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Author: Koturbash, Igor

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REVIEW

2017 Michael Fry Award Lecture
When DNA is Actually Not a Target: Radiation Epigenetics as a Tool to Understand and Control Cellular Response to Ionizing Radiation

Igor Koturbash[1]

Department of Environmental and Occupational Health, Fay W. Boozman College of Public Health, University of Arkansas for Medical Sciences, Little Rock, Arkansas

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Aside from the generally accepted potential to cause DNA damage, it is becoming increasingly recognized that ionizing radiation has the capability to target the cellular epigenome. Epigenetics unifies the chemical marks and molecules that collectively facilitate the proper reading of genetic material. Among the epigenetic mechanisms of regulation, methylation of DNA is known to be the key player in the postirradiation response by controlling the expression of genetic information and activity of transposable elements. Radiation-induced alterations to DNA methylation may lead to cellular epigenetic reprogramming that, in turn, can substantially compromise the genomic integrity and has been proposed as one of the mechanisms of radiation-induced carcinogenesis. DNA methylation is strongly dependent on the one-carbon metabolism. This metabolic pathway is central to the support of DNA methylation by means of providing the donor of methyl groups, as well as for the synthesis of amino acids. To better understand the mechanisms of radiation-induced health effects, we study how exposure to radiation affects DNA methylation and one-carbon metabolism. Also, a tight interaction that exists between DNA methylation and one-carbon metabolism allows us to simultaneously manipulate both cellular epigenetic and metabolic profiles to modulate the normal and cancerous tissue response to radiotherapy.

It is both a privilege and pleasure to speak here today as a recipient of a Michael Fry Award. I am truly humbled by this recognition and would like to thank the Award Committee and the Radiation Research Society for this Award.

[1] Address for correspondence: University of Arkansas for Medical Sciences, Fay W. Boozman College of Public Health, Department of Environmental and Occupational Health, 4301 W. Markham St, Slot #820-11, Little Rock, AR 72205-7199; email: ikoturbash@uams.edu.

The genotoxic properties of ionizing radiation that are exhibited as DNA strand breaks, the development of gene mutations and chromosomal aberrations have been known for many years. Subsequently, the damage to DNA has been considered as a central dogma in radiobiology. However, not all the effects of radiation can be attributed to DNA damage; for instance, radiation-induced large-scale dynamic changes in the expression of genes. Those changes are time- and tissue-specific and determine the tissue response to radiation. Furthermore, those differences in gene expression between the stable and unstable clonally expanded survived cells were also proposed to play the central role in the development of genomic instability (*1*). Indeed, the observed frequency of radiation-induced genetic instability is considerably higher than that observed for gene mutations at a similar dose; therefore, the latter was considered highly unlikely to be the initiating mechanism (*2–4*).

Expression of genes is governed by epigenetic mechanisms of regulation - the mechanisms that are not associated with alterations in underlying DNA sequence but that are rather exhibited as covalent DNA modifications. The latter are represented as methylation of DNA, post-translational histone modifications and nucleosome positioning along DNA. These covalent marks ensure appropriate structure and function of the epigenome and are applied by a number of specific enzymes, i.e., DNA and histone methyltransferases (the so-called ''writers''). Then, the proteins that recognize these marks, ''the readers,'' modulate the gene expression at particular genomic loci. In turn, the reversibility of the previously applied covalent marks is guaranteed by the ''erasures'' (*5*). Methylation of DNA is the most studied epigenetic modification; this is a covalent addition of a methyl group to the fifth position of carbon, enabled by a complex interplay between various enzymes, DNA methyltransferases, methyl-CpG-binding proteins, and associated with them, protein ubiquitin-like with PHD and RING finger domains 1 (UHRF1). DNA methylation is

considered a primary epigenetic mechanism of the expression of genetic information.

RADIATION EPIGENETICS: THE START

In 1989, groundbreaking work by Kalinich *et al.* set the stage for studies in radiation epigenetics (*6*). In that work, exposure to ^{60}Ca gamma radiation was shown to cause dose-dependent decreases in the levels of 5-methylcytosine (5-mC) in four cultured cell lines. This work was followed by Tawa *et al.*, who reported decreases in 5-mC content from the livers of C57BL/6NJcl mice after exposure to 4–10 Gy of X rays (*7*).

During my PhD studies in Olga Kovalchuk's laboratory, in parallel with Mark Plumb's work, we demonstrated that exposure to radiation at doses of 1 Gy and above causes detectable and persistent loss of global DNA methylation *in vivo* (*8, 9*). Importantly, DNA hypomethylation was observed in the target tissues (thymus and bone marrow) and was detectable without the presence of unrepaired DNA damage.

While the changes were observed on the whole genome level, it is important to identify whether they are uniformly distributed throughout the genome, or primarily stem from the particular genomic loci. More than one-half of the known eukaryotic genes and the vast majority of repetitive elements (REs) contain so-called CpG-rich regions, also known as CpG islands or CGIs. The differential regulatory functions of DNA methylation are dependent on the location of CGIs. For instance, methylation of DNA at CGIs located within the gene promoter/transcription start site is usually associated with gene silencing (*10*). DNA methylation of gene bodies either leads to stimulated elongation and splicing or prevention of initiation of aberrant transcription from alternative transcription start sites (*11, 12*).

Altered patterns of DNA methylation have been reported in numerous pathological states and diseases, including cancer. By today, virtually all human cancers have been characterized by the loss of global DNA methylation, which is one of the generally considered hallmarks of cancer (*13*). Furthermore, accumulating evidence clearly indicates that alterations in DNA methylation are not just the consequences of neoplastic transformation or passive bystanders in the process of carcinogenesis, but are the active players that shape the tumor landscape (*10, 14–16*). Indeed, altered DNA methylation can be detected very early during the process of carcinogenesis and may influence numerous biological processes. For instance, alterations in DNA methylation of particular genes may result in their aberrant transcriptional activity (in the case of oncogenes) or silencing (in the case of tumor-suppressor genes).

REPETITIVE ELEMENTS AS A TOOL TO STUDY RADIATION EPIGENETICS

Repetitive elements (REs) most likely represent the largest methylated body of coding and noncoding sequences in the genome. Indeed, REs shape about 50% of mammalian genomes, with some computational studies estimating up to two-thirds of the genome to be represented as REs (*17, 18*). Since the REs are primarily transposons and retrotransposons by nature, during the evolutionary arm race with the host, they were silenced by methylation of DNA to control their expression and potential (retro)transposition (*11, 17, 19*).

Long interspersed nuclear element-1 (LINE-1), the most abundant in mammalian genomes' RE, attracted my attention long ago. The DNA methylation capacity of this retrotransposon is tremendous, as LINE-1 comprises ~20% of mammalian genomes, roughly 10 times more than the entire protein-coding genes (*17, 20*). The full-length LINE-1 is comprised of the two open reading frames (ORF-1 and ORF-2), a 3′ untranslated region (UTR) and a CpG-reach 5′-UTR. The DNA methylation of LINE-1 5′-UTR is believed to be a critical mechanism in blocking transcription factor binding and the initiation of transcription, therefore playing a central role in silencing this retrotransposon (*11, 20*).

LINE-1 regulates the transcription by altering the chromatin structure, as well as functioning as an enhancer or promoter, and by generating new transcript isoforms (*42*). The loss of the DNA methylation control over LINE-1 may lead to its transcriptional reactivation and subsequent retrotransposition. The latter event can be associated with the disruptive insertional mutagenesis when a LINE-1 fragment is introduced within an ORF of a functional gene. DNA hypomethylation of LINE-1 and its retrotransposition may result in genomic instability and development of cancer (*20*); however, even without retrotransposition, aberrant LINE-1 DNA methylation can affect the tumor landscape by activating proto-oncogenes that have previously acquired LINE-1 insertions (*21, 22*).

Ionizing radiation has been shown to affect global DNA methylation, and DNA methylation of REs, in particular (*23*). Radiation-induced loss of DNA methylation within the REs, particularly LINE-1, have been shown to enhance their transcriptional activity and reactivation of transposable elements, leading to insertional mutagenesis and potentially to the development of genomic instability (*24–29*). The latter can also serve as a classic example of an epigenetically-driven potentially carcinogenic mutation event. Although the role of the RE-associated insertional mutagenesis as an independent and individual mechanism in carcinogenesis has not yet been proven, accumulating evidence indicates the plausibility of this event (*30, 31*).

In our initial studies, we have convincingly demonstrated that radiation-induced changes in DNA methylation stem primarily from the REs. For instance, exposure to low mean absorbed doses of heavy iron ions (^{56}Fe) resulted in dose-dependent DNA hypermethylation in the mouse lung (*32*). While there were no changes in gene-specific DNA methylation, including changes in the genes frequently hypermethylated in lung cancer, dose-dependent DNA

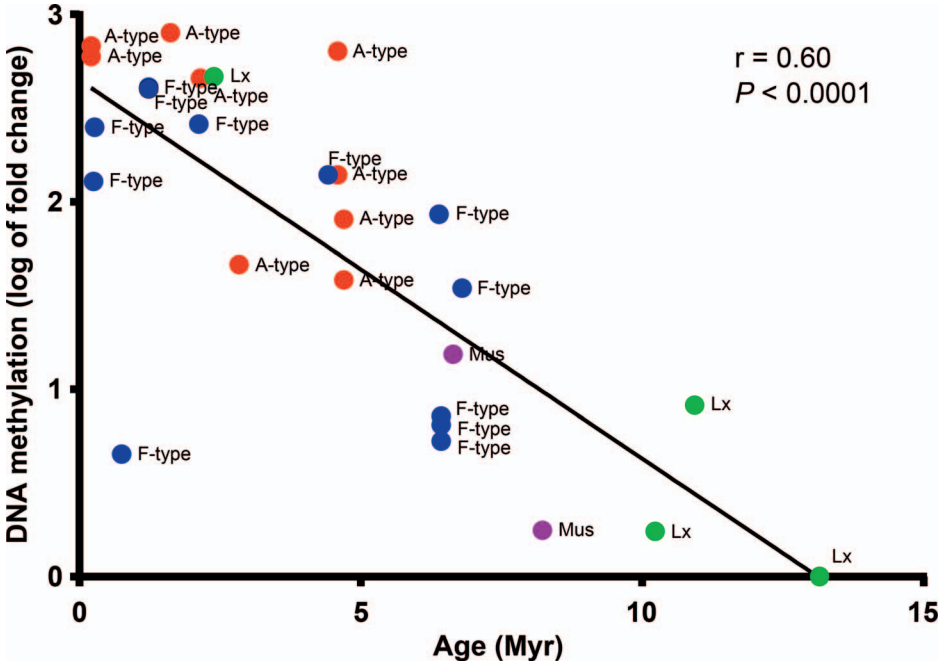

FIG. 1. The relationship between the age of LINE-1 elements (in Myr) and their DNA methylation. The age of the most abundant 27 LINE-1 elements is strongly associated with their evolutionary age, wherein older LINE-1 elements exhibit a higher degree of DNA demethylation within their 5′-untranslated regions (UTRs).

hypermethylation of REs was observed. Importantly, DNA hypermethylation was associated with dose-dependent decreases in the expression of those REs, suggesting potentially protective effects against radiation-induced reactivation of REs. Similar effects were observed in the mouse hematopoietic stem and progenitor cells, where the degree of the radiation-induced alterations in DNA methylation correlated with the extent of DNA methylation within the LINE-1 and another retrotransposon: short interspersed nuclear element-B1 (SINE-B1) (*25*).

With a growing interest in radiation epigenetics, a number of studies have shown that radiation-induced changes in DNA methylation are not unidirectional; LINE-1 DNA hypo- and hypermethylation can be observed, as well as an absence of changes in DNA methylation, especially at doses below 1 Gy (*33–37*). Those differences could be explained by the different types of radiation and doses, models used, as well as different methods of DNA methylation analysis. However, we recently demonstrated that effects of radiation on LINE-1 DNA methylation are dependent primarily on the following two factors: the evolutionary age/type of the LINE-1 promoter and the type of irradiated cell (*38, 39*).

Evolution of LINE-1 in mammals is characterized by the existence of a single lineage of this retrotransposon: after emergence and amplification to several hundreds to thousands of copies, the family would then become extinct and be replaced by a newly evolved one (*40*). Because both ORFs of various LINE-1 elements that belong to different families have a very high degree of homology, the lineages differ mainly in their 5′-UTRs. We have shown a relationship between the age (in Myr) of the LINE-1 elements and their DNA methylation, wherein older LINE-1 elements exhibited lower DNA methylation levels within their 5′-UTRs (Fig. 1). Importantly, this effect was organ- and cell-type independent as it was observed in the lung, intestine and the bone marrow, and in the cells of various lineages of the hematopoietic system (*38, 39, 41*). In general, the radiation-induced loss of DNA methylation was observed primarily from the evolutionary young LINE-1 elements that were enriched in methylated CGI sites. In contrast, older LINE-1 elements, with a number demethylated during the evolution CGI sites, were able to acquire additional methyl groups, leading to DNA hypermethylation in these loci. The DNA methylation status of the evolutionary-oldest elements remained often unchanged due to the initially hypomethylated phenotype on one hand and the loss of a substantial fraction of potential CGI sites due to spontaneous deamination on the other hand (*38, 39*).

We also demonstrated that even at doses as low as 0.1 Gy, exposure to radiation results in persistent changes in the DNA methylation of the selective LINE-1 elements in the mouse hematopoietic system. The most pronounced responses were detected in hematopoietic stem cells, and these effects were observed mostly in CBA/CaJ mice, the strain of mice prone to radiation-induced leukemia (*38*). Importantly, because hematopoietic stem cells reside at the top of the hematopoietic system hierarchy, epigenetic reprogramming of these cells may lead to epigenetic reprogramming of their differentiated progeny. Similar effects were also observed in the rat model as well, where the cranial exposure to 20 Gy of X rays resulted in the loss of LINE-1 DNA methylation in peripheral lymphocytes and

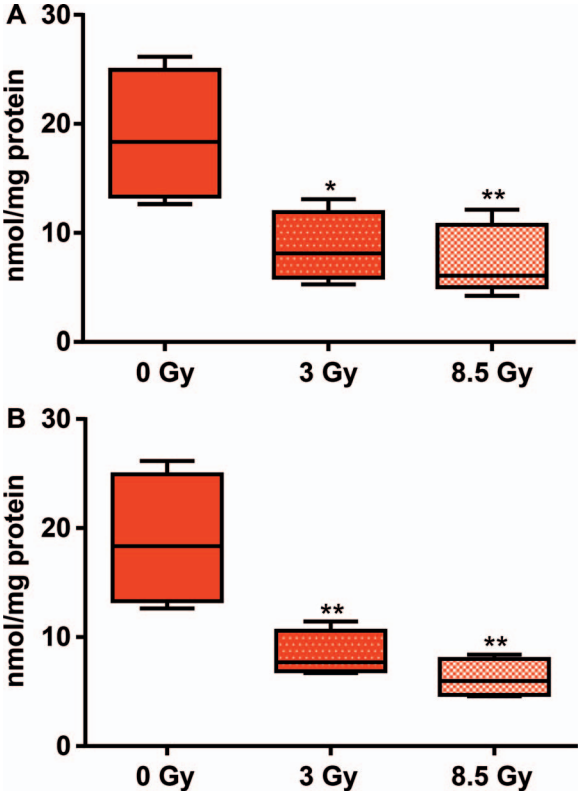

FIG. 2. Exposure to ionizing radiation causes substantial depletion in the methionine tissue concentrations. Analysis of the methionine tissue concentrations in the mouse jejunum after exposure to 3 and 8.5 Gy of ^{137}Cs, as measured by HPLC on day 3.5 (panel A) and day 6 (panel B). $*P < 0.05$, $**P < 0.01$ compared to control.

could be detected for at least 7 months after irradiation (*26*). Altogether, these findings suggest that the DNA methylation status of particular LINE-1 elements in peripheral blood may indicate previous exposures to radiation and may potentially be used for biodosimetry purposes (*23*).

With advances in the field of radiation epigenetics, the understanding of the mechanisms of those effects became critical for a number of reasons. First, this knowledge will certainly aid in preventing the radiation-induced epigenetic alterations and negative consequences associated with them. Second, understanding those mechanisms may also help in modulating the tissue response to radiation. Currently, normal tissue toxicity and cancerous cell radioresistance are the main obstacles in the treatment of many cancers. Therefore, modulation of the cellular epigenetic landscape may provide new opportunities in radiation oncology.

In this regard, a number of theories have been proposed and are being investigated to understand the epigenetic effects caused by radiation, including the altered function of DNA and histone methyltransferases, interference of DNA damage with the ability of DNA methyltransferases to methylate DNA, affected DNA damage and repair and radiation-induced proliferation [reviewed in (*19*)]. We have recently proposed another mechanism where the multifaceted interaction between the cellular metabolism, one-carbon metabolism in particular, and DNA methylation play an important role in the cellular response to exposure to radiation (*42*).

INTERACTION BETWEEN THE CELLULAR EPIGENOME AND METABOLOME

The one-carbon metabolism unifies a set of reactions surrounding the transfer of the methyl group from the S-adenosylmethionine (SAM) to acceptor molecules with the subsequent regeneration of SAM. This pathway, critical for nearly all cellular reactions, ties together the regulation of gene expression, synthesis of purine and pyrimidine and amino acids, and over a hundred biomethylation reactions. SAM and S-adenosylhomocysteine (SAH) are intermediate metabolites in one-carbon metabolism that coexist in a metabolic relationship. This relationship is characterized by the SAH-induced inhibition of SAM-dependent methyltransferases, where the former is accumulated in the presence of homocysteine. Some methyltransferases are much more susceptible to the inhibitory effects of SAH; for instance, the values of kinetic constants for histone methyltransferases determine their higher sensitivity to the SAM/SAH ratio compared to DNA methyltransferases (*43*). Accumulating evidence clearly indicates that SAM levels, as well as the SAM/SAH ratio, play key roles in the regulation of the chromatin state of the cell and can be modulated through the metabolic flux of the methionine cycle (*44–46*).

Methionine, the direct precursor of SAM, is the essential amino acid required for normal growth and development. Among its key functions is the regulation of stress resistance (i.e., by involvement in the synthesis of cysteine and glutathione), as well as its central role in the maintenance of the normal patterns of DNA and histone methylation by providing the donors of methyl groups. Depletion of methionine leads to changes in DNA and histone methylation (*44, 45, 47*). Exposure to radiation depletes internal methionine resources, leading to postirradiation loss of DNA methylation, as well as impairs synthesis of glutathione, needed to sustain overwhelming oxidative damage to DNA. For instance, even exposure to a relatively low 3 Gy dose of ^{137}Cs causes substantial and long-term intraintestinal methionine depletion in the mouse model (Fig. 2). Therefore, it has been previously hypothesized that prevention of the radiation-induced DNA hypomethylation and increased tolerance of the normal tissue to radiation may be achieved by modulation of the methionine dietary intake (*48, 49*).

FUTURE PROSPECTS: FROM TYING THE HOST'S EPIGENOME AND METABOLOME WITH THE GUT MICROBIOME TO TARGETING THE CANCER CELL METABOLOME

Radiation epigenetics, since it emerged as a separate field several decades ago, cannot be considered an isolated

discipline anymore. For instance, a very tight link exists between the critical epigenetic mechanisms, such as DNA and histone methylation, and cellular metabolism. Methionine, a key molecule in one-carbon metabolism, is not only important for the host's protein synthesis and for providing methyl (CH_3) groups for DNA methylation but is also indispensable for the gut microbiome. The latter itself affects the host's metabolome and epigenome, i.e., by providing amino acids (including methionine) for the host's needs (*50*).

While methionine supplementation may potentially exert some positive effects on the cellular epigenome, it must be considered that methionine is by far the most toxic among all the amino acids (*51*). As we have recently shown, dietary methionine overload may negatively affect the normal gut microbiome and the host's one-carbon metabolism, leading to the development of bacterial overgrowth of the small intestine (BOSI) in the mouse model (*41*). Furthermore, methionine overload causes shifts in the gut microbiome, resulting in the increased abundance of pathogenic bacterial species, decreased expression of the intestinal transmembrane proteins, as well as loss of crypt depth and mucosal surface (*41*). All these effects may not only contribute to the pathogenesis of intestinal inflammatory diseases but can sensitize the gut to exposure to other stressors, like radiation. On the other hand, dietary methionine deprivation has recently been demonstrated to exert protective effects on the gut by decreasing the inflammation and tightening epithelial barriers (*52*). This is particularly important, because Western populations consume 2-to-3.3-fold more methionine than recommended due to high levels of meat and dairy products in the diet, fortification of grains and a spiking increase in the consumption of methionine-containing dietary supplements (*53–55*). In the U.S. alone there are over six million patients who have received abdominal or pelvic radiotherapy and this number is expected to double by 2025 (*56*). A considerable proportion of the patients experience symptoms of acute and/or chronic bowel toxicity, and therefore methionine dietary modification during the course of radiotherapy may aid in alleviating these side effects. However, studies investigating the exact fate of dietary methionine in the irradiated proximal jejunum, the section of the gut specifically responsible for amino acid absorption, need to be performed. Furthermore, the host-intestinal microbiome axis needs to be taken into consideration, given the tight relationship that exists between the host's and the microbiome's amino acid metabolism and the radiation-induced changes in the gut microbiome (*50, 57–59*). Moreover, it has been demonstrated that the host's diet influences the production of xenometabolites (*60*).

A remarkable difference in the needs for methionine between normal cells and cancer cells exists, where the rapidly proliferating cancer cells require much higher levels of methionine to maintain their function. This difference is underlined by the normal cell's capacity for re-methylation and further utilization of methionine from homocysteine, and impaired ability of the cancer cell for the proper synthesis and utilization of endogenous methionine. Therefore, tumor cells were shown to be extremely sensitive to methionine restriction. For instance, plating normal fibroblasts and aggressive cancer cells on the same plate under the methionine-deficient/homocysteine-supplemented media conditions results in cell cycle arrest and death of tumor cells with the abundant overgrowth of normal fibroblasts (*61*). The potentiation of the chemotherapy effect by methionine deprivation has been investigated *in vitro* (*62*), *in vivo* (*63, 64*) and even in clinical trials (*65, 66*), while the potential combination of methionine dietary deprivation with radiotherapy has never been addressed before. At the same time, accumulating evidence indicates that cancer cells are radiosensitized by the deprivation of other methyl group donors (*67, 68*), suggesting methionine deprivation combined with radiotherapy may have beneficial effects for cancer treatment.

During the preparation of this lecture transcript, we were saddened to learn that Dr. Michael Fry passed away on November 24, 2017. He will be remembered by colleagues as a great scientist and a true gentleman. His life and his immense contributions to science remain a unique example for both current and new generations of radiation researchers to follow.

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I would like express my gratitude to everyone who was with me on this journey. Specifically, I would like to thank each member of my lab, past and present, whose diligent work allowed to perform this research. I would like to thank my colleagues for their continuous support and multiple research opportunities that turned into fruitful collaborations: Martin Hauer-Jensen and the entire Division of Radiation Health; Alan Tackett, Robert Griffin, and Stepan Melnyk (all, University of Arkansas for Medical Sciences); Gregory Nelson of Loma Linda University and Jacob Raber of Oregon Health and Sciences University for exciting studies on the effects of high-LET radiation; Nicolaas Deutz of Texas A&M University for collaborative studies on amino acids; and Amrita Cheema of Georgetown University for studies on radiation effects on one-carbon metabolism and microbiome. Last but not the least, I would like to thank my previous mentors, Olga Kovalchuk and Igor Pogribny, who immersed me in the world of radiation biology and epigenetics. The work presented in this lecture was generously supported by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant no. 1P20GM109005; Clinical and Translational Science Awards UL1TR000039 and KL2TR000063; the National Space Biomedical Research Institute (RE03701 through NCC 9-58); and the Arkansas Biosciences Institute.

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