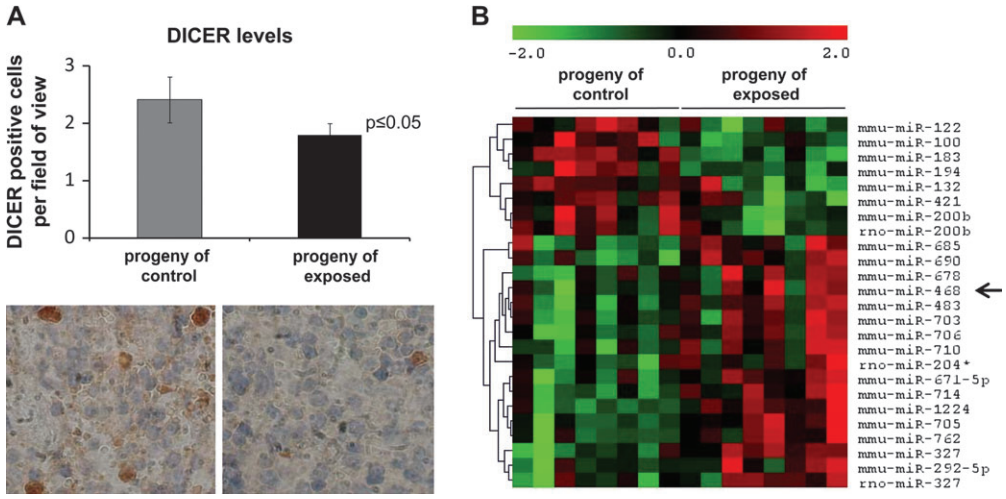

Fig. 3. Paternal radiation exposure results in microRNAome deregulation in the thymus tissue of the unexposed progeny. (**A**) Decreased levels of Dicer in the thymus tissue of the progeny of exposed animals as determined by immunohistochemistry (IHC). IHC was conducted as described before (27). Levels of Dicer-positive cells per field of view are shown, mean values ± SEM, $n = 10$ 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 RNA was extracted from the thymus tissue using TRIzol Reagent (Invitrogen, Burlington, Ontario, Canada). miRNA microarray analysis was performed by LC Sciences (Houston, TX). Red denotes high expression levels, whereas green depicts low expression levels.

murine LSH (Figure 4A). This prediction was further confirmed by the comprehensive microRNA.org data resource (51). 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 4B shows that miR-468 inhibited the luciferase activity of the construct containing the *Lsh*-3′-UTR segment (Figure 4B). There was no change in the luciferase reporter activity if cells were co-transfected 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 4B).

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 24 h after transfection. Transfection of NIH3T3 cells with miR-468 resulted in a decrease of LSH levels (Figure 5A). 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 5).

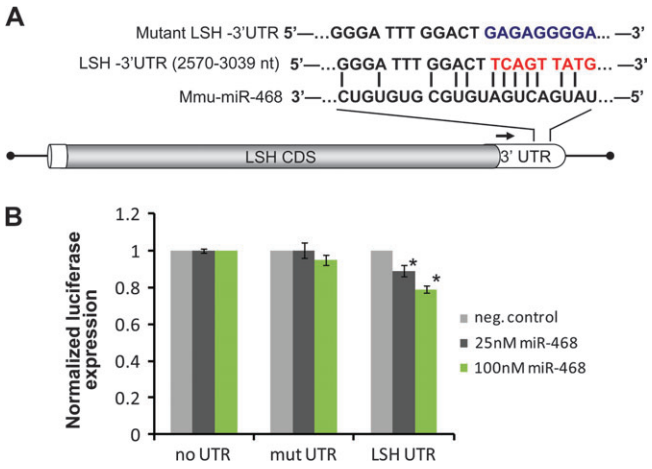

Fig. 4. mir-468 targets LSH. (**A**) Complementary site for miR-468 in the 3′-UTR of Lsh1. (**B**) A dose-dependent inhibition of *Lsh* expression in the luciferase assay. A 3′-UTR segment of the *Lsh* gene corresponding to a region of 366 nts (from 2642 nt through 3008 nt of the total transcript) of *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 (26,50). 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.

Discussion

Approximately 5% of human live births today have a birth defect, a *de novo* genetic disease or chromosomal abnormality (52). 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 causes decreased

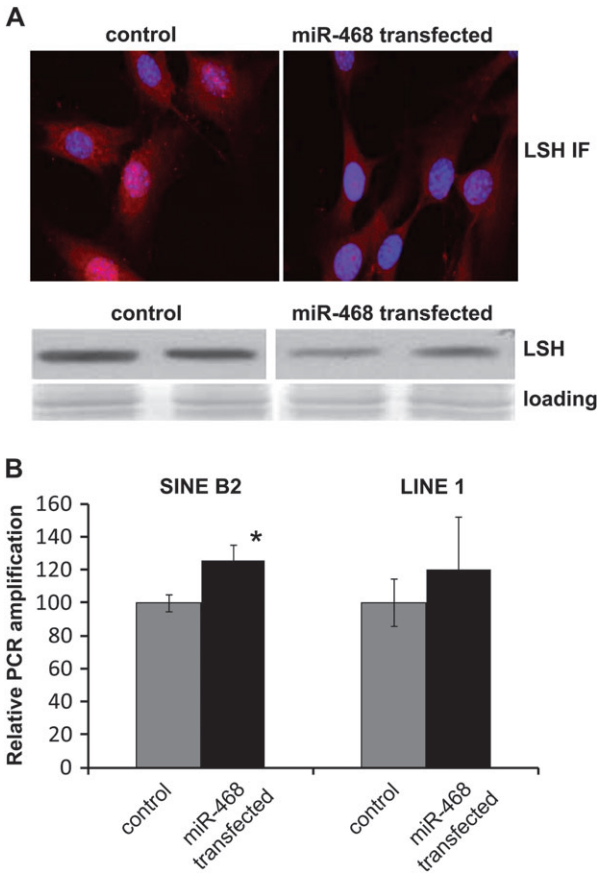

Fig. 5. miR-468 reduces cellular levels of LSH and affects DNA methylation. (**A**) Transfection of mouse NIH3T3 cells with miR-468 effectively reduces cellular levels of LSH. Levels of LSH were detected by immunofluorescence and by western immunoblotting using anti-LSH antibodies. Red indicates LSH 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.

expression of *de novo* methyltransferase DNMT3a (31) 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 DNA methylation in the male germline (18).

Importantly, epigenetic DNA methylation and microRNAome changes observed in the male germline led to deleterious 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 previously reported to be crucial for the maintenance of methylation and silencing of repetitive elements (39–41,43,53). LSH cooperates with DNMTs in setting DNA methylation patterns and silencing (54). 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 interactions 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 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 (30,40,53,55). This regulation may be specifically required when 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 (1,4,5,56). 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 (57,58). The epigenetic changes observed in sperm cells may interfere with post-fertilization epigenetic reprogramming, thus affecting fertilized eggs and leading to subsequent deleterious changes in the embryo (36). 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 exists 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 transgenerational effects. Therefore, this study may serve as a future roadmap for analyzing the role of methylation and microRNAome in transgenerational genomic instability.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/

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