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Exposure to Low-Dose ^{56}Fe-Ion Radiation Induces Long-Term Epigenetic Alterations in Mouse Bone Marrow Hematopoietic Progenitor and Stem Cells

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There is an increasing need to better understand the long-term health effects of high-linear energy transfer (LET) radiation due to exposure during space missions, as well as its increasing use in clinical treatments. Previous studies have indicated that exposure to ^{56}Fe heavy ions increases the incidence of acute myeloid leukemia (AML) in mice but the underlying molecular mechanisms remain elusive. Epigenetic alterations play a role in radiation-induced genomic instability and the initiation and progression of AML. In this study, we assessed the effects of low-dose ^{56}Fe-ion irradiation on epigenetic alterations in bone marrow mononuclear cells (BM-MNCs) and hematopoietic progenitor and stem cells (HPSCs). Exposure to ^{56}Fe ions (600 MeV, 0.1, 0.2 and 0.4 Gy) resulted in significant epigenetic alterations involving methylation of DNA, the DNA methylation machinery and expression of repetitive elements. Four weeks after irradiation, these changes were primarily confined to HPSCs and were exhibited as dose-dependent hypermethylation of LINE1 and SINE B1 repetitive elements [4.2-fold increase in LINE1 ($P < 0.001$) and 7.6-fold increase in SINE B1 ($P < 0.01$) after exposure to 0.4 Gy; n = 5]. Epigenetic alterations were persistent and detectable for at least 22 weeks after exposure, when significant loss of global DNA hypomethylation (1.9-fold, $P < 0.05$), decreased expression of Dnmt1 (1.9-fold, $P < 0.01$), and increased expression of LINE1 and SINE B1 repetitive elements (2.8-fold, $P < 0.001$ for LINE1 and 1.9-fold, $P < 0.05$ for SINE B1; n = 5) were observed after exposure to 0.4 Gy. In contrast, exposure to ^{56}Fe ions did not result in accumulation of increased production of reactive oxygen species (ROS) and DNA damage, exhibited as DNA strand breaks. Furthermore, no significant alterations in cellular senescence and apoptosis were detected in HPSCs after exposure to ^{56}Fe-ion radiation. These findings suggest that epigenetic reprogramming is possibly involved in the development of radiation-induced genomic instability and thus, may have a causative role in the development of AML. © 2014 by Radiation Research Society

INTRODUCTION

There is an increasing need to understand the long-term health effects of high-linear energy transfer (LET) radiation due to the high potential for exposure to high-LET radiation during space missions and its growing utilization in medicine. Comparative studies between X rays, protons and heavy iron ions such as ^{56}Fe ions have shown that the latter has the most deleterious effects on survival and levels of DNA damage (*1, 2*). Therefore, exposure to high-LET radiation such as ^{56}Fe ions may pose a significant hazard to space flight crews during and after space missions.

The damage to DNA caused by high-LET radiation is complex and includes clusters containing single- and double-strand breaks (SSBs and DSBs, respectively) and chromosomal aberrations [reviewed in Durante and Cucinotta (*3*)]. This damage is often lethal and consequently results in cell death within a relatively short time after exposure (*1*). Indeed, studies have reported a lack of persistent effects of exposure to ^{56}Fe ions, including a lack of chromosomal aberrations in lymphocytes, which is a validated biomarker for cancer risk in astronauts after a 2-year space mission (*4*). The vast majority of studies performed in the field have utilized relatively high doses of radiation (over 1 Gy), but little is known regarding the

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effects of low doses. It is becoming increasingly evident that at lower doses cells do not respond to radiation in a predictive dose-dependent fashion, which might be due to adaptive responses, bystander effects, genomic instability and/or low-dose hypersensitivity (*5*). These findings significantly complicate the identification of biomarkers of exposure to potentially dangerous carcinogenic doses of high-LET radiation.

Acute myeloid leukemia (AML) is one of the most frequent consequences of exposure to low-LET radiation and is characterized by the greatest morbidity risk from acute radiation exposure (*6*). In humans, the risk of developing AML after exposure to high-LET radiation and to ^{56}Fe ions, in particular, remains unknown. Previous studies have indicated that exposure to ^{56}Fe heavy ions results in increased rates of AML in mice (*7*). The molecular characteristics of ^{56}Fe ion associated AML in mice were similar to those detected in low-LET-radiation associated AML and appeared to involve biallelic mutations of the hematopoietic transcription factor gene *PU.1* (*Sfpi1*) and microsatellite instability (*8*). However, the exact molecular mechanisms underlying the development of AML after exposure to ^{56}Fe ions remain elusive.

Epigenetic alterations are involved in the pathogenesis of radiation-induced carcinogenesis (*7, 9, 10*). DNA methylation is the most studied mechanism of epigenetic regulation, and appears to play an important role during the development and maintenance of cellular homeostasis. In particular, DNA methylation is crucial for proper regulation of expression of genetic information in a sex-, tissue- and cell-type-dependent manner and in silencing of DNA repetitive elements (*11, 12*). Alterations in DNA methylation may lead to the reactivation of oncogenes and repetitive DNA sequences and to the silencing of tumor-suppressor genes (*13, 14*). These events can result in genomic instability and cancer and these alterations in DNA methylation and DNA methylation machinery have been observed in most cancers in animal models and humans, including leukemia and lymphoma (*15–22*).

The importance of epigenetic alterations in AML has been recognized. Numerous studies report alterations in DNA methylation, including loss of global and repetitive elements-associated DNA methylation as well as mutation-induced loss of function of genes involved in the regulation of normal patterns of DNA methylation (*15, 23, 24*). Moreover, the methylation status of LINE1 has recently been shown to predict a response in AML patients to epigenetic therapy with azacitidine (*25*). In hematopoietic tissue, exposure to low-LET radiation, aside from the strong genotoxic potential, has also been shown to affect DNA methylation (*9, 26, 27*). Recent studies in cell culture indicate that exposure to ^{56}Fe ions may cause epigenetic alterations, primarily associated with DNA methylation (*2, 28*). However, to the best of our knowledge, the epigenetic effects of ^{56}Fe ions in hematopoietic tissue in animal models and their persistence have never been addressed.

In this study, we investigated the molecular effects caused by exposure to low doses (600 MeV, 0.1, 0.2 and 0.4 Gy) of ^{56}Fe ions in the murine bone marrow (BM). Exposure to ^{56}Fe ions resulted in significant epigenetic alterations involving methylation of DNA, the DNA methylation machinery and reactivation of repetitive elements. These changes were primarily confined to hematopoietic progenitor and stem cells (HPSCs) rather than mononuclear cells (MNCs). Importantly, these epigenetic alterations were persistent and detected for at least 22 weeks after exposure. Irradiation did not result in accumulation of DNA damage or increased production of reactive oxygen species (ROS) in either MNCs or HPSCs. Furthermore, no significant alterations in cellular senescence and apoptosis were detected in HPSCs after exposure to ^{56}Fe ions. These findings suggest that epigenetic reprogramming is possibly involved in the development of radiation-induced genomic instability and thus may have a causative role in the development of AML. Additionally, methylation and expression of repetitive elements may be considered as biomarkers of exposure to potentially carcinogenic doses of cosmic radiation.

MATERIALS AND METHODS

Animals and Exposure

C57BL/6J male six-month-old mice (n = 80) purchased from the Jackson Laboratory (Bar Harbor, ME) were shipped to Brookhaven National Laboratories (BNL) in Upton, NY. After a one-week acclimation period, the mice were randomly assigned to experimental groups and were either sham irradiated (n = 10 mice per group) or given doses of 0.1, 0.2 or 0.4 Gy whole-body irradiation (600 MeV/n Fe ions). Dosimetry was performed by the Physics Dosimetry Group and BNL to ensure the quality of exposure. During the entire experiment, sham-irradiated mice were not housed together with irradiated mice. For each exposure, animals were individually placed into clear Lucite cubes (3 in × 1½ in × 1½ in) with breathing holes. Sham-irradiated mice were placed into the same enclosures for the same amount of time, since previous studies report no effect of sham irradiation on molecular end points. One week after irradiation, the mice were shipped to Oregon Health and Science University (OHSU) under climate-controlled conditions. At BNL and OHSU, the mice were housed under a constant 12 h light/dark cycle. Food (PicoLab Rodent Diet 20, no. 5053; PMI Nutrition International, St. Louis, MO) and water were provided *ad libitum*. Tissues were harvested 4 and 22 weeks after irradiation or sham irradiation. Five animals per group of treatment/time point were randomly selected per radiation dose and per time point. All the analyses were performed in duplicates. Data from two technical repeats were combined for the analysis. All procedures were approved by the Institutional Animal Care and Use Committee at OHSU and BNL.

Sample Coding

The researchers were all blinded to all parts of the experiments and decoding occurred after the final analyses were performed.

Isolation of Bone Marrow Mononuclear Cells Lineage-Negative Hematopoietic Progenitor and Stem Cells (HPSCs)

The femora and tibiae were harvested from mice immediately after they were sacrificed by cervical dislocation. Bone marrow cells were

flushed from the bones into Hank's balanced salt solution (HBSS, Mediatech Inc., Manassas, VA) containing 2% FCS using a 21-gauge needle and syringe. Bone marrow mononuclear cells (BM-MNCs) were isolated by Histopaque 1083 separation solution (Sigma, St. Louis, MO). For the isolation of HPSCs, BM-MNCs were incubated with purified rat antibodies specific for murine CD3e (clone 145-2C11), Mac-1 (clone M1/70), CD45R/B220 (clone RA3-6B2), Ter-119 (clone Ter-119) and Gr-1 (clone RB6-8C5) (all from BD Biosciences, San Jose, CA). The labeled mature lymphoid and myeloid cells were depleted twice by incubating with goat anti-rat IgG paramagnetic beads (Life Technologies, Grand Island, NY) at a bead:cell ratio of approximately 4:1. Cells binding the paramagnetic beads were removed with a magnetic field. The negatively isolated HPSCs cells were washed twice with 2% FBS (Atlanta Biologicals, Flower Branch, GA)/HBSS and resuspended in a complete medium (RPMI 1640 medium supplemented with 10% FBS, 2 m*M* L-glutamine, 10 μ*M* HEPES buffer and 100 U/mL penicillin and streptomycin) at 1×10^6 cells/mL.

Quantitative Analysis of 5-Methylcytosine (5-MC)

Total DNA was extracted from MNCs and HPSCs using the AllPrep DNA/RNA extraction kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. RNA concentrations and integrity were analyzed by the Nanodrop 2000 (Thermo Scientific, Waltham, MA). Only RNA samples with the 260/280 ratios between 1.8–1.9 and the 260/230 ratios above 1.5 were considered for further molecular analyses. Whole genome CpG methylation was assessed using a commercially available fluorescence-based immunoassay according to the manufacturer's protocol (Epigentek, Farmingdale, NY). Briefly, wells were coated with 100 ng of gDNA at 37°C for 90 min. A standard curve was built using suggested dilutions of the provided positive control. Samples of mouse DNA pretreated with 5-azacytidine, a potent demethylating agent, were used as negative controls. Wells were washed and the primary antibody was added and incubated for 60 min. Next, wells were washed again and the detection antibody was added and incubated for 30 min. Wells were washed and the enhancer solution was added and incubated for 30 min. Wells were washed, rinsed in phosphate buffered saline (PBS, Mediatech Inc.) and the developing solution added. The samples were read at 530EX/590EM and the results were expressed as relative fluorescent units.

Analysis of Methylation Status of DNA Repetitive Elements

The methylation status of LINE1 and SINE B1 were determined by methylation-sensitive McrBC- quantitative PCR (qPCR) assay (*29*). Genomic DNA (1 μg) was digested overnight with the methylation-specific restriction endonuclease McrBC that cleaves DNA containing methylcytosine on one or both strands (New England Biolabs, Ipswich, MA) (10 units McrBC, in the presence of 1× NEBuffer 2, 200 μg/ml BSA and 1 m*M* GTP) and then analyzed by qPCR on a ViiA 7 Real-Time PCR System (Applied Biosystems, Forrest City, CA). DNA samples not digested with the restriction enzyme served as positive control, while samples lacking the specific primers for DNA amplification and/or DNA template served as negative control. Primers for DNA methylation of specific repetitive elements can be found in Supplementary Table S1 (http://dx.doi.org/10.1667/RR13580.1.S1). The threshold cycle (Ct) was defined as the fractional cycle number that passes the fixed threshold. The Ct values were converted into the absolute amount of input DNA using the absolute standard curve method and further normalized towards rDNA readings.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from MNCs and HPSCs using the AllPrep DNA/RNA extraction kit (QIAGEN) according to the manufacturer's protocol. RNA concentrations and integrity were analyzed by the Nanodrop 2000 (ThermoScientific). Only RNA samples with the 260/280 ratios between 1.95–2.05 and the 260/230 ratios above 1.5 were considered for further molecular analyses. cDNA was synthesized using random primers and a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol (Life Technologies). The levels of gene transcripts for *Dnmt1* (Mm 01151063_m1), *Dnmt3a* (Mm 00432881_m1), *Dnmt3b* (Mm 01240113_m1), *MeCP2* (Mm01193537_g1) and *Uhrf1* (Mm00477868_mH) were determined by quantitative Real Time PCR (qRT-PCR) using TaqMan Gene Expression Assays (Life Technologies). Assays for determination of mRNA abundance of LINE1 and SINE B1 are provided in Supplementary Table S1 (http://dx.doi.org/10.1667/RR13580.1.S1). Each plate contained one experimental gene and a housekeeping gene. The threshold cycle for each sample was determined from the linear region of the amplification plot. The ΔCt values for all genes were determined relative to the control gene *β-actin* (Mm 00607939_s1, Life Technologies). The ΔΔCt were calculated using each exposed group means relative to control group means (*30*). The fold change data were calculated from the ΔΔCt values. All qRT-PCR reactions were conducted in triplicate and repeated twice.

LINE1 Copy Numbers Analysis

LINE1 copy number was assessed as described in Vitullo *et al.* (*31*). Briefly, LINE1 ORF1 was amplified by real-time quantitative PCR from 10 ng of gDNA. Sixteen of the 20 samples from the 22 weeks after exposure time-point were run in duplicates, due to the limited amount of available material ($n = 5, 3, 4, 4$, for the control, 0.1, 0.2 and 0.4 Gy groups, respectively). Relative abundance of the target in gDNA was normalized to 5S ribosomal DNA using the ΔΔCt method. The FAM/ZEN-conjugated primers with the probe sequence are shown in Supplementary Table S1 (http://dx.doi.org/10.1667/RR13580.1.S1) (Integrated DNA Technologies, Coralville, IA) and were used at a final concentration of 5 μ*M*. Amplification was performed for 40 cycles using conditions for the 2X Taqman Universal Master Mix as recommended by the manufacturer (Life Technologies). The total reaction volume was 20 μL.

Analysis of the Frequencies and Numbers of Different Hematopoietic Cell Populations by Flow Cytometry

BM-MNCs were preincubated with biotin-conjugated anti-CD3e, anti-CD45R/B220, anti-Gr-1, anti-CD11b and anti-Ter-119 antibodies (described above) and with anti-CD16/32 antibody (clone 2.4G2; Fcγ receptor blocker) to block the Fcγ receptors. They were then stained with streptavidin-FITC and anti-Sca1-PE-Cy7 (clone E13-161.7), c-Kit-APC-Cy7 (clone 2B8), CD150-APC and CD48-Pacific blue (BD-PharMingen, San Diego, CA). The frequencies of HPSCs were analyzed with a FACSAria™ II cell sorter (Becton-Dickinson, San Jose, CA). For each sample, approximately 5×10^5–1×10^6 BM-MNCs were acquired and the data were analyzed using BD FACSDiva 6.0 (BD Biosciences) and FlowJo (Tree Star Inc., Ashland, OR) software. The numbers of HPSCs in each mouse were calculated by multiplying the total numbers of BM-MNCs harvested from the two hind legs of each mouse with the frequencies of HPSCs in BM-MNCs.

Analysis of the Levels of Intracellular Reactive Oxygen Species

After staining with the appropriate cell surface marker antibodies (described above), HPSCs (1×10^6/mL) were suspended in PBS, supplemented with 5 m*M* glucose, 1 m*M* $CaCl_2$, 0.5 m*M* $MgSO_4$ and 5 mg/ml BSA and then incubated with 10 μ*M* 2′,7′-dichlorofluorescin diacetate (DCFDA, Life Technologies) for 30 min at 37°C. The levels of ROS in HPSCs were analyzed by measuring the mean fluorescence intensity (MFI) of 2′,7′-dichlorofluorescein (DCF) with an FACSAria II cell sorter. For each sample, a minimum of 200,000 HPSC cells was

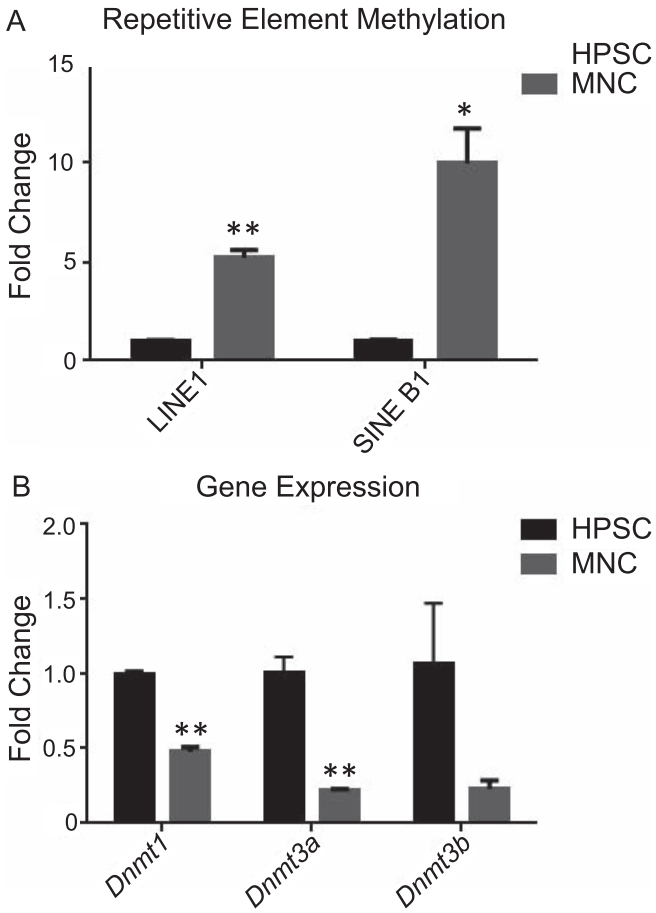

FIG. 1. Differences in DNA methylation profiles between the murine MNCs and HPSCs. Panel A: DNA methylation in LINE1 and SINE B1 was measured by methylation-sensitive McrBC-qPCR assay. Data are presented as mean ± SEM (n = 3). Asterisks denote significant (*$P < 0.05$) and (**$P < 0.01$) difference from control. Panel B: Analysis of expression of genes involved in maintenance of DNA methylation. The differential gene expression was determined by quantitative RT-PCR. Data are presented as mean ± SD (n = 3). Asterisks denote significant (*$P < 0.05$) and (**$P < 0.01$) difference from control (Dunnett's posthoc test).

acquired, and the data were analyzed using CellQuest software (Becton-Dickinson). PE and APC isotype controls were included, as appropriate.

DNA Damage Analysis

After HPSCs (approximately 4,000 sorted cells) were first stained with antibodies against specific cell-surface markers (described above), they were fixed and permeabilized using the fixation/permeabilization solution from BD Biosciences (San Diego, CA) followed by 0.2%Triton X-100 incubation for 10 min. Cells were then stained with Alexa Fluor 488 conjugated anti-Phospho-Histone H2AX (Ser139) or γ-H2AX rabbit antibody (1:100, Cell Signaling, Danvers, MA) for 1.5 h at 4°C and analyzed by flow cytometry. The levels of DNA damage were expressed by the mean fluorescence intensity of γ-H2AX with a FACSAria II cell sorter.

SA-β-gal Activity Analysis

SA-β-gal activity in HPSCs was measured by flow cytometry using an ImaGene Green™ C12FDG lacZ gene expression kit from Molecular Probes (Life Technologies), according to the manufacturer's instructions and protocols reported previously with the following modifications. Specifically, HPSCs were first stained with antibodies against the above-mentioned cell-surface markers and then incubated with 25 μ*M* chloroquine for 30 min at 37°C to induce lysosomal alkalinization and inhibit the basal levels of endogenous β-galactosidase activity in normal hematopoietic cells. After being washed with PBS, they were incubated with a β-galactosidase reaction buffer containing 16 μ*M* 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG) for 30 min at 37°C. The cells were washed again with PBS and were then analyzed immediately with an FACSAria II cell sorter. Dead cells were excluded from the assay by PI staining.

Apoptosis Assay

HPSCs were incubated with anti-CD16/32 at 4°C for 15 min to block the Fcγ receptors and then stained with antibodies against the above-mentioned cell surface markers in the dark. After annexin V staining with a kit from BD Pharmingen (San Diego, CA) according to the manufacturer's instructions, apoptotic cells were analyzed with an FACSAria II cell sorter.

Statistical Analysis

All data are presented as mean ± standard error of means of at least five independent biological samples per radiation dose. With 5 animals per comparison group, there was 80% power to detect effect sizes of approximately 2 standard deviations in a two-tailed test with a 5% significance level. All assessed parameters were measured within the same batch of animals. Linear correlation relationships did not reveal statistically significant associations between any of the measured end points. Statistically significant differences for each treatment compared to the control (at $\alpha = 95\%$) were assessed using one-way ANOVAs followed by Dunnett's or Tukey's posthoc tests. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software Inc., LaJolla, CA).

RESULTS

Differences in DNA Methylation Profiles between the Murine MNCs and HPSCs

First, we assessed whether the levels of DNA methylation differ between the populations of mature and less differentiated cells by measuring 5-mC in MNCs and HPSCs. We found that HPSCs exhibit 19% higher levels of 5-mC than MNC (data not shown). To further validate this finding, we investigated the methylation patterns of two repetitive elements, LINE1 and SINE B1, using the McrBC-qRT PCR approach. These two repetitive elements that are silenced by DNA methylation comprise over 20% of the mouse genome (*29, 32*). Therefore, their methylation status is a generally accepted surrogate biomarker for the evaluation of global DNA methylation. The extent of LINE1 and SINE B1 methylation was also significantly higher in HPSCs. Specifically, in HPSCs methylation of LINE1 was 5.3-fold higher ($P < 0.01$) and methylation of SINE B1 was 10.1-fold higher ($P < 0.05$) than in MNCs (Fig. 1A). This finding is in good agreement with a recent study, in which the less differentiated cells in bone marrow had higher levels of DNA methylation (*33*). Congruently with the levels of global and repetitive elements-associated

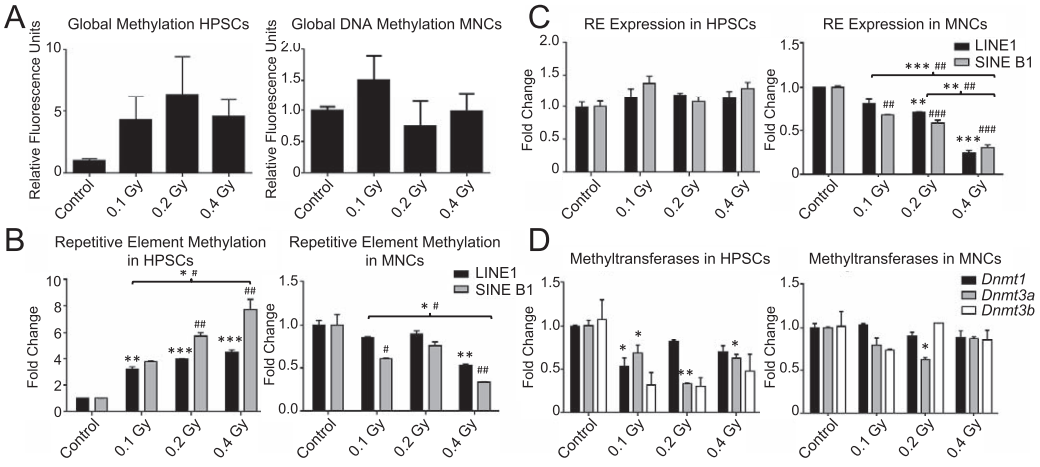

FIG. 2. Epigenetic effects of ^{56}Fe-ion irradiation in MNCs and HPSCs 4 weeks after exposure. Panel A: Global DNA methylation was measured by quantification of the 5-mC levels in DNA of HPSCs and MNCs using the fluorescence-based immunoassay. Data are presented as mean ± SD (n = 5). Panel B: DNA methylation in LINE1 and SINE B1 was measured by methylation-sensitive McrBC-qPCR assay. Data are presented as mean ± SEM (n = 5). Asterisks and pound signs denote significant ($^{*,\#}P < 0.05$), ($^{**,\#\#}P < 0.01$) and ($^{***,\#\#\#}P < 0.001$) difference from control, and from other groups (brackets) (Tukey's test). Panel C: The differential expression of repetitive elements was determined by quantitative RT-PCR. Data are presented as mean ± SD (n = 5). Asterisks and pound signs denote significant ($^{*,\#}P < 0.05$), ($^{**,\#\#}P < 0.01$) and ($^{***,\#\#\#}P < 0.001$) difference from control and from other groups (brackets) (Tukey's test). Panel D: Analysis of expression of genes involved in maintenance of DNA methylation. The differential gene expression was determined by quantitative RT-PCR. Data are presented as mean ± SD (n = 5). Asterisks denote significant ($^{*}P < 0.05$) and ($^{**}P < 0.01$) difference from control (Dunnett's test).

DNA methylation, the levels of expression of the maintenance DNA methyltransferase *Dnmt1* were twofold higher in HPSCs ($P < 0.01$) (Fig. 1B). Finally, the expression of both *de novo* methyltransferases *Dnmt3a* and *Dnmt3b* in HPSCs was higher than in MNCs (4.6-fold, $P < 0.01$ and 4.4-fold, $P < 0.1$, respectively).

Effects of ^{56}Fe-Ion Irradiation on DNA Methylation in MNCs and HPSCs Four Weeks after Exposure

Exposure to various environmental stressors, including ionizing radiation, may result in alterations in the cellular epigenome. To determine whether or not exposure to high-LET radiation may lead to stable alterations in DNA methylation in bone marrow cells and whether MNCs and HPSCs will respond differently, methylation of DNA was analyzed 4 weeks after exposure. Previous studies have shown that this time is sufficient for the repair of radiation-induced damage, but not for the restoration of normal patterns of DNA methylation, if the latter occur (*9*). We did not find significant alterations in the levels of 5-mC in MNCs after exposure to ^{56}Fe-ion radiation. There was a post-exposure increase in 5-mC levels in HPSCs, but that did not reach significance (Fig. 2A). Both repetitive elements, LINE1 and SINE B1, were hypomethylated in MNCs and hypermethylated in HPSCs in a dose-dependent manner, with the highest degree of hypermethylation observed after exposure to 0.4 Gy [4.2-fold increase in LINE1 ($P < 0.001$) and 7.6-fold increase in SINE B1 ($P < 0.01$) methylation, Fig. 2B].

Exposure to ^{56}Fe Ions Results in Differential Expression of Repetitive Elements in HPSCs and MNC

Taking into consideration that DNA methylation is involved in silencing of repetitive elements, we measured the expression of LINE1 and SINE B1 after exposure to ^{56}Fe-ion radiation. The prominent hypermethylation observed in LINE1 and SINE B1 upon irradiation was associated with unchanged (silenced) status of both examined repetitive elements in HPSCs (Fig. 2C). In contrast, significant and dose-dependent loss of expression of LINE1 and SINE B1 was observed in MNCs, with the most pronounced effects detected after irradiation to 0.4 Gy (3.9-fold, $P < 0.001$ for LINE1 and 3.3-fold, $P < 0.001$ for SINE B1) (Fig. 2C). These alterations in the expression of repetitive elements suggest that mechanisms other than DNA methylation may be involved in post-exposures regulation of repetitive elements in MNCs.

DNA Methylation Machinery in HPSCs but Not MNCs is Affected by ^{56}Fe-Ion Exposure

Based on the pronounced changes in DNA methylation associated with exposure to ^{56}Fe ions, we assessed the expression patterns of DNA methyltransferases *Dnmt1*, *Dnmt3a* and *Dnmt3b*. While minor changes were observed in the expression of these genes in MNCs, ^{56}Fe-ion irradiation decreased the mRNA abundance of all three genes in HPSCs. The most pronounced effects were associated with *de novo* methyltransferase *Dnmt3a*. Its

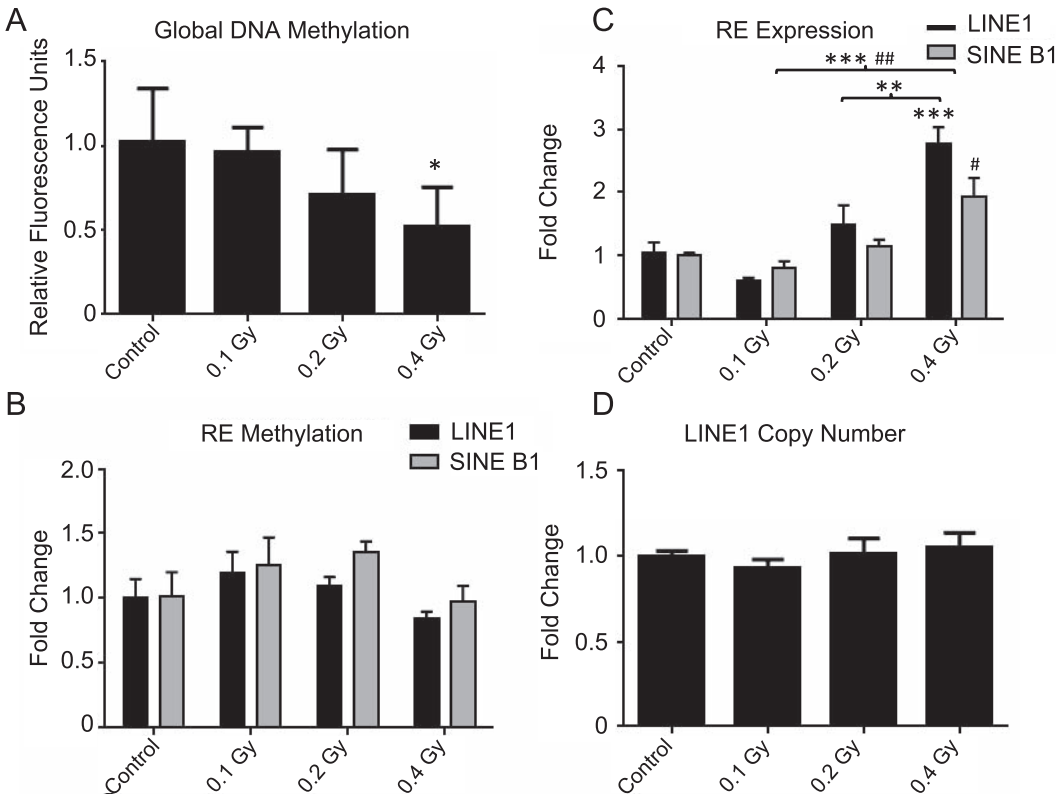

FIG. 3. Epigenetic effects of ^{56}Fe-ion irradiation in MNCs and HPSCs 22 weeks after exposure. Panel A: Global DNA methylation was measured by quantification of the 5-mC levels in DNA of HPSCs and MNCs using the fluorescence-based immunoassay. Data are presented as mean ± SD (n = 5). Asterisks denote significant ($^{*}P < 0.05$) difference from control (Dunnett's test). Panel B: DNA methylation in LINE1 and SINE B1 was measured by methylation-sensitive McrBC-qPCR assay. Data are presented as mean ± SEM (n = 5). Panel C: The differential expression of repetitive elements was determined by quantitative RT-PCR. Data are presented as mean ± SD (n = 5). Asterisks and pound signs denote significant ($^{*,\#}P < 0.05$), ($^{**,\#\#}P < 0.01$) and ($^{***,\#\#\#}P < 0.001$) difference from control, and from other groups (brackets) (Tukey's test). Panel D: Analysis of LINE copy numbers by qRT-PCR. Expression was normalized to 5S ribosomal DNA using the $\Delta\Delta C_t$ method.

expression was significantly diminished in all irradiated groups in a dose-independent fashion (Fig. 2D).

No Changes Associated with Bone Marrow Injury: Analyses of DNA Damage, Reactive Oxygen Species, Apoptosis and Senescence in HPSCs Four Weeks after Exposure to ^{56}Fe Ions

Exposure to ionizing radiation results in multiple effects in bone marrow through DNA damage induction, directly or indirectly, by means of ROS production. If the damage cannot be repaired, the cell will undergo apoptosis or senescence. Previous studies have reported acute effects of ^{56}Fe-ion irradiation on bone marrow (*34*). The long-term molecular effects, however, have never been evaluated. Therefore, we next investigated whether exposure to ^{56}Fe ions induces persistent DNA damage, increased production of ROS, as well as apoptosis and senescence in HPSCs 4 weeks after exposure. As shown in Supplementary Fig. S1 (http://dx.doi.org/10.1667/RR13580.1.S1), no profound effects were detected, suggesting that the observed epigenetic effects were not associated with persistent damage to DNA.

Persistence of Alterations in Global and Repetitive Elements-Associated DNA Methylation in HPSCs

Previous studies have reported long-term epigenetic alterations associated with exposure to low-LET radiation (*26*). To evaluate the persistence of epigenetic alterations in HPSCs associated with high-LET radiation exposure, the analysis of DNA methylation 22 weeks after ^{56}Fe-ion irradiation was performed. Contrary to hypermethylation effects observed 4 weeks after exposure, we noted significant loss of global DNA methylation in HPSCs after exposure to 0.4 Gy (1.9-fold, $P < 0.05$). Despite the general trend towards hypomethylation, no significant changes in methylation of LINE1 and SINE B1 repetitive elements were detected (Fig. 3A–B).

Expression of Repetitive Elements and Copies Number

Expression of both repetitive elements was dramatically increased after exposure to 0.4 Gy of ^{56}Fe ions (2.8-fold, *P*

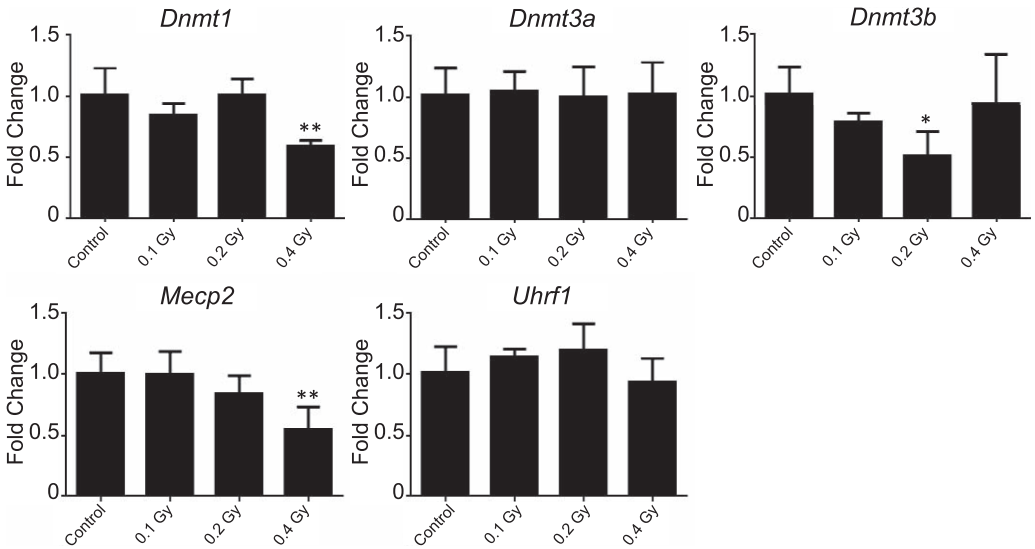

FIG. 4. Analysis of expression of genes involved in maintenance of DNA methylation. The differential gene expression was determined by quantitative RT-PCR. Data are presented as mean ± SD (n = 5). Asterisks denote significant (*$P < 0.05$) and (**$P < 0.01$) difference from control (Dunnett's test).

< 0.001 for LINE1 and 1.9-fold, $P < 0.05$ for SINE B1, Fig. 3C). Both LINE1 and SINE B1 are retrotransposons by nature, and their aberrant reactivation may result in subsequent ''copy-paste'' based increase in their copy numbers in the genome. This may lead to genome amplification, insertions, deletions and alterations in neighboring genes expression (*35, 36*). Of particular concern in this regard is LINE1, which comprises about 20% of mammalian genomes (*32*) and whose protein machinery is needed for transcription of short interspersed elements, such as SINE B1 (*37*). Therefore, we further measured the LINE1 copy numbers in HPSCs 22 weeks after ^{56}Fe-ions irradiation and found no significant changes (Fig. 3D).

Analysis of DNA Methylation Machinery in HPSCs 22 Weeks after ^{56}Fe-Ion Exposure

To further investigate the possible mechanisms of DNA hypomethylation observed in HPSCs, we measured the expression of DNA methyltransferases. While the expression of the *de novo* methyltransferases *Dnmt3a* and *Dnmt3b* was not affected by the exposure to 0.4 Gy of ^{56}Fe ions, the 1.9-fold decrease ($P < 0.01$) of *Dnmt1* expression was detected (Fig. 4A–C). Additionally, we measured the expression of *Uhrf1* and *Mecp2*. The former is responsible for recruiting *Dnmt1* to the replication fork and hemi-methylated sites (*13*) and has been also implicated in facilitation of response to radiation-induced DNA damage (*38*), while the latter is a methyl-CpG-binding protein that binds specifically to methylated DNA and is involved in regulation of gene expression. We detected a 1.9-fold loss of *Mecp2* expression after exposure to 0.4 Gy of ^{56}Fe ions, but no significant changes were detected in expression of *Uhrf1* (Fig. 4D–E).

Persistent Epigenetic Alterations are not Associated with Long-Term Bone Marrow Injury

Although the radiation-induced myelosuppression is usually rapidly resolved, there is a high probability of the development of residual long-term bone marrow injury (*39*). To determine whether or not the observed epigenetic alterations are associated with the delayed manifestation of bone marrow injury, we addressed the levels of DNA damage, production of reactive oxygen species, cellular senescence and apoptosis in HPSCs 22 weeks after exposure to 0.1, 0.2 and 0.4 Gy of ^{56}Fe ions. No significant changes were detected in HPSCs at this time point (Supplementary Fig. 2; http://dx.doi.org/10.1667/RR13580.1.S1).

DISCUSSION

In this study, we showed that epigenetic alterations can be detected in the murine bone marrow upon exposure to low doses of ^{56}Fe ions. First, we analyzed the DNA methylation in HPSCs and MNCs 4 weeks after irradiation. This time is usually sufficient to repair the DNA damage caused by ionizing radiation, but not for the restoration of alterations in DNA methylation in the radiation target tissue (*9*). Indeed, we did not observe any significant increases in DNA damage, oxidative stress, apoptosis or senescence. However, significant alterations in DNA methylation were detected in bone marrow in a cell type-specific and dose-dependent manner. Specifically, loss of LINE1 and SINE B1 repetitive elements-associated but not global DNA methylation was detected in MNCs that primarily consist of mature terminally differentiated cells with a limited lifespan. Absence of changes in global DNA methylation patterns in MNCs paralleled by decreased methylation of repetitive

elements may be associated with the redistribution of 5-mC within the genome, where demethylation of the pericentromatic chromatin (enriched in repetitive elements) is observed on one side and hypermethylation of coding sequences (primarily, promoter-first exon regions of tumor suppressor genes) is observed on the other side. Conversely, HPSCs that initially exhibited higher levels of DNA methylation responded to ^{56}Fe-ion irradiation by even more progressive hypermethylation. The latter was clearly associated with the hypermethylation of repetitive sequences, since a profound, dose-dependent increase in methylation was observed in two of the most abundant in the mammalian genome repetitive elements, LINE1 and SINE B1 (the latter corresponds to *Alu* elements in humans). The levels of 5-methylcytosine (5-mC) were also increased in HPSCs in response to ^{56}Fe-ion irradiation, although insignificantly. DNA methylation is crucial for the silencing of repetitive elements, and thus the observed hypermethylation in HSPCs may be considered as a protective mechanism preventing their reactivation. Interestingly, repetitive elements-associated hypomethylation, observed in MNCs, did not correlate with their expression, suggesting the silencing role of histone modifications and/or small regulatory RNAs, such as PIWI-interacting RNAs in MNCs (piRNAs) (*29, 40, 41*).

There is a high level of uncertainty in the long-term biological effects of exposure to high-LET radiation. Even though experimental evidence suggests higher RBE values for high-LET irradiation and formation of complex DNA damage, there is a lack of knowledge in regard to the long-term effects of radiation, especially those associated with low-dose exposures. For instance, only a few complex chromosomal aberrations are found in bone marrow one week after exposure to 1 GeV ^{56}Fe-ion radiation (doses of 0, 0.1, 0.5 and 1.0 Gy) (*34*) and minimal changes can be detected in the murine bone marrow one month after exposure to 1 GeV ^{56}Fe ion radiation (doses of 0, 0.5, 2 and 3 Gy) (*27*). Furthermore, the yield of chromosomal aberrations in blood lymphocytes in astronauts that spent about 2 years in a space mission, is very close to the initial levels detected before the first flight (*4*). In this study, we show that epigenetic alterations can be observed not only relatively short term after exposure (4 weeks), but that they persist and are detectable for at least 22 weeks after exposure. While changes at the earlier time point were associated with epigenetic alterations after all three doses (0.1, 0.2 and 0.4 Gy), the epigenetic effects 22 weeks postirradiation were confined primarily to the highest dose. Exposure to lower doses might be characterized by transitory epigenetic effects, while the exposure to a higher, leukemogenic dose of 0.4 Gy of ^{56}Fe ions (*7*) might be associated with persistent epigenetic alterations.

Another intriguing finding at this time point was the dose-dependent loss of global DNA methylation. DNA hypomethylation is a common epigenetic alteration observed in a majority of human cancers (*42*). Moreover, accumulating evidence clearly demonstrates that loss of global DNA methylation can be observed during cancer development, suggesting its causative role in carcinogenesis (*43, 44*). Interestingly, a few longitudinal studies performed in animal models suggest a carcinogenic potential of high-LET radiation. In particular, it has been reported that exposure to ^{56}Fe ions results in the development of AML and malignant lymphomas in different mouse strains (*7, 35*). Also, loss of global DNA and LINE1 methylation has been reported in humans occupationally exposed to benzene, a known cause of AML (*45*) and benzene's metabolites *in vitro* (*46*).

Global DNA hypomethylation, observed in HPSCs 22 weeks after exposure to ^{56}Fe ions, was associated with nonsignificant loss of DNA methylation in LINE1 and SINE B1. This is possibly due to the limited areas in the repetitive elements sequences in which methylation of DNA was assessed. However, expression of LINE1 and SINE B1 was dramatically increased and, together with the loss of global DNA methylation, may also be explained by the lower levels of *Mecp2*, the methyl-binding protein that has been recently shown to regulate LINE1 transcription (*47*).

Several mechanisms may be involved in the loss of global DNA methylation. It is well accepted that global DNA hypomethylation may be due to the repair of radiation-induced DNA damage and incorporation of cytosine, but not methylcytosine, during the repair process (*9*). However, this scenario seems unlikely since the damage caused by ^{56}Fe ions is complex and usually resolved by apoptosis rather than DNA repair (*48*). Additionally, it is highly unlikely for repair processes to occur 22 weeks after irradiation, especially in the absence of oxidative stress and DNA strand breaks. Another possible mechanism of loss of global DNA methylation is the impaired function of the DNA methylation machinery. For instance, the altered function of DNA methyltransferases may lead to imbalance in maintenance of the normal methylation patterns, resulting in alterations in DNA methylation (*44*). Previous studies reported decreased levels of *Dnmt1* after exposure to both high (*10, 26*) and low doses of low-LET radiation (*49*). Additionally, the deletion of one of the DNA methyltransferases may result in profound loss of DNA methylation at both LINE1 and SINE B1 (*50*). Congruent with those findings, we found significant twofold decreases ($P < 0.01$) in the expression of a maintenance DNA methyltransferase *Dnmt1*, as well as a methyl-binding protein *Mecp2*.

Another interesting finding of this study is that the cells with distinct epigenetic profiles, in particular, patterns of DNA methylation, respond to irradiation differently. This effect was previously observed when mice with distinct patterns of DNA methylation responded differently to administration of the methyl-deficient diet (*51*). The status of DNA methylation in the target cells and its role in response to exogenous stressors needs further investigation, since modulation of epigenetic parameters may aid in the development of effective radioprotectants and countermea-

TABLE 1
Epigenetic Effects of Exposure to 0.4Gy of ^{56}Fe Ions in Hematopoietic Progenitor and Stem Cells

2.57 p	4 weeks (fold change)	22 weeks (fold change)
Global DNA methylation		
5-mC	4.6	0.5*
Repetitive elements methylation		
LINE1	4.2***	0.8
SINE B1	7.6**	0.9
Repetitive elements expression		
LINE1	1.1	2.8***
SINE B1	1.2	1.9*
DNA methylation machinery expression		
Dnmt1	0.7	0.5**
Dnmt3a	0.6*	1
Dnmt3b	0.5	1

Notes. Data are presented as mean ± SD (n = 5). Asterisks denote significant (*$P < 0.05$), (**$P < 0.01$) and (***$P < 0.001$) difference from control (Dunnett's test).

sures. The limitations of this study are the utilization of male mice only, since gender differences in epigenetic response to radiation exposure have been previously reported (*52, 53*), and the assumption that sham irradiation would not result in any effects that were not observed in absolute control animals.

In conclusion, we posit that cells at earlier stages of differentiation, like HPSCs, are more prone to epigenetic alterations caused by HZE high-LET radiation such as ^{56}Fe ions than terminally differentiated MNCs. The epigenetic alterations after ^{56}Fe-ion irradiation are characterized by hypermethylation of repetitive elements at earlier time points and loss of global DNA methylation and reactivation of repetitive elements as a delayed consequence of exposure. These alterations were primarily observed after exposure to a leukemogenic dose of 0.4 Gy and associated with sustained loss of function of DNA methylation machinery (Table 1). Together, these findings suggest that epigenetic alterations might contribute to the development of high-LET radiation-induced leukemia.

SUPPLEMENTARY INFORMATION

Supplementary Fig. S1. Analyses of the levels of (panel A) intracellular reactive oxygen species (as measured by fluorescence intensity of 2′7'-dichlorofluorescein), (panel B) DNA damage (as measured by fluorescence intensity of γ-H2AX, (panel C) apoptosis (as measured with the Annexin V staining) and (panel D) senescence (as measured by SA-β-gal activity) in HPSCs 4 weeks after exposure to ^{56}Fe ions.

Supplementary Fig. S2. Analyses of the levels of (panel A) intracellular reactive oxygen species (as measured by fluorescence intensity of 2′7'-dichlorofluorescein), (panel B) DNA damage (as measured by fluorescence intensity of γ-H2AX, (panel C) apoptosis (as measured with the Annexin V staining) and (panel D) senescence (as measured by SA-β-gal activity) in HPSCs 22 weeks after exposure to ^{56}Fe ions.

Supplementary Table S1. Primers utilized in the current study for the analysis of repetitive elements.

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