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The Epigenetic Effects of Radiation Exposure

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Abstract: **The Epigenetic Effects of Radiation Exposure**

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Exposure to high linear energy transfer (LET) radiation in space, which consists of high energy protons and high charge and energy nuclei, is a primary health concern for space travelers. Complex DNA damage caused by high LET radiation can lead to mutations that alter oncogene and tumor suppressor gene expression, promoting carcinogenesis. However, the precise mechanisms by which radiation promotes cancer is unknown. Epigenetic deregulation, which involves changes in DNA methylation and histone modifications, is one way genes can be mis-expressed in cancers. Therefore, we sought to determine how radiation elicits epigenetic changes associated with tumorigenesis. Specifically, we looked at the effect of microRNA-21 (miR-21), an oncogenic microRNA that plays a central role in radiation stress responses. We hypothesized that aside from translationally suppressing a number of tumor suppressor genes, miR-21 may also target DNA methyltransferase 1 (DNMT1), the enzyme responsible for maintaining DNA methylation, and thereby disrupt a facet of epigenetic regulation. We attempted to elucidate the relationship between miR-21 and DNMT1 by overexpressing miR-21 both stably and transiently. DNMT1 levels did not appear to be affected by either consistently or transiently high miR-21 levels, but further experimentation with greater transfection efficiency is required to validate these results. We also investigated the time- and dose-dependent effects of low LET radiation exposure on DNA methyltransferases (DNMTs) and a DNA hydroxymethylation enzyme (TET2). Low LET radiation stimulated increased expression of DNMT1, DNMT3a, and TET2 over one week. Furthermore, DNMT3a gel migration patterns were altered in irradiated samples, implying radiation may promote alternate splicing or post-translational modification of DNMT3a. Increasing radiation dose appeared to impact DNMT1

negatively, but DNMT3a, DNMT3b, and TET2 positively. Our results indicate that irradiation elicits expression changes in key DNA modifying proteins, suggesting that aberrations in DNA methylation patterns as a result of these expression changes could be one mechanism that contributes to radiation-induced oncogenesis. Further experiments will help clarify the role of miR-21 in the mis-regulation of DNMT1 and the significance of mis-expressed DNMT and TET proteins, allowing a better understanding of the risks of space travel and radiation exposure in general.

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TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

Cancer is a Risk for Astronauts

Before astronauts can explore space in long-duration missions, the National Aeronautics and Space Administration must assess the risks explorers may encounter so that they can be mitigated or at least accounted for. Among these risks, space radiation-induced carcinogenesis is "the main hindrance to interplanetary travel" (Durante and Cucinotta 2008). Space radiation consists of high-energy protons and high charge (Z) and energy (E) nuclei (HZE), and is characteristically different from terrestrial radiation (Durante and Cucinotta 2008). Specifically, space and other high linear-energy-transfer (LET) (dense) radiation create types of DNA damage that are more complex and clustered than low LET (sparse) radiation (Ponomarev and Cucinotta 2006). This complex DNA damage is known to contribute to genomic instability and carcinogenesis (Little 2000). Exposure to high LET radiation elicits unique gene expression profiles (Ding, Shingyoji et al. 2005). Strong evidence shows that the risk of lung cancer increases proportionally to the dose of terrestrial ionizing radiation, which has a low linear-energy-transfer (LET) rating (Wakeford 2004). This suggests that high-LET particle induced lung cancer may present a major risk for space travelers (Durante and Cucinotta 2008). However, the risks associated with exposure to high-LET radiation are understudied.

Epigenetics and Cancer

Understanding the unique radiobiology of the cellular response to space radiation and the mechanisms of carcinogenesis is crucial to estimating the risk of space travel. Cancer has traditionally been defined as a genetic disorder through which a cell acquires abilities to sustain unhealthy proliferation and metastasis. The past decades of cancer research have been spent

teasing out the intricacies of this complex multistep process. The "hallmarks of cancer", as described by Hanahan and Weinberg, is an acquired set of "distinctive and complementary capabilities…that provide a foundation for understanding the biology of cancer" (Hanahan and Weinberg 2011). A growing body of research shows that epigenetics, loosely defined as the study of cellular phenotypes caused by mechanisms other than changes in DNA sequence, plays a significant role in all stages of neoplastic progression. Specifically, DNA and histone modifications compartmentalize the genome into domains of varying transcriptional capacity; this method of transcriptional regulation can become deregulated and promote or even induce cancer development. Abnormal gene expression programs partially arising from an altered epigenome are well documented in human cancers. Gradual genome-wide DNA hypomethylation seen in many cancers can contribute to tumorigenesis through decreased chromosome stability, loss of imprinting, overexpression of proto-oncogenes, and de-repression of transposons and miRNAs (Eden, Gaudet et al. 2003). Concurrently, de novo DNA methylation of the promoter regions in tumor suppressors and other genes, along with the assembly of repressive chromatin, causes aberrant gene silencing (Eden, Gaudet et al. 2003). In addition, deregulation of the enzymes that methylate DNA, the DNA methyltransferases (DNMTs) DNMT1, DNMT3a, and DNMT3b, has been shown in a number of cancer types (Baylin and Jones 2011). DNMT3a mutations have also been implicated in some forms of aggressive leukemia (Baylin and Jones 2011).

MicroRNA-21 as an 'Onco'microRNA

Aberrant transcriptional regulation of the cancer genome through altered DNA methylation patterns can lead to the mis-expression of microRNAs (miRNAs), a type of translational

regulation. miRNAs are short noncoding RNAs that typically hybridize with complementary sequences in the 3' untranslated region of mRNAs, resulting in mRNA degradation or the blocking of protein synthesis (Baer, Claus et al. 2013). miRNAs undergo several modifications after transcription before they are considered active. Most primary miRNAs go through RNase cleavage machinery in the nucleus, mediated by Drosha, creating precursor miRNA (pre-miR), which is then exported to the cytosol and processed by Dicer, an endoribonuclease (Baer, Claus et al. 2013). The mature microRNA of length 21-22 nucleotides acts to regulate protein expression after hybridization by recruiting the RNA-induced silencing complex (RISC), which can both lead to mRNA degradation or physically block the translation apparatus, thereby preventing protein production (Van Wynsberghe, Chan et al. 2011).

Overexpressed microRNA-21 (miR-21) may provide a molecular mechanism for the alteration and aberration of the epigenome seen in lung cells exposed to space radiation. It has been shown that miR-21 is significantly upregulated in lung cells irradiated with high LET particles (Zhu, Yu et al. 2010). MiR-21 was initially dubbed an "oncomir" because it was found to target a number of tumor suppressor genes, including PDCD4 and PTEN (Papagiannakopoulos, Shapiro et al. 2008). This microRNA has been shown to target other pathways associated with cancer progression as well, including proteins with roles in the cell cycle, migration, differentiation, and survival (Li, Liang et al. 2012). In fact, miR-21 is the most commonly upregulated microRNA in both solid and hematological cancers (Li, Liang et al. 2012).

Like most miRNAs, miR-21 is initially transcribed as a pri-miR, and subsequently processed through a series of cleavage events to a mature stem-loop structure that can be processed into two strands, miR-21-3p (22bps) and miR-21-5p (21bps), respectively

(Papagiannakopoulos, Shapiro et al. 2008). Each of these strands has the potential to target specific mRNAs and promote their translational repression (Winter, Jung et al. 2009).

Preliminary Data

Preliminary experiments in the Vertino lab compared the DNA methylation status of 27,000 methylation sites in liver cells exposed to either high or low LET radiation. While they had both unique and shared sites of methylation changes, these changes also persisted over a period of weeks. Importantly, this implies an "epigenetic memory" of acute irradiation, wherein changes in DNA methylation can become fixed and allow for altered and potentially carcinogenic gene expression profiles. In a follow-up experiment, lung cells were exposed to various doses and types of high LET radiation, and samples were collected over the period of about 4 months. The results showed that acute high LET radiation exposure does indeed induce changes in DNA methylation, and also, that high LET radiation appears to accelerate age-dependent methylation drift. The molecular mechanisms driving the changes in epigenetic profiles associated with exposure to space radiation, as well as those related to carcinogenesis in general, have not yet been fully explored.

Sequence alignment predictions suggest that DNA methyltransferase 1 (DNMT1) is a potential target of miR-21-3p, given that the miR-21-3p strand has significant sequence homology with DNMT1. The sequence alignments and miR-21's predicted position relative to the processed DNMT1 message is shown in Figure 1. DNMT1 is responsible for maintaining methylation patterns by predominately methylating hemi-methylated DNA after replication, while DNMT3a and DNMT3b provide de novo methylation. DNA methylation is a chemical modification of DNA that occurs on the 5' position on cytosines, typically in the context of CpG

dinucleotides. DNMTs act to methylate cytosine residues using S-adenosyl methionine as a donor, as illustrated in Figure 2. Therefore, microRNA-21 may play a role in controlling DNA methylation through the inhibition of DNMT1 expression. A decrease in expression of DNMT1 may contribute to the global changes in methylation patterns observed in cells exposed to high LET radiation, and ultimately contribute to space radiation-induced lung carcinogenesis.

Altered DNA hydroxymethylation patterns may also play a role in radiation-induced carcinogenesis. DNA hydroxymethylation occurs at methylated cytosines, and is believed to be an intermediate in oxidative DNA de-methylation pathways (Kinney and Pradhan 2013). DNA hydroxymethylation is performed by the ten-eleven-translocation (TET) enzymes. TET enzymes require 2-oxoglutarate for the oxidative step in their catalysis. Like DNMTs, TET proteins are believed to play a crucial role in development and differentiation (Kinney and Pradhan 2013). As their name implies, the TET family of enzymes is also subject to mutation in some neoplasms; specifically, TET1 translocations often occur in acute myeloid leukemia, and a number of TET2 mutations have been identified in myeloproliferative neoplasms (Kinney and Pradhan 2013).

While elucidating the epigenetic consequences of high LET radiation exposure is crucial to estimating the risk of space travel, it also important to study the effects of terrestrial low LET radiation such as X-rays used in radiotherapy and diagnostics. Like high LET radiation, low LET radiation is known to contribute to carcinogenesis (Wakeford 2004). Low LET radiation also elicits a unique biological response, including altered gene expression programs and epigenetic consequences. Furthermore, the response to low LET radiation exposure may share features with the response to high LET exposure. Altered DNMT and TET expression may provide insight to the mechanisms by which epigenetic patterns are altered in human cancers caused by both high LET and low LET radiation.

Objectives

The overarching goal of this study was to define the epigenetic determinants of radiation exposure and its role in lung cancer tumorigenesis (Figure 3). We have shown that exposure to high LET or low LET radiation elicits epigenetic patterns that persist for weeks, yet we are not sure mechanistically how these responses are mediated. A major part of the response to high LET exposure is the overexpression of miR-21, a known oncomir that potentially targets a key epigenetic protein. Thus, the first part of this project concerns determining the microRNA-target relationship between miR-21 and DNMT1. A second major goal of this study was to elucidate the epigenetic response to low LET radiation by observing its short term and long term effects on DNMT and TET expression. Taken together, these studies will further our knowledge of the epigenetic mechanisms of response to radiation, provide insights into the processes of cancer development in general, and clarify the risks of extraterrestrial travel.

MATERIALS AND METHODS

Cell Types and Culture Conditions

NL20

NL20 is a non-tumorigenic human bronchial epithelial cell (HBEC) line immortalized with SV40 Large T antigen. Dr. Ya Wang's lab at Winship Cancer Institute transduced the NL20s with a lentiviral construct (pMIF-cGFP-ZeomiR21, obtained from System Biosciences) with or without miR-21 primary RNA. In the figures, NL20+(1) and NL20+(2) refer to NL20 cells independently transduced with the primary miR-21 RNA, while NL20- was transduced with the vector only. NL20 cells were cultured in Ham's F-12 medium containing 4% FBS, 1.5g/L sodium bicarbonate, 2.7g/L glucose, 2.0 mM L-glutamine, 0.1mM nonessential amino acids, 0.005mg/ml insulin, 10ng/ml EGF, 0.001 mg/ml transferrin, and 500ng/ml hydrocortisone.

3KT

3KT is a non-tumorigenic telomerase-immortalized HBEC line. 3KT were maintained in keratinocyte serum-free media supplemented with bovine pituitary extract and epithelial growth factor.

MCF7

MCF7 is a breast cancer cell line derived from a primary ductal carcinoma. MCF7 were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and L-glutamine.

293T

293T is a human embryonic kidney (HEK) cell line which contains the SV40 Large T-antigen. 293T was also grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and L-glutamine.

All cells were cultured and maintained at 37 ˚C in a 5% CO2 atmosphere.

Cell Treatment

MicroRNA Mimic Transfection

miRNAs mimics were purchased from Sigma-Aldrich. miRNA mimics are dsRNA oligonucleotides that act as mature miRNAs. Mimic-induced downregulation experiments can be used to help demonstrate microRNA-target relationships. Negative controls mimics are based on *C. elegans* sequences and have been shown to elicit no identifiable effects. Before transfection, 3KT or MCF7 cells were cultured until about 60% confluent. For transfection, cells were transfected with the mimic using Oligofectamine (Invitrogen) reagent according to the manufacturer's instruction. Cells were incubated under the conditions described above for 48 hours, and then protein and/or RNA was collected and analyzed. Transfection efficiency was not measured. We used hsa-miR-21 (catalog no. HMI0371), has-miR-21* (catalog no. HMI0372), and Negative Control 2 (catalog no. HMC0003) in our mimic transfection experiments.

Transduction with Lentiviruses

Dr. Ya Wang's lab generously provided fully packaged lentiviral particle encoding the precursor miR-21 and GFP for immediate use. Lentivirus transfection was performed by Dr. Wang's lab using the Lenti-Pac HIV Expression Packaging Kit (Genecopoeia, catalog no. HmiR0284-MR03) under the manufacturer guidelines. 3KT cells were infected with the lentiviral particles using Polybrene (Sigma-Aldrich) at 10ug/ml. Media was replaced within 24 hours to remove Polybrene. Cells were later visualized to confirm the presence of GFP and to determine the relative efficiency of transfection. Cells were collected at 48 or 72 hours and lysates were prepared for western blot analysis.

X-ray Irradiation

3KT cells were plated in 10cm dishes and allowed to recover overnight (3x10^6 for the week time course, and 4x10^6 for the day time course). Subsequently, they were exposed to 0, 1, or 4 Gy gamma radiation using the X-RAD 320 x-ray system. Cells were collected at various times and immediately lysed using standard procedures.

Cell Visualization

Microscopy was performed using an Olympus IX51inverted microscope. Images were acquired at various magnifications using a Hamamatsu ORCA-ER monochrome digital camera (Hamamatsu photonics, Bridgewater, NJ) and modified for presentation using ImageJ (U. S. National Institutes of Health, Bethesda, Maryland).

Western Blot

Cells were lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× protease inhibitors (Roche #13145700)) on ice for 10 min, and lysates were clarified by centrifugation. Protein content was quantified using the Bio-Rad Bradford protein dye assay and electrophoresed for 1 hour at 200V on an SDS-PAGE minigel. Immunoblot analysis was performed by the transfer of proteins onto a polyvinylidene difluoride (PVDF) membrane overnight at 25V, followed by blocking in TBS containing 0.1% Tween and 5% bovine serum albumin (BSA) or 5% non-fat milk for approximately 1 hour. The membranes were incubated overnight with primary antibodies and then washed and subsequently incubated with secondary antibodies conjugated with HRP for about 1 hour at room temperature. After extensive washing, the bands were visualized using

standard HRP chemiluminescent procedures. The same membranes were stripped and re-probed repeatedly with other primary antibodies. The following primary antibodies were used: GAPDH (Abcam; catalog no. 8245), DNMT1 (Vertino Lab), DNMT3a (Active Motif; catalog no. 39206), DNMT3b (Active Motif; catalog no.39207), TET 2 (Active Motif; catalog no.61389, 61390), programmed cell death protein 4 (PDCD4) (Rockland; catalog no. 600-401-965), PTEN (Cell Signaling).

RT-qPCR

RNA was isolated using Qiagen RNeasy Mini Kits (Qaigen; catalog no. 74104) and was subsequently reverse transcribed to cDNA using random hexamer priming and Moloney murine leukemia virus reverse-transcriptase (MMLV-RT). cDNA was amplified with primers against DNMT1, PDCD4, PTEN, and 18s using SYBR Green assay performed in triplicate on a Bio-Rad MyIQ optical module instrument under standard conditions. Starting RNA quantities were determined relative to a common standard curve generated using A549 cDNA. The relative expression levels were calculated by standardizing to the housekeeping gene 18s rRNA.

Forward and reverse primer sequences, respectively:

DNMT1: CTCAGCCTCCCAAGTAACTG, GGCTCTTTCAGACTCTTCCTG

PTEN: CTAAGTGCAAAAGATAACTTTATATCA,

ACAACATAGTACAGTACATTCATACCTAC

18s: GAGGGAGCCTGAGAAACGG, GTCGGGAGTGGGTAATTTGC

PDCD4: GACTACCAAAGAAAGGTGGTGCAGGAGG,

CATAAACACAGTTCTCCTGGTCATCATCATAGTTAGGAT

RESULTS

MicroRNA-21 May Target DNA Methyltransferase 1

Effect of Stably-Overproduced MiR-21 on DNMT1

We began studying the effects of miR-21 on DNMT1 in a setting where the microRNA is stably overproduced. A non-tumorigenic human bronchial epithelial cell line (NL20) was transduced by Dr. Wang's lab with a stably expressing plasmid containing miR-21 or with an empty plasmid, NL20+ or NL20-, respectively. According to data shared by Dr. Wang's lab, miR-21 levels in the NL20+ were approximately 6 times higher than that in the NL20- (Figure 4.) Protein lysates from frozen cell pellets and isolated RNA shared by Dr. Wang's lab was subjected to western and RT-qPCR analysis, respectively. Figure 5 is representative of the western data collected from the NL20s. DNMT1 levels appear relatively consistent in both NL20+ and NL20- cells, while PDCD4 and PTEN appear to be slightly downregulated. PDCD4 and PTEN are known targets of miR-21 and act as positive controls in the transfection experiments. Figure 6 are representative graphs of the mRNA levels of DNMT1, PDCD4, and PTEN in NL20. The mRNA data seems to mirror the protein data; DNMT1 levels are relatively consistent, while PDCD4 and PTEN are both downregulated. In sum, this experiment suggests that DNMT1 levels are not greatly affected by the stable overproduction of miR-21.

To verify that the results from the NL20+ were reproducible, Dr. Wang's lab provided us with another set of NL20+ which had undergone identical transduction procedures. Protein cell lysates and RNA from the NL20+ cells was collected using procedures as described. Similar to the first experiment, the relative protein (Figure 7) and mRNA level of DNMT1 (Figure 8) of

DNMT1 appears consistent between samples. PTEN and PDCD4 mRNA levels are both lower in the NL20+. Table 1 illustrates the fold change in mRNA levels in the two experiments.DNMT1 levels appear slightly reduced, while PTEN and PDCD4 are greatly reduced. In each case, the known targets of miR-21 (namely PDCD4 and PTEN) were consistently downregulated, which is consistent with expression of miR-21. In contrast, neither DNMT1 protein nor mRNA appeared to be significantly downregulated. Taken together, the NL20 experiments indicate that miR-21 likely does not have an effect on DNMT1 expression when the microRNA is stably overexpressed.

Effect of Transiently Increased MiR-21 Levels on DNMT1

Given that there may be mechanisms of DNMT1 recovery that negate the effect of stably high miR-21 levels, and that the NL20+ cell lines have a tendency to lose their potency to overexpress miR-21, we next attempted to look at the effect of miR-21 on DNMT1 in transient settings. To accomplish this task we first utilized microRNA mimics, which act as mature microRNAs upon transfection. 3KT cells were transfected with microRNA mimics, and protein and RNA samples were collected at various times post-transfection for western and RT-qPCR analysis. Figure 9 and Figure 10 are representative results that illustrate the protein and message levels of DNMT1 and PDCD4 after transfection of miR-21 mimics, respectively. DNMT1 expression (as measured by protein or message level) does not appear to be significantly altered with the addition of miR-21-3p, the miR-21 product strand predicted to target DNMT1. Likewise, PDCD4 levels were relatively consistent between treatments, and did not appear lower with the addition of miR-21-5p, the miR-21 strand known to target PDCD4. Variations of this experiment were repeated with similar results. The lack of response in the positive control (PDCD4) suggests that the

transfections were not efficient. Therefore, we attempted to transfect MCF7, a breast cancer cell line, which is more susceptible to transfection. Figure 11 shows that while DNMT1 levels are not discernible, the transfection does appear more efficient than previous experiments since PDCD4 levels appear lower in the miR-21-5p treatment.

We have also attempted to induce miR-21 overexpression in 3KT cells by infecting them with a lentivirus that expresses the miR-21 pre-miR and GFP. This allows us to visualize the efficiency of infection, thereby reducing the uncertainty experienced with mimic transfections. 3KT were exposed to varying amounts of viral supernate containing lentiviral particles, and images were acquired at various times after infection. The concentration of viral supernatant and time was found to be more efficient at 100ul virus supernate per 500ul media, as shown in Figure 12 A. Thus, 3KT cells were incubated with 20% v/v viral supernatant for 24 hours, and harvested at 60 hours. Western analysis showed that DNMT1 levels were not significantly different between cells treated with the viral supernatant or the mock virus supernatant (data not shown). It is likely that this is due to the low infection efficiency (Figure 12, B).

X-Ray Irradiation Elicits Changes in DNMT, TET Expression

In the next set of experiments we tested the effect of both dose and time of exposure to gamma radiation and its effect on the expression of key epigenetic proteins, namely DNA modifiers DNMT1, DNMT3a, DNMT3b, and TET2. Cells were exposed to either 1Gy or 4Gy of gamma radiation (1a and 1b are biological replicates of 1Gy exposure), or left unexposed. Both longer term and short term changes in protein expression were monitored by western blot analysis. Protein levels were measured either daily over the period of 1 week (including days 1, 3, 5, and 7), or every few hours (2, 4, 8, 12) over the course of 1 day.

Time-Dependent Effects of Irradiation

Some aspects of the epigenetic protein expression response to radiation exposure exhibit a time dependency. Figures 13, 14, 15, and 16 show protein levels of DNMT1, DNMT3a, DNMT3b, and TET2, respectively, up to one week after exposure to radiation. Figure 13 indicates that DNMT1 levels appear to increase in the irradiated cells versus the non-irradiated over the week. As Figure 14 illustrates, total DNMT3a expression appears to increase over the period of 7 days in irradiated cells. Furthermore, the migration of DNMT3a appears to shift in response to irradiation over time. DNMT3a is known to undergo certain posttranslational modifications, including sumoylation, which has been shown to modify its interactions with histone deacetylases (Ling, Sankpal et al. 2004). However, the functions of most DNMT3a modifications are not understood. It is also possible that this represents an alternate isoform produced by alternative splicing. DNMT3b levels remain relatively constant in both the treated and the untreated cells over the weeklong time course. TET2 however, shows a dramatic upregulation in the 4 Gy treatments, especially after day 4. In conclusion, these experiments indicate there are time dependent effects of exposure to gamma radiation over the course of 1 week. Specifically, DNMT1, DNMT3a, and TET2 levels increase over time; DNMT3a migration shifts are also altered a week after initial exposure.

Dose-Dependent Effects of Irradiation

Some changes in epigenetic protein expression also exhibit a dose dependency to radiation exposure. Figure 17 illustrates some of the dose effect of gamma radiation. While DNMT1 levels are only available for days 1 and 3, it appears as if DNMT1 is consistent between the untreated and the cells exposed to 1 Gy of gamma radiation, but lower in the cells exposed to 4 Gy.

DNMT3a however, by days 3 and 5, appears upregulated after both 1 Gy and 4 Gy exposures. It is interesting to note in Figure 15 that both the increase in expression of DNMT3a and the alterations in its migration are especially pronounced in the 4Gy treatment as opposed to the 1 Gy treatments, implying both total expression and migration of DNMT3a are affected by radiation dose. Figure 14 shows that by day 5, the slower migrating DNMT3a band, perhaps a modified form of DNMT3a, is more apparent in the treated samples. By day 7, there is a distinctively positive correlation between the slow migrating DNMT3a band and dose of radiation. This implies that increased dose may lengthen the period during which these DNMT3a alterations are apparent. TET2 levels are consistent between samples up until day 5. On days 5 and 7, the 4 Gy treatments appear to be greater than the 1 Gy or the untreated cells. In conclusion, these experiments indicate that there is a dose response to radiation exposure that manifests in altered epigenetic protein expression. In general, as dose increases, DNMT1 protein levels appear to decrease while DNMT3a, DNMT3b, and TET2 levels seem to increase; dose also seems to affect DNMT3a gel migration patterns.

DISCUSSION

High LET radiation has been shown to induce carcinogenesis, and is a potential risk for space travelers (Durante and Cucinotta 2008; Zhu, Yu et al. 2010). This study aims to investigate the epigenetic consequences of radiation exposure so that we can better estimate and combat the cancer risks of space travel. Previous studies indicate that miR-21 plays a primary role in the carcinogenic response elicited by high LET radiation (Zhu, Yu et al. 2010). Preliminary data suggest that DNA methylation patterns are altered in cells exposed to radiation. We hypothesized that miR-21 may play a role in disrupting epigenetic patterns by targeting DNMT1. Furthermore, it was suspected that the expression of key DNA modifying proteins, such as DNMT1, DNMT3a, DNMT3b, and TET2 may be altered in response to irradiation. Thus the primary goals of this study were to test the potential microRNA-target relationship between miR-21 and DNMT1, and to determine the changes in epigenetic protein expression that occur in response to gamma radiation exposure.

We utilized several methods to determine the relationship between miR-21 overproduction and DNMT1 levels in an effort to explain the altered methylation patterns seen in cells exposed to high LET radiation. In experiments using lung cells stably overexpressing miR-21, protein and mRNA DNMT1 levels seemed relatively unaffected. We next looked at transient miR-21 overexpression since there could be mechanisms of DNMT1 recovery that offset the effect of stable miR-21 levels overproduction. In the transient miR-21 overexpression experiment where we transfected miR-21 mimics, protein levels of DNMT1 appeared to be lower than the mock transfection after 48 h, but PDCD4 levels, our positive control, were unaltered indicating that the transfections may be inefficient. The transfection procedure was further optimized using a cell line that is more receptive to transfection. Finally, in another

transient miR-21 overexpression model, we utilized a lentiviral construct that expresses the miR-21 pre-miR and GFP. Several infection conditions have been tested to optimize the lentiviral dose and time of infection. Western analysis suggested DNMT1 levels were not affected, but this is likely due to the low infection efficiency. Taken together, our data suggest that while stably overexpressed miR-21 may play a significant role in space radiation promoted tumorigenesis by targeting tumor suppressors such as PDCD4 and PTEN, it does not appear to affect DNMT1 levels in that setting. Thus, the epigenetic alterations seen in cells exposed to high LET radiation may not be mediated by miR-21 targeting DNMT1. It is possible that miR-21 may directly or indirectly suppress the expression of other key epigenetic proteins, or that epigenetic protein expression is altered in a miR-21 independent fashion.

We also examined the changes in expression of key DNA modifiers to clarify the mechanisms of DNA methylation pattern alterations that occur in cells exposed to gamma radiation. To carry this out, cells were exposed to gamma rays and DNMT1, DNMT3a, DNMT3b, and TET2 levels were measured using western blot analysis. Changes were observed as factors of both time after irradiation and dose given. The time-dependent effects of exposure to gamma radiation include increasing levels of DNMT1, DNMT3a, and TET2 over a weeklong period. The dose-dependent effects of radiation include decreasing DNMT1 levels, and increasing DNMT3a, DNMT3b, and TET2 levels as dose increased. Interestingly, we observed a shift in the migration pattern of DNMT3a in response to radiation exposure. A slower-migrating DNMT3a band is revealed in cells exposed to radiation. Furthermore, this band seems to correlate with radiation dose. This finding is especially noteworthy since it implies that the DNMT3a protein itself is being altered either in its size or charge. This could occur through alternate splicing or posttranslational modification. A number of alternate DNMT3a transcripts

have been isolated, although the difference in function of these isoforms is not understood. Likewise, DNMT3a has been shown to undergo a number of posttranslational modifications, but these are also understudied. Whether by posttranslational modifications or other means, changes in DNMT3a gel migration indicate that DNMT3a is affected by exposure to radiation; these changes may play a role in promoting radiation induced cancers. In summary, the expression of key DNA modifying proteins, including a number of DNA methyltransferases and TET2, is shown to respond to irradiation. These expressions changes may play a role in altering DNA methylation patterns and influence radiation induced cancer formation.

FUTURE DIRECTIONS

While these experiments have shed light on the epigenetic protein response to irradiation and may provide mechanisms for which DNA methylation patterns are altered in radiation induced carcinogenesis, much of the biological response to radiation is still unknown. Further studies are required to fully elucidate the effect of miR-21 on DNMT1 expression. Repeating the mimic transfection experiments with cell lines more amiable to transfections may clarify the potential miR-target relationship. If there appears to be a correlation between the addition of miR-21-3p, the miR-21 product that is predicted to target DNMT1, and DNMT1 levels, a luciferase reporter assay can be used to assess the effect of the predicted binding site of DNMT1 on luciferase transcript stability and translation efficiency. The infection experiments can be further optimized in a cell line that is more prone to infection, and infection procedures can be modified to increase efficiency. Finally, we can execute a rescue experiment where cells are transfected with an anti-miR-21 oligonucleotide, which effectively decreases cellular miR-21 content. These experiments would further elucidate the relationship between miR-21 and DNMT1 levels.

It is also necessary to explore the significance of the altered DNA modifying proteins seen in the gamma radiation exposure experiments. Altered DNMT and TET levels may influence global DNA methylation levels and distribution. The Vertino lab has already began to look at the long-term changes in DNA methylation patterns in lung cells exposed to high LET radiation, but has yet to repeat similar experiments in a low LET environment. Furthermore, the mechanistic relationship between altered DNA methylation patterns and changing DNMT and TET expression has yet to be studied. Knockout experiments and overexpression programs could be used to investigate the role these proteins play in changing DNA methylation patterns, altered gene expression profiles, and carcinogenesis. It would also be useful to explore the mechanisms

which lead to the altered DNA modifying protein expression that occurs in response to radiation exposure. This could yield insight as to how exactly an “epigenetic memory” of irradiation is created, and perhaps even how it can be prevented or manipulated. Ultimately, these experiments will not only allow us to better estimate the risks of space travel or exposure to terrestrial radiation, they will also benefit our understanding of the field of epigenetics and its complex role in carcinogenesis.

FIGURES

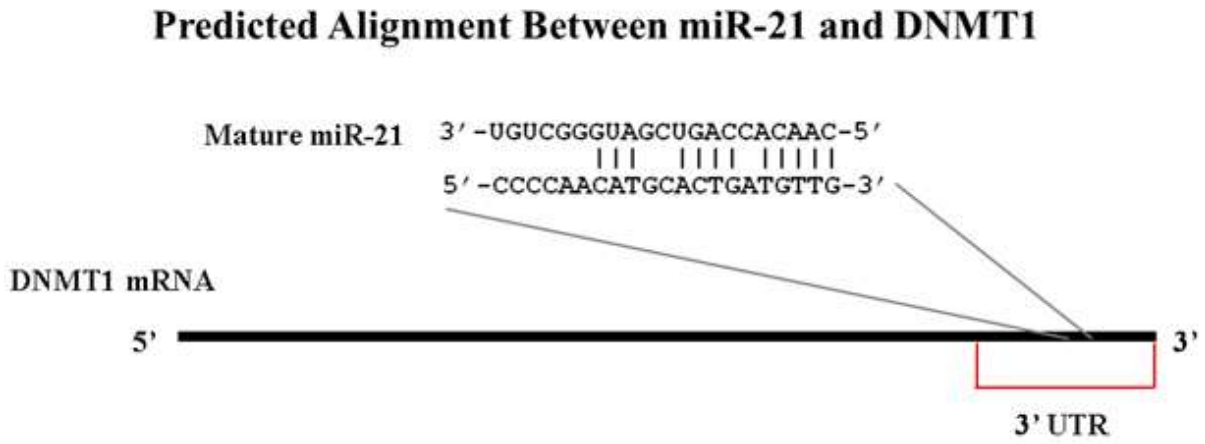


Figure 1. Predicted *miR-21 Target sequence in the 3' untranslated region of DNMT1.* The sequence alignments and predicted position of miR-21 relative to the processed DNMT1 message. miR-21 is predicted to bind in the 3'UTR of DNMT1. Figure taken from Emory NSCOR Proposal.

Figure 2. *DNA Methylation and DNA methyltransferases.* DNA methylation occurs on cytosine residues in the context of CG dinucleotides. The methylation reaction requires S-adenosylmethionine (SAM). Figure shared by Dr. Paula Vertino.

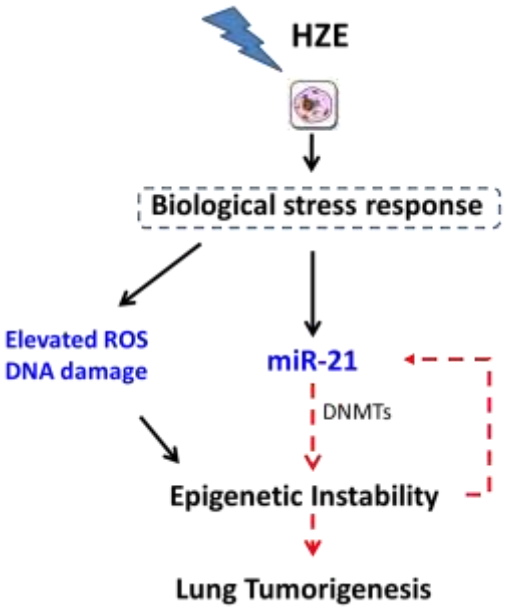


Figure 3. *Schematic of hypothesized cellular response to high LET exposure*. Irradiation with high LET particles elicits a unique biological stress response, including upregulated miR-21. Figure taken from Emory NSCOR Proposal.

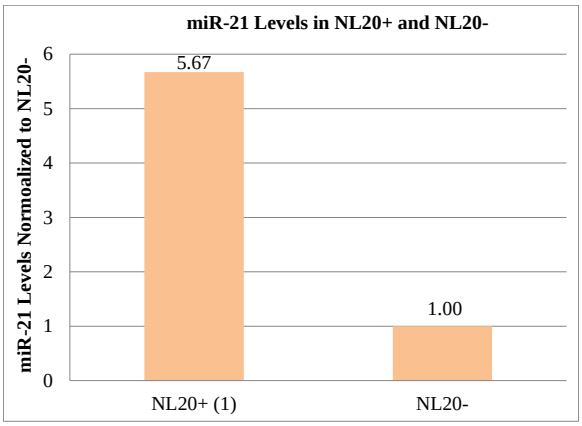


Figure 4. *miR-21 Levels in NL20+ and NL20- cells.* miR-21 levels are approximately 6x higher in cells transduced with a stably producing plasmid (NL20+) containing miR-21, compared to those transduced with an empty plasmid (NL20-). Data shared by Dr. Ya Wang's lab.

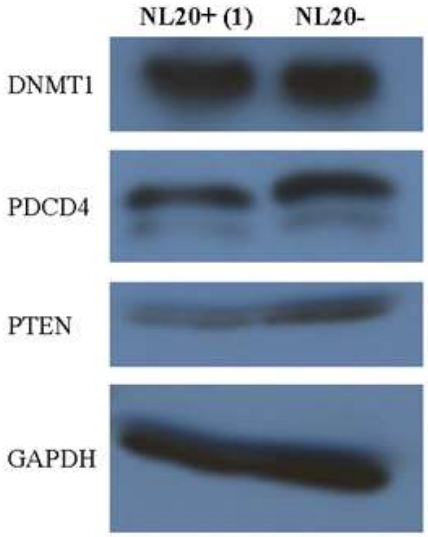


Figure 5. *Protein Levels of DNMT1, PDCD4, and PTEN in NL20 cells.* DNMT1 levels are consistent while PDCD4 and PTEN appear to be lower in the NL20+ cells. Protein made from frozen cell pellet donated by Dr. Ya Wang's lab.

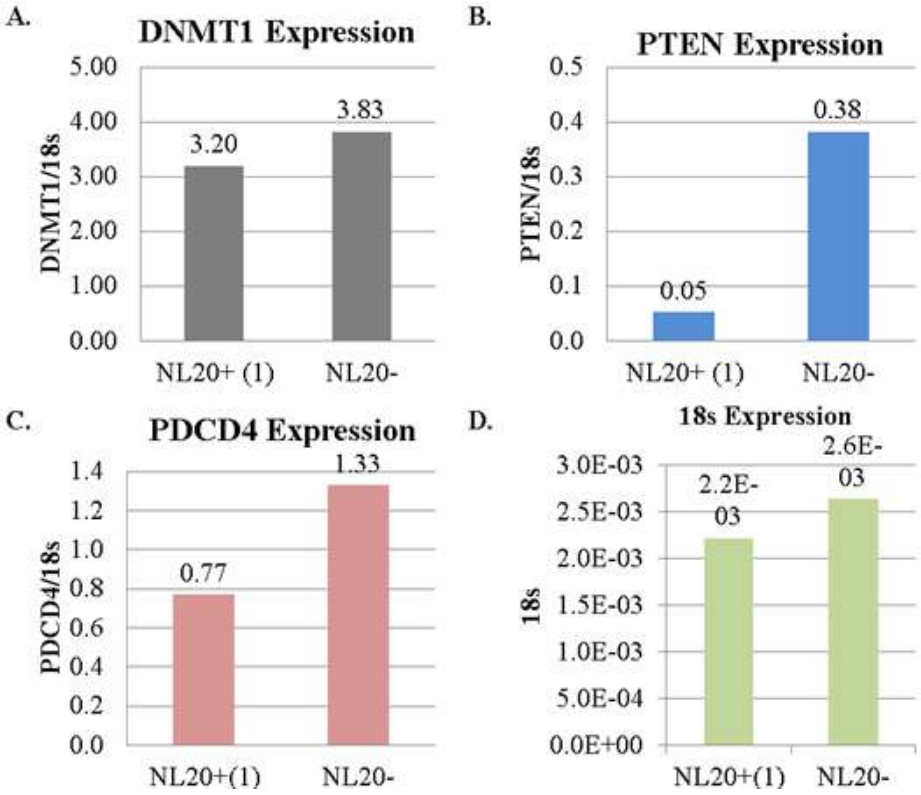


Figure 6. *mRNA levels of DNMT1, PDCD4, and PTEN in NL20 cells (1).* A: DNMT1 appears to be nominally less (~16%) in NL20+ compared to NL20-. B-C: Both PTEN and PDCD4 are decreased (PTEN-86%, PDCD4-52%). D: 18s levels are relatively consistent considering experimental uncertainty. (18s is a housing keeping gene that serves as an internal control).

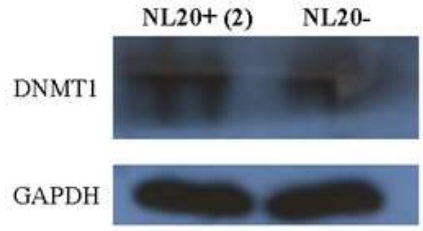


Figure 7. *Protein Levels of DNMT1 in NL20+ and NL20- cells.* DNMT1 protein analyzed using NL20+ and NL20- cell lysates appear consistent between cell types. PTEN and PDCD4 data is unavailable.

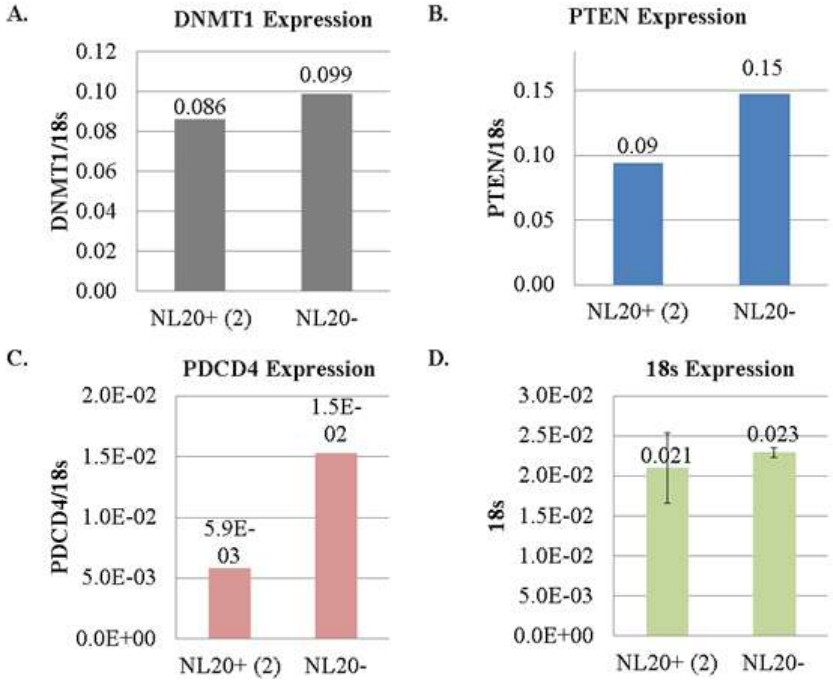


Figure 8. *mRNA levels of DNMT1, PDCD4, and PTEN in NL20+ and NL20- cells (2).* A: DNMT1 appears to be slightly downregulated (~13%) in NL20+ compared to NL20-. B-C: PTEN and PDCD4 are acknowledged targets of miR-21 and are used as positive controls. Both are decreased substantially (PTEN-46%, PDCD4-62%). D: 18s levels are approximately equal considering experimental uncertainty, and are used as an internal standard.

	Fold Change		
	DNMT1	PTEN	PDCD4
NL20+(1)/NL20-	0.84	0.14	0.48
NL20+(2)/NL20-	0.87	0.64	0.38

Table 1. *Fold Change in Protein Levels.* DNMT1, PTEN, and PDCD4 levels in NL20+ relative to NL-20- after normalization to a loading control. DNMT1 levels are reduced, but not to the same extent that PTEN and PDCD4 are reduced.

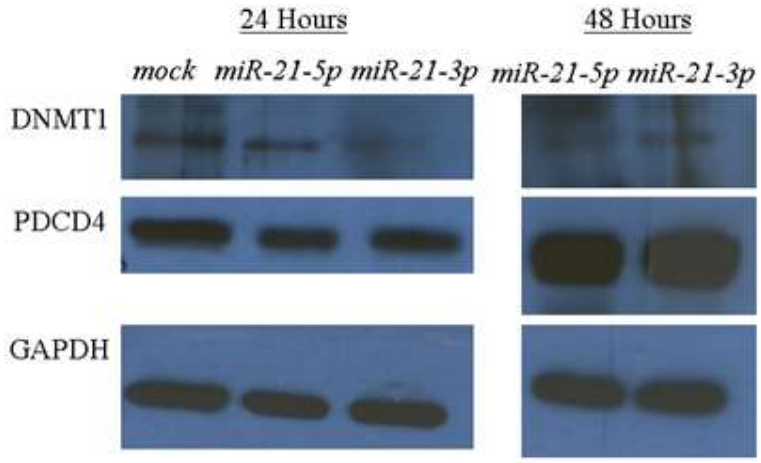


Figure 9. *DNMT1 and PDCD4 Protein Levels in miR-Mimic Transfected 3KT Cells.* 3KT cells were transfected with 50nmols of mock microRNA (a *C. elegans* miR shown to shown to elicit no identifiable effects), miR-21-5p, or miR-21-3p. Cells were collected at 24 and 48 hours post-transfection. At 24 hours, DNMT1 protein levels appear to below lower in the miR-21-3p treatment, however, PDCD4 protein levels remain consistent between samples. At 48 hours, DNMT1 levels appear to be consistent in both miR-21-5p and miR-21-3p treatments, as does PDCD4.

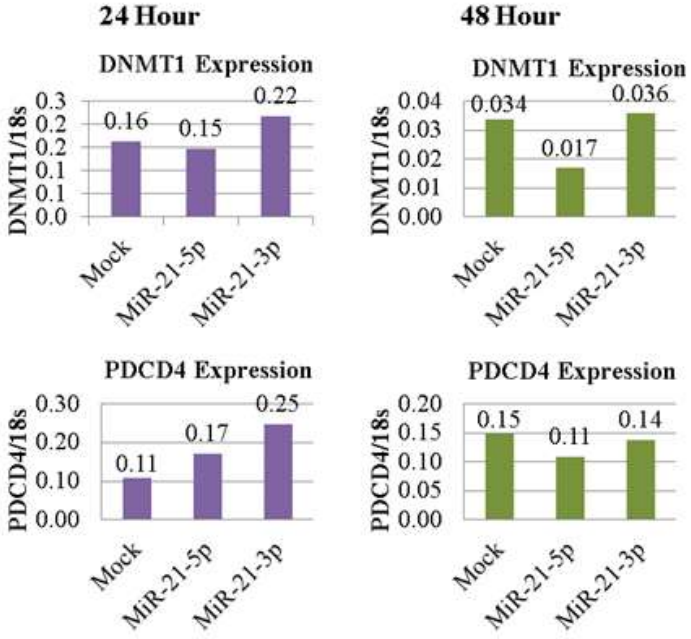


Figure 10. *DNMT1 and PDCD4 mRNA Levels in miR-Mimic Transfected 3KT Cells.* RNA was collected in tandem with the experiment in Figure 9, reverse transcribed, and subjected to real time PCR. At 24 hours, DNMT1 message levels are consistent, and PDCD4 is not reduced in the miR-21 treatments. At 48 hours, DNMT1 message level appears to be lowered in the miR-21-5p, but PDCD4 levels are not significantly reduced.

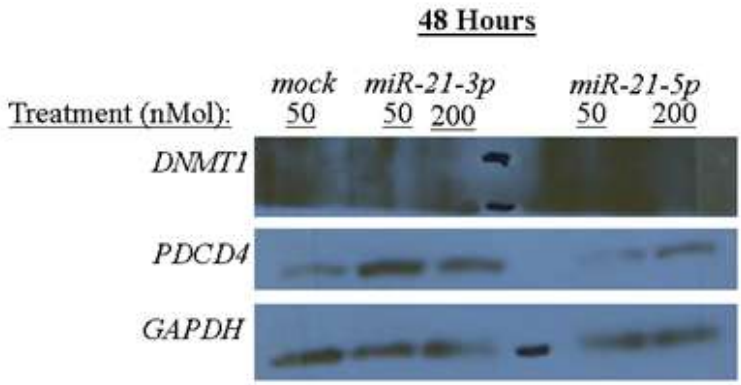
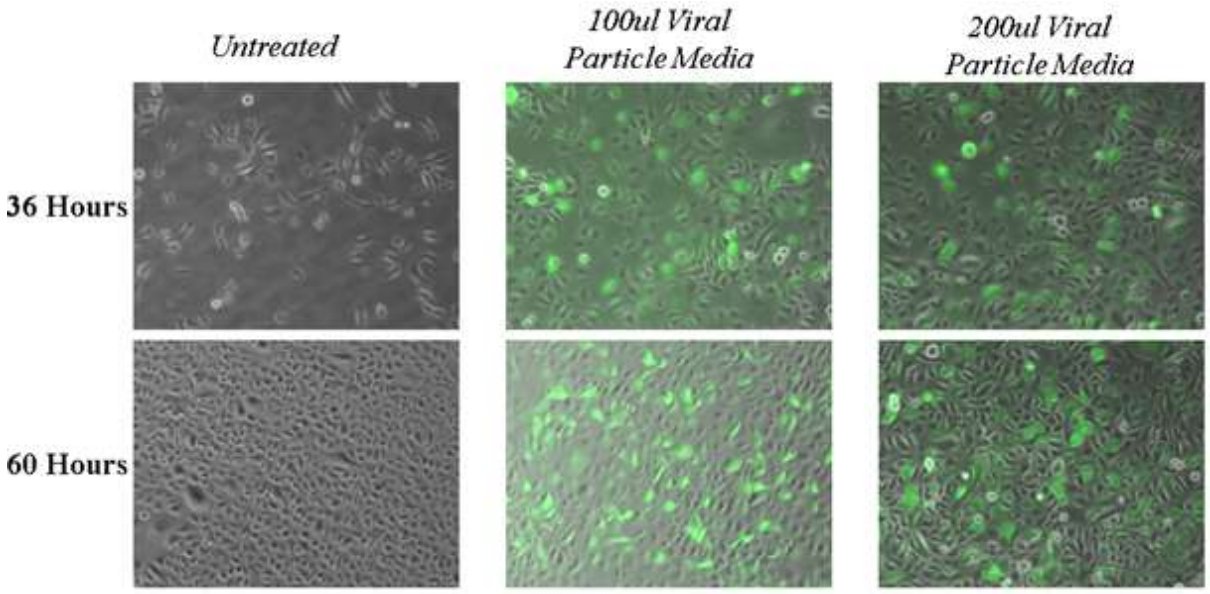


Figure 11. *DNMT1 and PDCD4 Protein Levels in miR-Mimic Transfected MCF7 Cells.* MCF7 cells were transfected with 50nmols of mock microRNA, or either 50nmols or 200nmols of miR-21-5p or miR-21-3p. Cells were collected and 48 hours post-transfection. DNMT1 levels are not discernible, but PDCD4 appears to be reduced in the higher treatment of the miR-21-5p, indicating that the transfection may have been efficient.

A.



B.

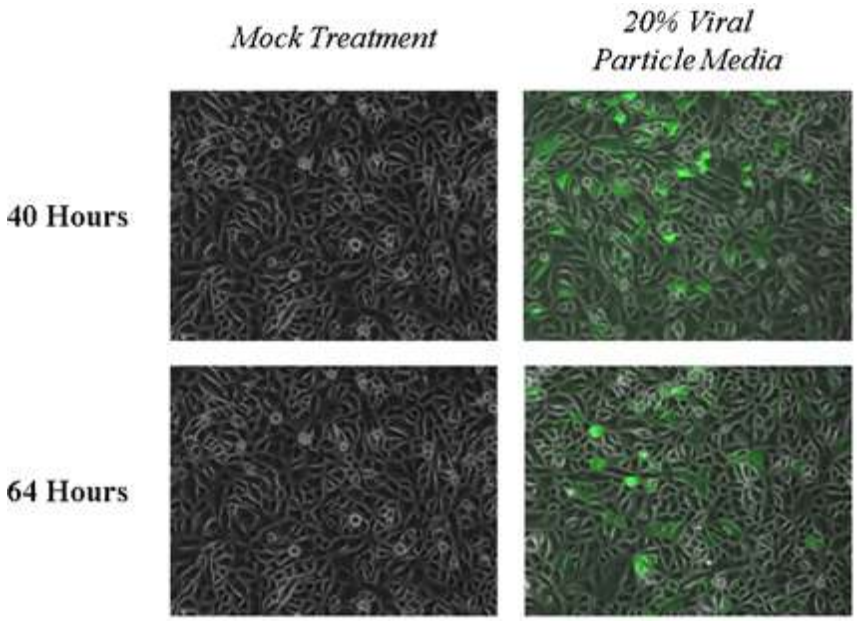


Figure 12. *3KT Lentiviral Infection Efficiency*. A: To measure infection efficiency, 3KT were infected with 100ul (20% of the total media) or 200 ul (40%) viral media which held viral particles containing precursor miR-21 and GFP. At both 36 and 60 hours, 100ul viral particle treatments seemed to have higher infection efficiency as suggested by the merged GFP and bright field images above. Infection efficiency did not appear to increase after 60 hours (not pictured). B: 3KTwere infected with 20% viral particle media and collected at 64 hours post-infection.

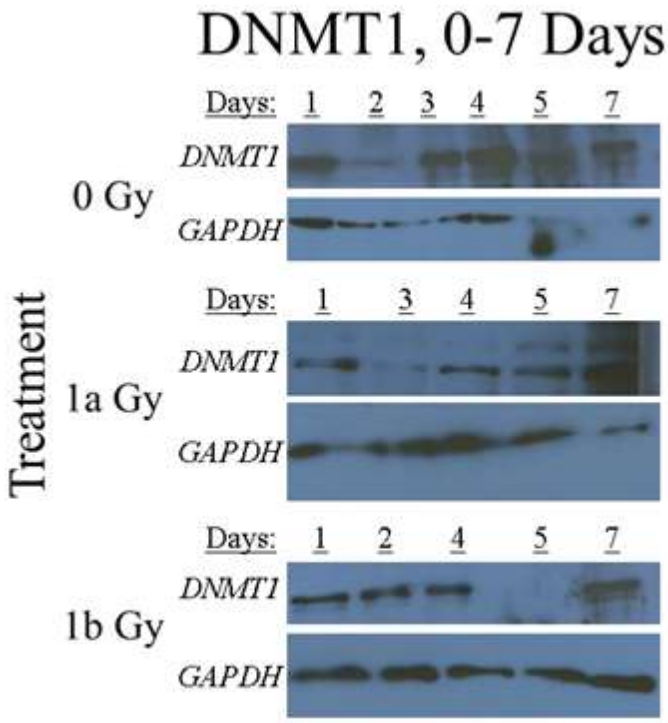


Figure 13. *Time-effects of radiation on DNMT1*. Protein lysates from 3KT cells exposed to varying amounts of radiation were collected over 1 week and analyzed by western. DNMT1 levels appear relatively consistent in the untreated cells, while DNMT1 levels appear to increase slightly over the period of 7 days in the cells treated with 1Gy radiation (1b especially).

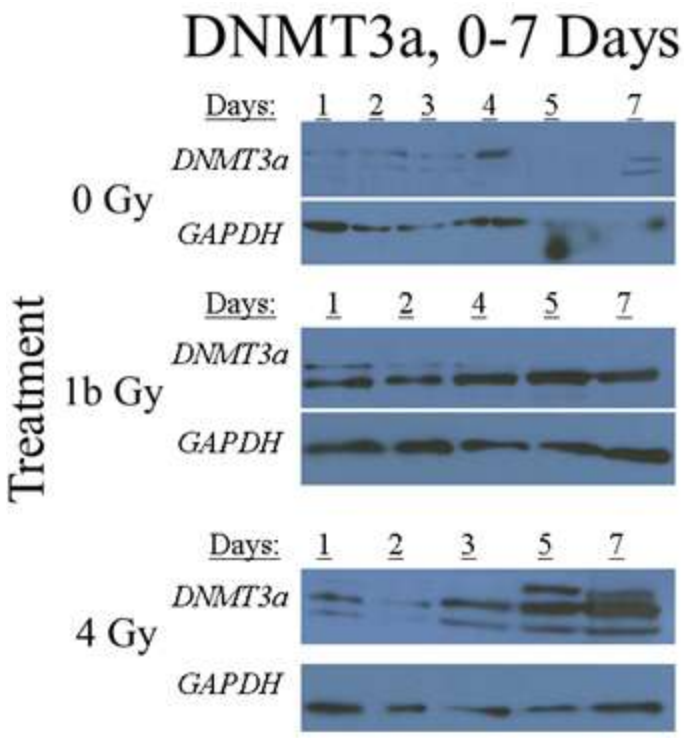


Figure 14. *Time-effects of radiation on DNMT3a.* DNMT3a levels appear consistent in the non-irradiated cells. In both radiation treatments, DNMT3a expression appears to increase over the period of 7 days. Furthermore, the distribution DNMT3a gel migration appears to change in the irradiated cells. The migration distributions are especially pronounced in the 4Gy treatment as opposed to the 1Gy treatment, implying a dosage effect.

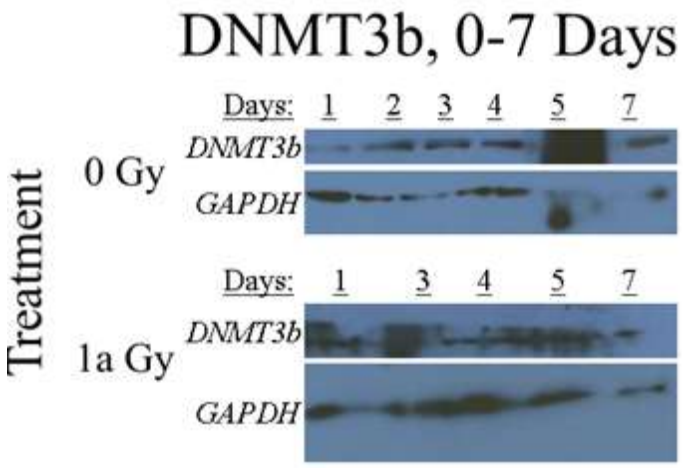


Figure 15. *Time-effects of radiation on DNMT3b.* DNMT3b protein levels appear relatively unchanged in both the non-irradiated and the cells irradiated with 1 Gy.

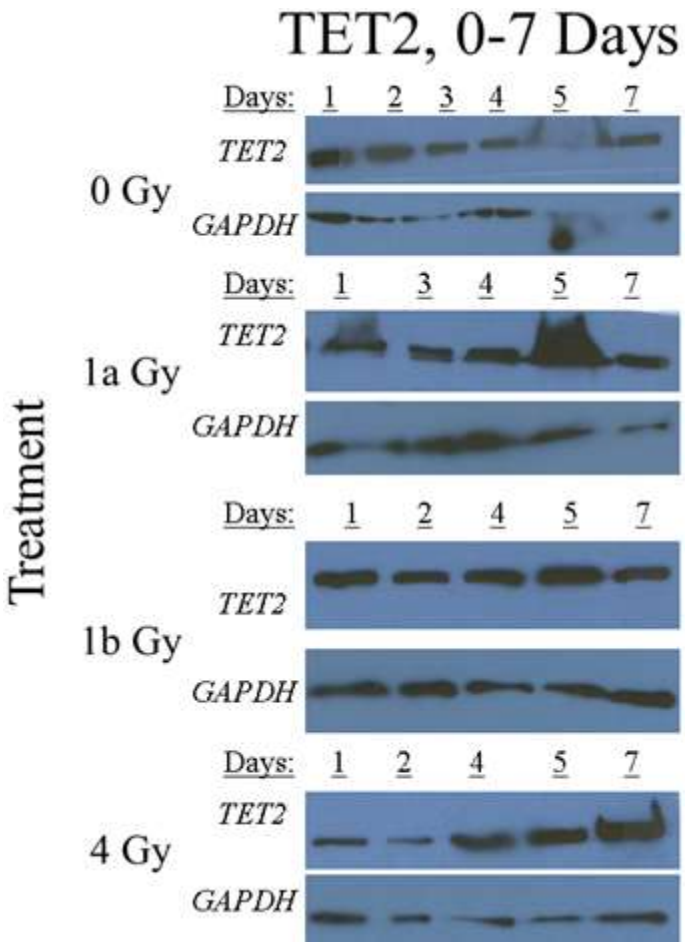


Figure 16. *Time-effects of radiation on TET2.* TET2 protein levels are consistent over a period of 7 days in both the non-irradiated and the cells irradiated with 1 Gy of gamma radiation. TET2 levels increase greatly by day 7 in the 4 Gy treated cells.

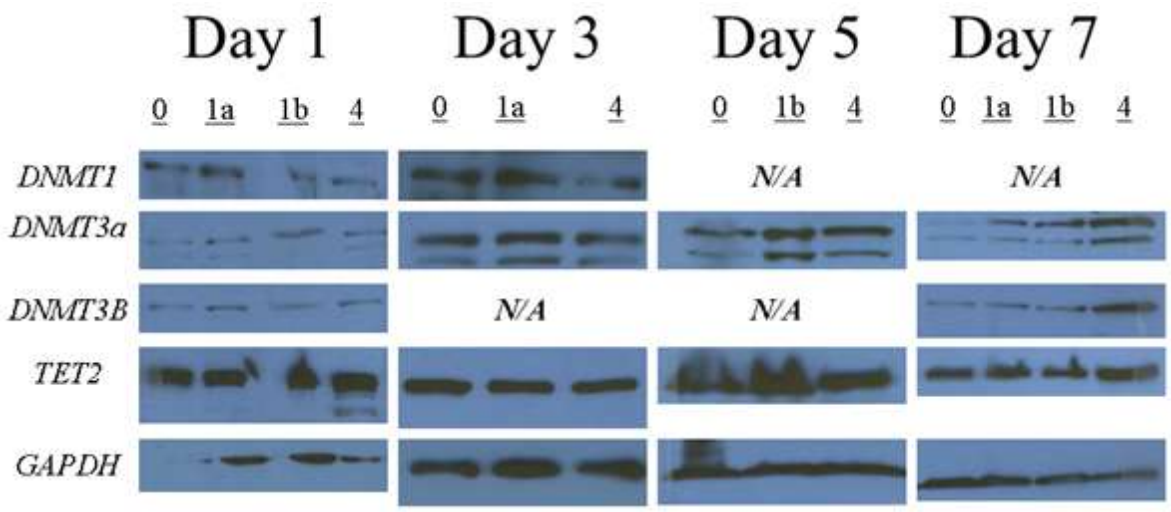


Figure 17. *Dose-effects of radiation on DNA modifying proteins*. Dose-response of DNMT1, DNMT3a, DNMT3b, and TET 2 expression in cells irradiated with 0, 1 or 4 Gy gamma radiation. DNMT1 protein levels appears to decrease with increasing dose. DNMT3a, DNMT3b, and TET2 protein levels appear to increase with increasing expression. DNMT3a gel migration patterns are also different between doses.

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