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Role of epigenetic effectors in maintenance of the long-term persistent bystander effect in spleen *in vivo*

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Radiation therapy is a primary treatment modality for brain tumors, yet it has been linked to the increased incidence of secondary, post-radiation therapy cancers. These cancers are thought to be linked to indirect radiation-induced bystander effect. Bystander effect occurs when irradiated cells communicate damage to nearby, non-irradiated 'bystander' cells, ultimately contributing to genome destabilization in the non-exposed cells. Recent evidence suggests that bystander effect may be epigenetic in nature; however, characterization of epigenetic mechanisms involved in bystander effect generation and its long-term persistence has yet to be defined. To investigate the possibility that localized X-ray irradiation induces persistent bystander effects in distant tissue, we monitored the induction of epigenetic changes (i.e. alterations in DNA methylation, histone methylation and microRNA (miRNA) expression) in the rat spleen tissue 24 h and 7 months after localized cranial exposure to 20 Gy of X-rays. We found that localized cranial radiation exposure led to the induction of bystander effect in lead-shielded, distant spleen tissue. Specifically, this exposure caused the profound epigenetic dysregulation in the bystander spleen tissue that manifested as a significant loss of global DNA methylation, alterations in methylation of long interspersed nucleotide element-1 (LINE-1) retrotransposable elements and down-regulation of DNA methyltransferases and methyl-binding protein methyl CpG binding protein 2 (MeCP2). Further, irradiation significantly altered expression of miR-194, a miRNA putatively targeting both DNA methyltransferase-3a and MeCP2. This study is the first to report conclusive evidence of the long-term persistence of bystander effects in radiation carcinogenesis target organ (spleen) upon localized distant exposure using the doses comparable with those used for clinical brain tumor treatments.

Introduction

Radiation therapy is the number one treatment for almost all brain tumor types (1), malignant (2–4), as well as benign (5). Despite the markedly better prognosis and increased patient survival rates after radiation therapy, a serious drawback of induction of secondary tumors arose. Numerous cases of secondary cancer development following radiation therapy have been reported (6,7), yet mechanisms of secondary cancers remain enigmatic. Recent studies attribute secondary carcinogenesis to indirect radiation effects, particularly to the radiation-induced bystander effect.

Abbreviations: DNMT, DNA methyltransferase; IR, ionizing radiation; LINE-1, long interspersed nucleotide element-1; MeCP2, methyl CpG binding protein 2; miRNA, microRNA; ORF1, open reading frame 1; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; RT, reverse transcription.

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Bystander effect is a phenomenon whereby irradiated cells communicate the damage to the non-irradiated nearby bystander cells, thus contributing to their genome destabilization and carcinogenesis (8–10). This enigmatic phenomenon is considered by some researchers to be a negative complication in radiation oncology, whereas others refer to it as an important protective homeostatic response (8,10–13).

The majority of the bystander effect data currently come from the studies in cell cultures, whereas *in vivo* data are scarce (8,14,15). Mechanistically, current models link radiation-induced bystander effects with the radiation-induced genome instability (15–18). Radiation-induced genomic instability that encompasses a wide range of end points such as gross genome rearrangements, chromosome aberrations and gene mutation (18,19) was recently suggested to have an epigenetic nature, yet the contribution of known epigenetic mechanisms to its generation has to be defined (19,20).

Epigenetic changes are heritably stable alterations and include DNA methylation, histone modifications and RNA-mediated silencing (21). Aberrant cytosine DNA methylation is associated with genomic instability, increased genome rearrangement rates and is linked to cancer development (21–23). A variety of DNA-damaging agents, including ionizing radiation (IR), are known to affect genome DNA methylation pattern (24–26). DNA methylation changes are closely connected with the alterations in the other components of chromatin structure, primarily histone modifications, which are also affected by IR exposure (27). One of the mechanisms of chromatin modification is the involvement of small regulatory RNAs (28). Regulatory RNAs were associated with cellular differentiation, proliferation, apoptosis, as well as with predisposition to cancer (29–31). These molecules have the ability to change the pattern of gene expression or target epigenetic modifications in various regions of the genome (24–27). The role of RNA-mediated silencing, specifically microRNAs (miRNAs), in bystander effects has never been addressed.

We have recently confirmed the existence of *in vivo* bystander effects using a mouse skin model whereby half of the animal body was exposed to radiation whereas the other half was protected by the medical grade shield (32). The data showed that X-ray exposure to one side of the animal body caused profound epigenetic changes in the unexposed bystander body half 4 days after exposure. The potential of the localized body part exposures to induce bystander effects has never been addressed in detail.

The data obtained in the mouse skin model showed that bystander effects were the most prominent when spleen was in the field of irradiation, suggesting a possible role of spleen in the bystander effects (32). Spleen is an important radiation target organ (24,25), yet the existence and molecular mechanisms of the radiation-induced bystander effects in spleen have never been addressed.

Also, to be relevant for carcinogenesis, the bystander effects should accumulate and/or persist over a long period of time. Yet, the long-term persistence of the distant bystander lesions in somatic organs upon irradiation has never been addressed.

To investigate the possibility that localized X-ray irradiation induces bystander effects in the distant tissue, we monitored the induction of epigenetic changes (i.e. DNA methylation, histone methylation and miRNA expression) in spleen tissue 24 h and 7 months after the localized cranial irradiation. Here, we report that localized cranial radiation exposure leads to decreased levels of global DNA methylation, alters the levels of key proteins known to modulate methylation patterns and silencing (i.e. *de novo* methyltransferase DNA methyltransferase (DNMT)-3a and methyl-binding protein MeCP2) and contributes to reactivation of long interspersed nucleotide element-1 (LINE-1) retrotransposons in the bystander spleen, located at least 16 cm from the irradiation site. We also show that radiation exposure

results in the altered levels of miR-194 in plasma and spleen of animals subjected to cranial X-irradiation. Importantly, the changes persisted for 7 months after exposure. These are some of the first data to clearly demonstrate that bystander effects occur *in vivo* in distant tissue and, most importantly, persist over a long period of time and are epigenetically regulated.

Materials and methods

Model and irradiation of animals

In this study, we examined the genetic and epigenetic alterations in rat spleen following *in vivo* cranial irradiation exposure. Rats (3-month-old male Long Evans animals) were randomly assigned to different treatment groups. Handling and care of animals were in the strict accordance with the recommendations of the Canadian Council for Animal Care and Use. The procedures have been 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 whole body-exposed cohort (six animals) received 20 Gy (5 cGy/s) of X-rays (90 kV, 5 mA) applied as two doses of 10 Gy in two consecutive days. The animals were killed 24 h after the second exposure. The head-exposed cohort (18 animals) received 20 Gy (5 cGy/s) of X-rays (90 kV, 5 mA) to the hippocampal area of the scull, applied as two doses of 10 Gy in two consecutive days. The rest of the animal body protected by an ∼3 mm thick lead shield, the same type as used for the human body protection in diagnostic radiology. The protection of shielded bystander tissue was complete, as verified by careful dosimetry using RAD-CHECK™ monitor (Nuclear Associates Division of Victoreen, Carle Place, NY). Six animals were humanely killed 24 h after exposure to address the early bystander effects. Twelve animals were humanely killed 7 months after exposure. This time point is sufficient to see the persistence, if any, of the radiation-induced changes, as 7 months in rat life correspond to 10 human years (33). Control rats (12 animals) were sham treated. For the sham treatment the animals were placed into the irradiator machine and completely shielded by lead. No radiation leakage through the shield has occurred as verified by dosimetry using RAD-CHECK™ monitor (Nuclear Associates Division of Victoreen). Six control animals were killed 24 h after exposure, whereas the other six animals 7 months after exposure. Spleen tissue was sampled upon killing, snap frozen and processed for further molecular studies. Upon killing, blood from *vena cava* was collected into the polypropylene tubes with heparin and plasma was obtained by centrifugation (440*g*, 10 min). Plasma was carefully removed and snap frozen.

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

Total DNA was prepared from spleen tissue of exposed and control animals using Qiagen DNAeasy kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's protocol. The cytosine extension assay and the determination of the absolute percent of double-stranded unmethylated CCGG sites were conducted as described previously (27,32,34). DNA methylation changes in the exposed cohorts were related to the age-matched controls. No statistically significant age-related changes were noted.

Western immunoblotting

Western immunoblotting for DNMT1, DNMT3a, DNMT3b, MeCP2 and proliferating cell nuclear antigen (PCNA) was conducted using spleen tissue of exposed and control animals as described before (27,32) using the following antibodies—DNMT1 (1:1000, Abcam, Cambridge, MA), DNMT3a, DNMT3b (1:500, Abgent, San Diego, CA), MeCP2 (1:1000, Abcam) and PCNA (1:500, Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) and the enhanced chemiluminescence plus immunoblotting detection system (Amersham, Baie d'Urfé, Québec, Canada). Chemiluminescence was detected by Biomax MR films (Eastman Kodak, New Haven, CT). Signals were quantified using NIH ImageJ 1.63 Software and normalized to both glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the Mr 50 000 protein that gave consistent results. Protein levels in the exposed cohorts were related to the age-matched controls.

Analysis of LINE-1 methylation status by COBRA assay

Methylation status of LINE-1 was determined by combined bisulfite restriction analysis (COBRA) assay, which consists of a standard bisulfite modification of genomic DNA, subsequent polymerase chain reaction (PCR) amplification and digestion of PCR product with appropriate restriction endonuclease (35–37). The combination of sodium bisulfite treatment and PCR amplification results in methylation-dependent creation of new restriction endonuclease sites, such as RsaI, or methylation-dependent retention of pre-existing sites, such as BstUI. Briefly, genomic DNA was treated with sodium bisulfite as described previously in detail (37). Bisulfite-modified DNA was PCR amplified with primers corresponding to the regulatory region of rat LINE-1 sequence (37). The sense primer was 5′-TTTGGTGAGTTTGGGATA-3′ and the anti-sense primer was 5′-CTCAAAAATACCCACCTAAC-3′. The PCR products were digested with RsaI or BstUI restriction endonuclease (New England Biolabs, Beverly, MA). The digested products were separated on 3% high-resolution agarose gels (Sigma, St Louis, MO), stained with ethidium bromide, photographed and the band intensity was analyzed by NIH ImageJ 1.63 Software.

mRNA isolation and real-time reverse transcription–PCR

Total mRNA was isolated from frozen spleen tissue of control and exposed animals by using TRIzol reagent (Invitrogen, Burlington, Ontario, Canada) and first-strand cDNA template was synthesized using RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Burlington, Ontario, Canada) according to the manufacturer's instructions. The primers used in this study were synthesized by Operon Biotechnologies (Huntsville, AL). The LINE-1 open reading frame 1 (ORF1) primer sequences were as follows: LINE-1 ORF1 forward 5′-AAGAAACACCTCCCGTCACA-3′ and LINE-1 ORF1 reverse 5′-CCTCCTTATGTTGGGCTTTACC-3′. The beta actin primers were as follows: beta actin forward 5′-CCTCTGAACCCTAAGGCCAA-3′ and beta actin reverse 5′-AGCCTGGATGGCTACGTACA-3′. The data for LINE-1 ORF1 were standardized against the actin data. Control wells containing SYBR Green PCR master mix and primers without sample cDNA emitted no fluorescence after 40 cycles.

Histone extraction

The acid extracts were prepared from frozen spleen tissues using lysis buffer containing 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol and 1.5 mM phenylmethylsulfonyl fluoride, followed by addition of HCl to a final concentration of 200 mM. Cell lysates were centrifuged at 14 000*g* for 10 min at 4°C and the acid-insoluble pellets were discarded. The supernatant fractions, which contain the acid soluble proteins, were purified by sequential dialysis against 100 mM acetic acid and H_2O. Protein concentrations were determined by Bradford assay (Pierce, Rockford, IL). Equal amount of total histones (40 μg) were resolved on 15% polyacrylamide gel. Proteins were transferred onto polyvinylidene difluoride membranes (27).

Analysis of histone modifications

Equal amount of total histones were resolved on 15% polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. Membranes were incubated using anti-trimethyl-histone H3 lysine 9 (1:1000), anti-trimethyl-histone H4 lysine 20 (1:2000), anti-histone H3 (1:1000) and anti-histone H4 (1:1000) primary antibodies (Upstate, Charlottesville, VA). Upon secondary alkaline phosphatase-labeled antibody treatment chemifluorescence was detected with the ECF substrate for Western Blotting (GE Healthcare, Piscataway, NJ) and measured directly by Storm Imaging System (Molecular Dynamics, Sunnyvale, CA).

miRNA microarray expression analysis

Total RNA from frozen plasma was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA from rat spleen was also extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, size fractionated (<200 nt) by using the *mir*Vana kit (Ambion, Austin, TX) and labeled with Cy3 and Cy5 fluorescent dyes. Dye switching was performed to eliminate the dye bias. Pairs of labeled samples were hybridized to dual-channel microarrays. Microarray assays were performed on a μParaFlo microfluidics chip with each of the detection probes containing a nucleotide sequence of coding segment complementary to a specific miRNA sequence and a long non-nucleotide molecule spacer that extended the detection probe away from the substrate. The maximum signal level of background probes was 180. A miRNA detection signal threshold was defined as twice the maximum background signal. Normalization was performed with a cyclic LOWESS (locally-weighted regression) method to remove system-related variations. Data adjustments included data filtering, log2 transformation and gene centering and normalization. The *t*-test analysis was conducted between control and exposed sample groups. miRNAs with P values < 0.05 were selected for cluster analysis. Two independent microarray hybridizations were performed, each involving four control and four exposed samples to insure the reproducibility of the results. The miRNA microarray analysis was performed by LC Sciences (Austin, TX).

Quantitative real-time reverse transcription–PCR miRNA expression analysis

Reverse transcription (RT)–PCRs on total spleen and plasma RNA were performed by using SuperTaq Polymerase (Ambion) and the *mir*Vana qRT–PCR

miRNA Detection Kit™ (Ambion) following the manufacturer's instructions. Reactions contained *mir*Vana qRT–PCR Primer Sets™ specific for hsa-miR-194 and human 5S rRNA as positive controls. Real-time RT–PCR was performed on a SmartCycler (Cepheid, Sunnyvale, CA) and end-point reaction products were also analyzed on a 3.5% high-resolution agarose gel stained with ethidium bromide.

Statistical analysis

Statistical analysis (Student's *t*-test, Bonferroni correction, Tukey–Kramer test and Dunnett's test) was performed using the MS Excel 2000 and JMP5 software packages.

Results

In this study, we used a rat spleen model to gain further insight into the occurrence, possible persistence and molecular underpinnings of the somatic bystander effect *in vivo* after a localized cranial irradiation. The animals received either a whole-body exposure of 20 Gy or the localized cranial exposure of 20 Gy to the hippocampal area of the brain, whereas the rest of the body was shielded by lead. Shielding was complete as verified by careful dosimetry. We examined the role of epigenetic factors in the generation of bystander effect *in vivo* in the rat spleen. This important organ is frequently affected following therapeutic radiation exposures and is one of the targets of the radiation oncology side effects (38).

Significant and persistent loss of global genome DNA methylation in bystander spleen

Initially, we investigated the IR-induced changes in global cytosine DNA methylation in control, exposed and bystander spleen tissue 24 h and 7 months after radiation treatment. To determine the absolute percent of methylated HpaII sites in DNA, we used the HpaII/MspI-based cytosine extension assay that measures the proportion of CCGG that had lost methyl groups on both strands. While HpaII cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands, its isoschizomer MspI which cleaves CCGG sites in DNA regardless of CpG methylation status. The absolute percent of double-stranded unmethylated CCGG sites can be calculated by relating the data of HpaII and MspI digests (34). Because the vast majority of the frequently occurring HpaII tetranucleotide recognition sequences are constitutively methylated *in vivo*, an increase in cleavage at these sites is an indicator of genome-wide hypomethylation (34). We found that whole body X-ray exposure resulted in a significant increase in the absolute percent of unmethylated CCGG sites in rat spleen as compared with controls (Figure 1A). Localized head exposure also led to a significant increase in the absolute percent of unmethylated CCGG sites in the shielded bystander spleen as compared with the control (Figure 1A and B). This effect, indicative of a profound global DNA hypomethylation, was seen in the bystander spleen 24 h and 7 months after cranial irradiation (Figure 1A and B).

Hypomethylation-related reactivation of LINE-1 retroelements in bystander spleen

Having seen the significant and persistent global DNA hypomethylation in rat spleen 7 months after cranial irradiation, we decided to further analyze the nature of the DNA methylation changes and the possible hypomethylation target loci. Several studies in humans and rodents have shown that global DNA demethylation resulted in activation of transposable elements contributing to genome instability (39,40). With this in mind, we analyzed the levels of DNA methylation of the LINE-1 transposable retroelements that constitute almost 23% of rat genome (41). Figure 2 shows the status of the LINE-1 regulatory region methylation in spleen of the control rats and the rats exposed to cranial irradiation, as determined by the COBRA assay (35,37). The assay is based on digestions of the PCR fragment amplified from the bisulfite-converted DNA, where all non-methylated cytosines are converted to thymines. LINE-1-specific primers amplified the fragment of 163 nt from the bisulfite-converted DNA (Figure 2A). This fragment contains two sequences that can be recognized by

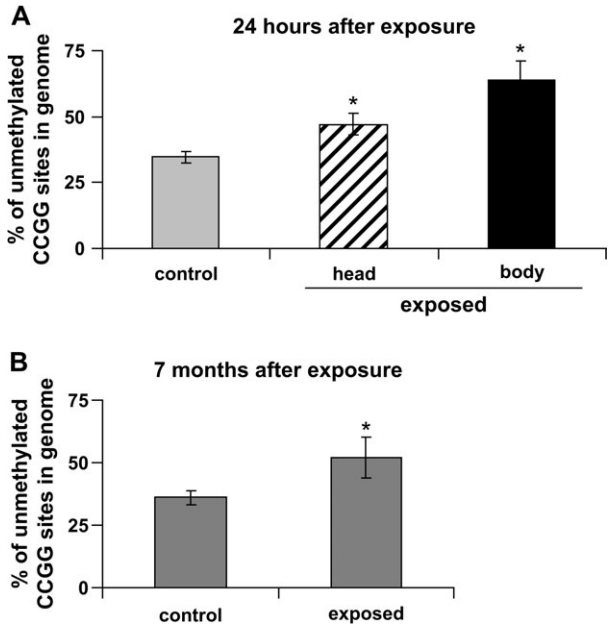

Fig. 1. Pronounced loss of DNA methylation in rat spleen after the whole body and the localized cranial irradiation. Levels of the global genome DNA methylation in spleen tissue were measured by the HpaII/MspI cytosine extension assay. HpaII that cleaves CCGG sequences when internal cytosine residues are unmethylated on both strands. MspI is an isoschizomer of HpaII that cleaves CCGG sites in DNA regardless of CpG methylation status. The absolute percent of double-stranded unmethylated CCGG sites was calculated by relating the data of HpaII and MspI digests. Data are presented as mean values ± SD, $^{*}P < 0.01$, Student's *t*-test, Dunnett's test and Tukey–Kramer test. (**A**) DNA methylation changes observed 24 h after the whole body and head exposure and (**B**) DNA methylation changes seen 7 months after the head exposure.

BstUI and RsaI endonucleases, upon retaining and modifying the original sequence after bisulfite conversion. Digestion with BstUI is only possible if both cytosines at recognition sequence CGCG are methylated and thus protected from conversion. Complete methylation will lead to the complete cleavage resulting in two bands of 80 and 83 nt long (seen as one band on the gel; Figure 2B), whereas the loss of methylation at any of the CpG cytosines would prevent the cleavage and contribute to 'uncut' 163 nt band (Figure 2B). The recognition sequence GTAC for the RsaI enzyme can be formed from GGCACG sequence in the 163 nt fragment of LINE-1 promoter when non-CpG cytosine is completely unmethylated and CpG cytosine is completely methylated (Figure 2C). Previous papers showed that methylation at the non-CpG sites at LINE-1 promoter is extremely rare, with 98.2% of all the non-CpG cytosines been unmethylated (37,42). Thus, it is safe to suggest that the RsaI recognition site is influenced primarily by the loss of methylation at CpG cytosine (Figure 2C).

COBRA assay revealed that LINE-1 promoters were indeed hypomethylated in the bystander rat spleen 7 months after cranial irradiation. There was a 5–10% decrease in the cleavage of the PCR products using BstUI and RsaI enzymes in the group of exposed versus group of control animals (Figure 2D and E). These phenomena, indicative of LINE-1 reactivation, can in turn contribute to the gross genome rearrangements and genome instability resulting in the neoplastic cell transformation. LINE-1 activation was previously documented in tumor cells and was paralleled by the global genome hypomethylation (43).

To prove our hypothesis, we analyzed the steady-state level of LINE-1 ORF1 RNA. RT–PCR analysis of spleen tissue revealed significant 60% increase in expression of LINE-1 in the bystander spleens of the head-exposed rats as compared with the controls (Figure 2F).

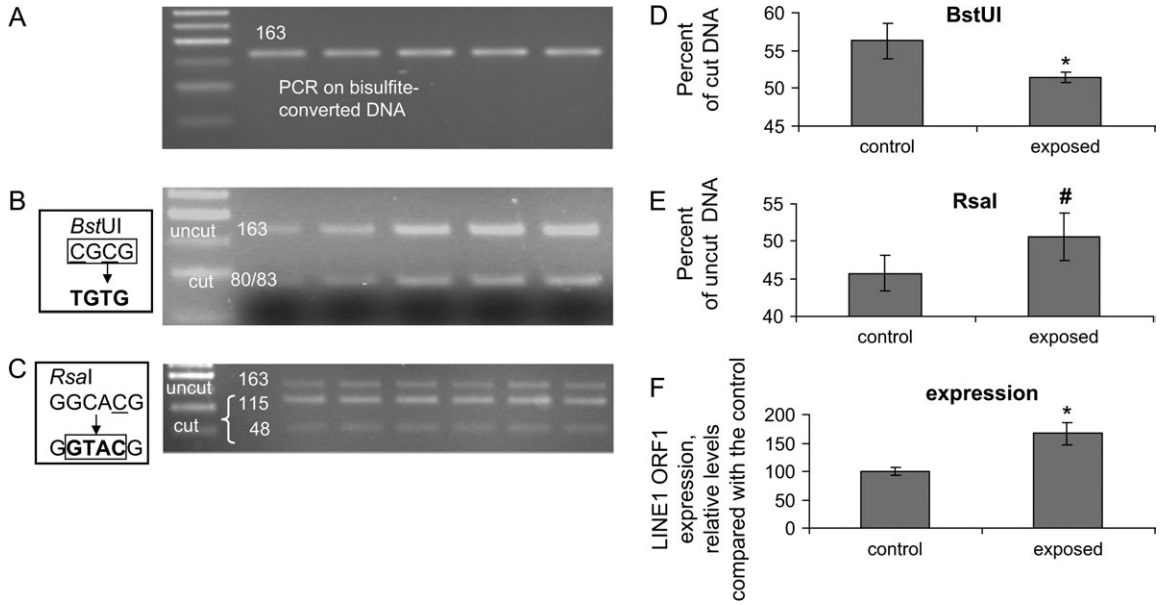

Fig. 2. Cranial irradiation leads to altered methylation of LINE-1 promoter, resulting in elevated expression of LINE-1 ORF1 in the spleen of exposed rats. Methylation status of LINE-1 was determined by COBRA assay, involving bisulfite modification of genomic DNA, subsequent PCR amplification and digestion of PCR product with BstUI and RsaI enzymes. Sizes of the DNA fragments were determined using the 50 bp DNA Step Ladder (Promega, Madison, WI). (**A**) PCR amplification of 163 nt fragment from LINE-1 promoter. (**B**) Methylation dependent retention of pre-existing BstUI restriction sites. Unmethylated CpG cytosines (underlined) at CGCG recognition sequence (squared) can be lost by bisulfite conversion (sequence in bold), resulting in uncut bands (163 nt). Methylation at both sites allows the cleavage, resulting in fragments of 80 and 83 nt (seen as one band). (**C**) CpG methylation-dependent retention of cytosine (underlined) at GGCACG sequence results in generation of RsaI recognition site (squared and in bold), leading to cleavage of the 163 nt fragment into fragments of 48 and 115 nt. Loss of methylation at CpG cytosine will prevent the cleavage. (**D**) Quantification of BstUI cut fragments. The *y*-axis shows the percent of cleaved 163 nt fragment presented as an average (with SD). (**E**) Quantification of RsaI cut fragments. The *y*-axis shows the percent uncleaved 163 nt fragment presented as an average (with SD). (**F**) Levels of LINE-1 ORF1 transcript detected by RT–PCR; steady-state RNA levels relative to those of control animals are shown as the mean ± SD, *significant, 95% confidence limit, $P < 0.05$ and #90% confidence limit, $P < 0.1$ (Student's *t*-test).

Altered levels of DNA methyltransferases and methyl-binding proteins in bystander spleen

Having seen the pronounced and persistent DNA hypomethylation in the exposed and bystander spleen, we proceeded with the analysis of the possible mechanisms of this phenomenon. We addressed the radiation-induced changes in the expression levels of the maintenance (DNMT1) and *de novo* (DNMT3a and DNMT3b) methyltransferases (44) in the control, exposed and bystander spleen tissue 24 h and 7 months after irradiation.

We noted that the levels of methyltransferases DNMT1, DNMT3a and DNMT3b and methyl-binding protein MeCP2 were statistically significantly down-regulated 24 h upon the whole-body as well as the localized head exposure as compared with the controls (Figure 3). We also found that the whole-body exposure resulted in a significant down-regulation of the PCNA 24 h after exposure (Figure 3). Contrarily, PCNA levels were not altered in the bystander spleen 24 h after exposure (Figure 3). These data are in good agreement with the whole-body irradiation-induced DNA hypomethylation and reduced expression of DNMTs and MeCP2 in mouse spleen reported previously by our group (24,25).

Importantly, altered protein levels were also observed 7 months after cranial radiation exposure (Figure 4). We found that DNMT1 levels were statistically significantly decreased in the spleen of the bystander group 7 months after cranial irradiation (Figure 4).

At the same time, we noted that the levels of *de novo* methyltransferase DNMT3a were also statistically significantly down-regulated in bystander spleen 7 months after exposure (Figure 4). We have shown previously that DNMT3a expression was reduced following IR exposure, and correlated with post-irradiation DNA hypomethylation (24,25). Also, DNMT3a expression was previously found to be down-regulated in the bystander skin (32).

In addition to the DNA methylation loss and down-regulation of the key methyltransferases, we also noted a significantly lower level of methyl-binding protein MeCP2 in the bystander spleen 7 months after irradiation (Figure 4). This protein was shown to interact with methylated DNA and affect the methylation-mediated chromatin remodeling and gene silencing (21,45). The contribution of MeCP2 to the IR-induced genome instability and bystander effects have yet to be further analyzed.

Since DNA replication is one of the most active mechanisms of partial loss of methylation, it was important to show that cell proliferation was not induced in bystander spleens either 24 h or 7 months after cranial exposure (Figures 3 and 4). Western blot analysis did not find any changes in the level of the PCNA, confirming that the observed changes were not linked to the increase in cellular proliferation (Figure 3).

The reduced levels of DNMTs and MeCP2 seen 7 months after exposure may be linked to the LINE-1 hypomethylation and reactivation seen at the same time (Figure 2). Previously, down-regulation of methyl-CpG-binding protein MeCP2 was shown to pre-dispose the cells to elevated levels of mutation and LINE-1 retrotransposon reactivation, and result in genome instability and aberrant gene expression rates, leading to carcinogenesis (46). Also, altered levels of DNMT1, DNMT3a or 3b significantly reduced DNA methylation levels in the LINE-1 repetitive elements (47).

Undoubtedly, DNA methylation changes and altered expression/activity of DNA methyltransferases may in part be linked to the DNA damage induced by the reactive oxygen species generated upon radiation exposure (24,25,27). Reactive oxygen species were implicated in the bystander effects in the cell cultures, yet their exact role in the bystander effects *in vivo* still need to be addressed.

Lack of histone methylation changes in bystander spleen

Recent studies indicate that the global loss of DNA methylation may be paralleled by changes in histone methylation, specifically histones

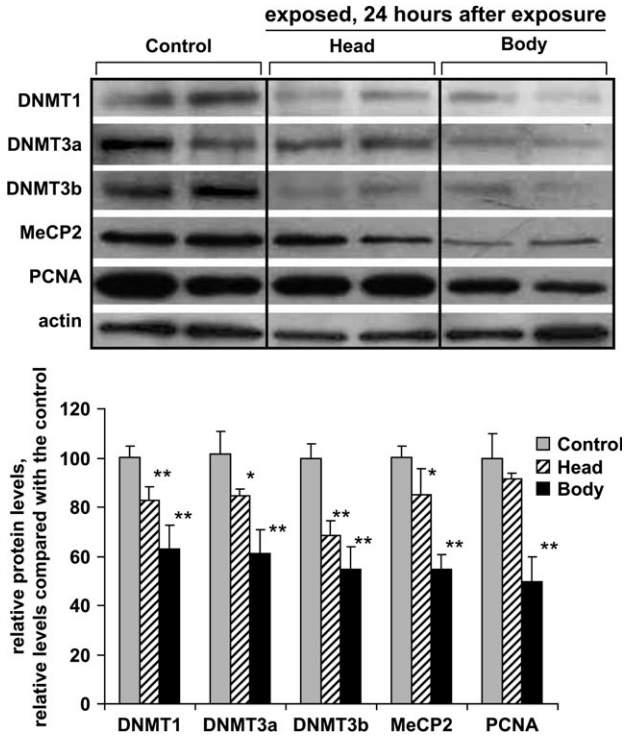

Fig. 3. Altered expression of DNA (cytosine-5) methyltransferases, methyl-binding protein MeCP2 and PCNA in spleen of rats 24 h after whole-body or localized cranial irradiation. Lysates of spleen tissue were subjected to immunoblotting using antibodies against DNMT1, DNMT3a, DNMT3b, MeCP2, PCNA and actin (loading control). Protein levels relative to those of control animals are shown as the mean ± SD, $^{*}P < 0.0167$, $^{**}P < 0.01$ Student's *t*-test with Bonferroni correction for multiple comparisons, Dunnett's test and Tukey–Kramer test. Representative western blots from among five independent technical repeats of the experiments are shown.

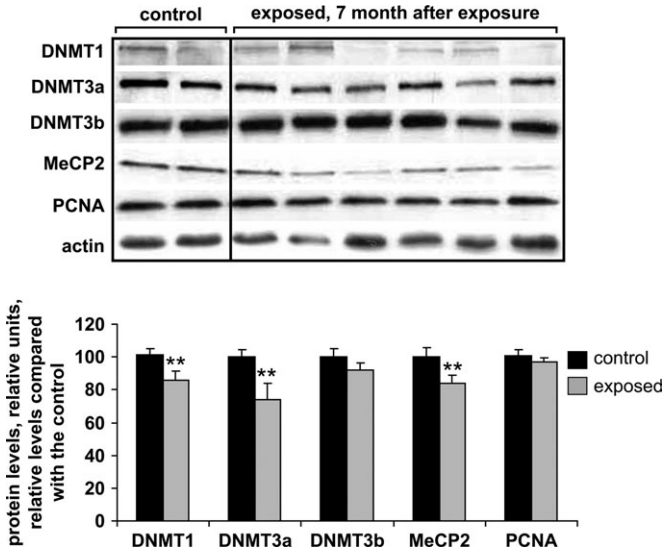

Fig. 4. Altered expression of DNA (cytosine-5) methyltransferases, methyl-binding protein MeCP2 and PCNA in spleen of rats 7 months after localized cranial irradiation. Lysates of spleen tissue were subjected to immunoblotting using antibodies against DNMT1, DNMT3a, DNMT3b, MeCP2, PCNA and actin (loading control). Protein levels relative to those of control animals are shown as the mean ± SD, **significant, 99% confidence limit, $P < 0.01$, Student's *t*-test, Dunnett's test and Tukey–Kramer test; *significant, 95% confidence limit, $P < 0.05$, Student's *t*-test, Dunnett's test and Tukey–Kramer test. Representative western blots from among five independent technical repeats of the experiments are shown. Black bars, control animals and gray bars,animals subjected to cranial irradiation.

H3 and H4 hypomethylation (48). The combined loss of DNA methylation and trimethylation of histone H3 lysine 9 (H3K9me3) and H4 lysine 20 (H4K20me3) were reported to be signs of carcinogenesis. The interplay between radiation-induced DNA methylation changes and histone modifications is still under investigation (27). The roles of histone modifications in bystander effects have never been addressed. Thus, the pronounced DNA methylation changes have prompted us to investigate the changes in histones H3K9 and H4K20 trimethylation in control, exposed and bystander spleen 24 h and 7 months after exposure. We noted that whole-body exposure to 20 Gy of X-rays resulted in a significant decrease in the levels of H4K20me3 (Figure 5A). This was in agreement with the loss of H4K20 trimethylation upon irradiation in mouse thymus tissue reported previously by our group (27). Yet, we did not observe any significant changes in histone H3K9me3 and histone H4K20me3 levels in the bystander spleen tissue either 24 h or 7 months after irradiation (Figure 5A and B).

miRNA expression in bystander tissue

Another mechanism of epigenetic control is through the involvement of small regulatory RNAs. Among those of special interest are miRNAs. miRNA molecules are small non-coding RNAs that function as key regulators of RNA interference pathway (29–31). miRNAs can reduce the levels of their target transcripts as well as the amounts of protein encoded by their transcripts (29–31). By doing so miRNAs regulate RNA stability and translatability, thus contributing to gene silencing. In parallel, their roles as regulators of epigenetic status and chromatin structure have also been proposed (28). Thereafter, miRNAs play crucial roles in regulating cellular differentiation, proliferation and apoptosis. Further to this, miRNA expression changes are common occurrence in cancers and were suggested to play important roles in pathogenesis of human tumors (49,50).

Thus, in a search for the possible regulators of the long-term persistent bystander effects, we analyzed the role of miRNAs. We set out to determine the expression patterns of known miRNAs in spleen of control and head-exposed animals 7 months after irradiation using the miRNA microarray technology. Two independent miRNA microarray hybridizations were performed, each involving four control and four bystander spleen samples. We identified that miR-194 was significantly up-regulated in bystander spleen 7 months after exposure. We also noted a subset of miRNAs that exhibited an up-regulation trend (90% confidence limit) (data not shown). To confirm the result of microarray analysis, we carried out RT–PCR of miR-194. The RT–PCR was a solid validation of the microarray result (Table I).

We next proceeded to address the early changes in *miR*-194 expression upon whole-body and cranial irradiation using the RT–PCR approach. We found that the levels of miR-194 were significantly elevated in the spleen of whole-body and head-exposed animals 24 h after irradiation (Table II). Furthermore, the elevated levels of miR-194 were also seen in plasma of the whole-body and head-exposed animals 24 h after irradiation (Table II), suggesting the possible spread on miRNAs by blood.

We next proceeded with identification of putative miR-194 targets using the known algorithms (51). To our surprise, we found that *miR-194* may be targeting DNMT3a and MeCP2, the proteins that were significantly down-regulated in the exposed and bystander spleen tissue 24 h and 7 months after irradiation (Figures 3 and 4).

Thus, up-regulation of miR-194 may be seen as a putative step in maintenance of the low levels of DNMT3a and MeCP2, the key regulators of DNA methylation. The low levels of the aforementioned proteins may consequently lead to global genome hypomethylation and locus-specific DNA methylation loss in LINE-1 retrotransposons (Figure 2). Thus, miR-194 may be one of the putative players in maintenance of epigenetic bystander response in rat spleen. This miRNA can also regulate a variety of other targets influencing cellular stress responses.

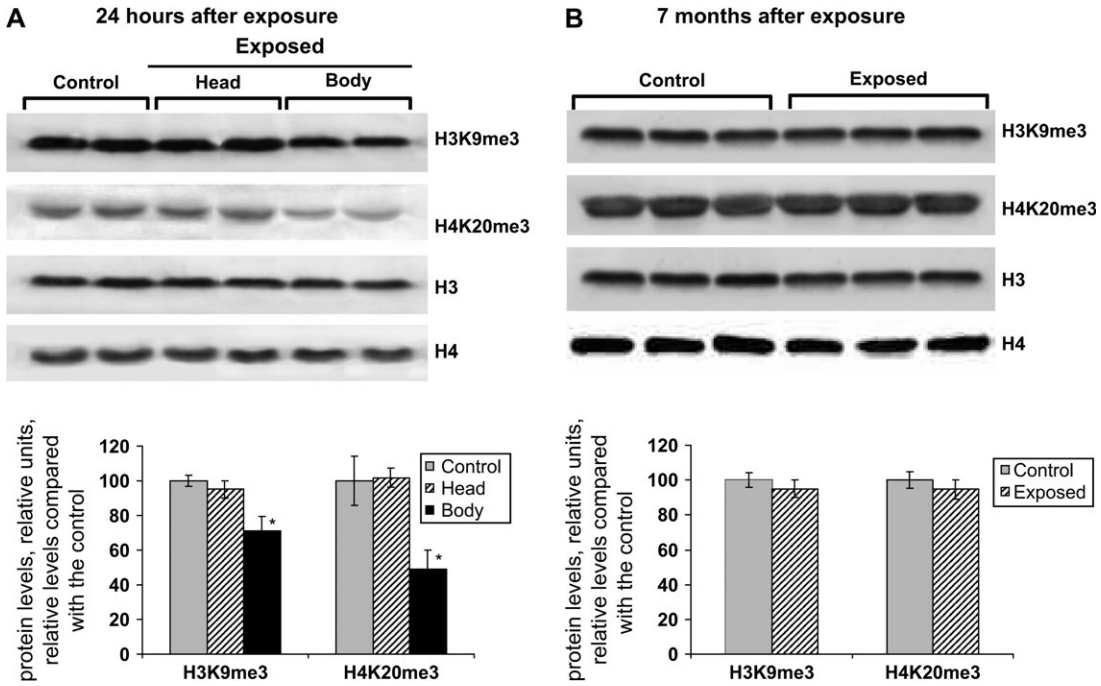

Fig. 5. Histone methylation levels in rat spleen upon whole body and localized cranial irradiation. Acidic spleen tissue lysates enriched in histones were subjected to immunoblotting using antibodies against histone H3K9me3, H4K20me3 and total histones H3 and H4 as loading controls. Levels relative to those of control animals are shown as the mean ± SD. Representative blots from among three independent experiments are shown. $^{*}P < 0.0167$, Student's *t*-test with Bonferroni correction for multiple comparisons, Dunnett's test and Tukey–Kramer test. (**A**) Histone methylation changes observed 24 h after whole body and head exposure and (**B**) Histone methylation changes seen 7 months after head exposure.

Table I. Relative miR-194 expression values in folds in the bystander rat spleen in comparison with those of the control rat spleen as analyzed by miRNA microarray and quantitative real-time PCR

miR-194 expression change	Fold	*P* value
Microarray	1.3	0.017
RT–PCR	1.8	0.041

Significance of the differences was analyzed by the Student's *t*-test, Dunnett's test and Tukey–Kramer test.

Table II. Relative miR-194 expression values in folds in the exposed and bystander rat spleen and plasma 24 h after irradiation in comparison with those of the control rat spleen and plasma as analyzed by the quantitative real-time PCR

miR-194 expression change	Fold	
Tissue	Whole body exposed	Bystander
Spleen	**1.9**	**1.4**
Plasma	**1.5**	*2.0*

Bold figures: significant, 95% confidence limit, $P < 0.05$; italic figures: 90% confidence limit, $P < 0.1$ (Student's *t*-test, Dunnett's test and Tukey–Kramer test).

Discussion

In this report, we describe a significant and persistent *in vivo* bystander effect that occurs in the rat spleen upon distant cranial irradiation. The main findings of the present study are as follows: (i) localized cranial radiation exposure leads to the induction of bystander effect in the lead-shielded distant spleen tissue; (ii) epigenetic dysregulation in the bystander spleen tissue manifested as a significant loss of global DNA methylation and alterations in the methylation of LINE-1 retrotransposable elements; (iii) irradiation leads to the significant down-regulation of DNA methyltransferases and methyl-binding protein MeCP2; (iv) irradiation significantly alters the expression of miR-194, a miRNA that putatively targets DNMT3a and MeCP2 and (v) profound epigenetic alterations can be seen 24 h after the exposure and persist for 7 months.

Overall, the firm and conclusive evidence that IR-induced bystander effects *in vivo* are operational limited (8,14,15,32). The clinical literature is scarce and thus does not provide any support for or against the somatic bystander effect *in vivo*, even though numerous studies suggest a functional bystander effect *in vitro* reviewed in ref. (52).

To bridge this knowledge gap, our laboratory has recently developed an *in vivo* model whereby bystander effects were studied in the skin of animals subjected to half-body or head exposure, whereas the rest of the body was protected by a medical grade lead shield. Using this model, we have pioneered the studies of the role of epigenetic mechanisms in the bystander effects in cutaneous tissue *in vivo*, and generated the first data to clearly demonstrate that bystander effects occur *in vivo* in distant tissue and are epigenetically mediated (32). Importantly, the data obtained in the mouse skin model showed that bystander effects were the most prominent when the spleen was in the field of irradiation, suggesting a possible role of the spleen in bystander effects (32).

As it is shown in the present study, spleen, an important radiation target organ, is also an important target organ for the bystander effect. The bystander effect observed in the shielded bystander spleen 24 h and 7 months after head exposure was characterized by the profound epigenetic dysregulation. This is in agreement with our previous report on the *in vivo* bystander effect in skin where epigenetic alterations also played a key role (32). In both systems, we noted a down-regulation of *de novo* DNA methyltransferase DNMT3a in bystander spleen tissue. *De novo* methyltransferases function primarily as regulators of a cell fate and differentiation. Their de-regulated

expression/activity may lead to deleterious effects and contribute to carcinogenesis (44,53).

We have also noted a significant decrease in the level of maintenance DNA methyltransferase DNMT1 and methyl-binding protein MeCP2 in bystander spleen. These results somewhat contradict our previous observations in the skin model. Yet, in our previous study, we were analyzing the bystander effects that occurred after 1 Gy of X-ray exposure, whereas the dose used in the current study was 20 times higher. Also, in the current study, we addressed the persistent long-term effects 7 months after exposure. Thus, the differences in DNMT1 and MeCP2 expression patterns observed in the two studies may to a certain extent reflect the dynamics of changes that occur over time. Furthermore, the discrepancy could also be due to the tissue specificity of bystander changes. Further studies are clearly needed to address the tissue specificity and dose responsiveness of the bystander effect induction.

It should be noted that the reduction of the levels of DNA methyltransferases and methyl-binding protein MeCP2 in the exposed and bystander spleen ranges between 15 and 50%. DNA methyltransferases are responsible for setting and maintaining DNA methylation patterns in mammalian cells (44). Importantly, it was shown that both maintenance (DNMT1) and *de novo* (DNMT3a and 3b) methyltransferases played a crucial role in the development of mammalian organisms, and their loss had lethal consequences (54–56). Thus, these seemingly 'small' methyltransferase expression changes can significantly affect the cellular functions. The exact impact of DNMTs and methyl-binding proteins on radiation-induced bystander effects has to be further elucidated.

Of special interest and potential importance were the loss of DNA methylation and reactivation of LINE-1 retroelements. Hypomethylation of LINE-1 sequences have been reported in many cancers (57). It was suggested that hypomethylation of LINE-1 sequences may promote the genomic instability and facilitate tumor progression (58). However, it has not yet been established whether the epigenetic changes, including hypomethylation and reactivation of LINE-1 elements often found in tumors, play a causative role in carcinogenesis, or they are merely a consequence of the transformed state. Recently, a progressive hypomethylation of LINE-1 regulatory region in liver has been found at very early stages of carcinogenesis prior to full-fledged tumor formation (37). The result of our present study that shows the loss of LINE-1 CpG methylation in bystander spleen may be viewed as the genome instability event predisposing the cells to future carcinogenic transformations.

As X-rays penetrate tissue, they can react with biological matter resulting in the deflection of its trajectories. This is referred to as scatter radiation (32). Low doses, close to the scatter-dose range levels, were shown to induce bystander effects (59–63). Yet, congruent with the previous study, the bystander effects observed in this study in the rat spleen were not due to insufficient shielding or radiation scattering. We used the same animal body shielding that was described previously (32). The scatter dose for rat spleen was miniscule (∼0.0012 Gy), slightly less than the previously reported one for the mouse model (32). Exposure of animals to the scatter dose did not affect DNA methylation and protein expression 24 h after exposure (data not shown).

The important outcome of this study is the observation of the altered levels of miR-194 in spleen and plasma of the whole-body and head-exposed animals. miRNAs are involved in the post-translational gene regulation and gene silencing. Although the precise biological role of miRNAs has not been fully understood, they are suggested to regulate various developmental and physiological processes, such as apoptosis, cellular differentiation and proliferation. Their altered expression may contribute to diseases. Recently, the importance of miRNAs in gene silencing and chromatin dynamics has attracted scientists' increasing attention (28). Profiling miRNA expression under various conditions as well as correlating the profiles with the physiological changes may allow investigators to uncover the exact role of miRNAs in cellular life. Here, we found that up-regulated levels of miR-194 in the exposed and bystander tissue are correlated with the down-regulated levels of its putative targets—*de novo* methyltransferase DNMT3a and methyl-binding protein MeCP2. The decreased levels of these proteins were linked to the significant global and LINE-1 locus-specific hypomethylation, thus contributing to the genome destabilization and possibly further to carcinogenesis.

It should be mentioned that miR-194 is the only miRNA that is statistically significantly up-regulated in bystander rat spleen tissue 24 h and 7 months after exposure. It is also strongly up-regulated in the spleen and plasma of rats subjected to the whole-body irradiation (Table II). Overall, miR-194 seems to play some role in the maintenance of the long-term response where, probably, the altered levels of just a few RNAs are enough.

Further studies are clearly needed to achieve the in-depth understanding of the complete dynamics of miRNAome patterns in the bystander effects.

Most recent report by Kemppainen *et al.* (64) proved that miRNAs are very abundant in biofluids—plasma, serum, urine and saliva—and exhibit high donor-to-donor consistency (64). Thus, even though their means of dispersion into biofluids are illusive, miRNAs in plasma, serum, saliva and urine can be used as biomarkers for cancer and other diseases (64).

Our study is the first that has shown the altered levels of miRNA in the plasma upon radiation exposure. More studies are needed to understand the ways of miRNA penetration/secretion into plasma and the role of blood miRNAs in mediation of the distant radiation responses. Overall, this is the first report suggesting the possible role of miRNA in bystander effects. More studies are needed to confirm and dissect the miRNA role in this enigmatic phenomenon in detail.

Most importantly, this study is the first report that provides the conclusive evidence of the long-term persistence of the bystander effects in radiation carcinogenesis target organ (spleen) under the localized distant cranial exposure. The doses and exposure regimes used in this study are close to those used in clinic for brain therapy treatments (1). Radiotherapy is one of the most common treatments for brain tumors (1–4). Nevertheless, the risk of the secondary radiation-induced complications, including secondary cancers that peak 10 years after exposure, has been reported (1,14,15). Seven months in the life cycle of a rat corresponds to ∼10 years in human life (33). Thus, the data obtained in this study using a rat model can be potentially extrapolated to humans. Further studies are required to delineate the possible contribution of radiation-induced bystander effects to secondary carcinogenesis, to identify and characterize the nature of the enigmatic bystander signal and to address the impact of reactive oxygen species and DNA damage on the mediation of the immediate and delayed bystander effects *in vivo*.

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