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 The Emory University-Georgia Health Sciences University NSCOR will investigate the mechanisms by which high charge and energy (HZE) particles, a component of space radiation, induce lung cancer. HZE exposure elicits complex DNA damage, together with a broader cell/tissue stress response that likely includes changes in expression of tumor suppressor proteins, persistent elevation of reactive oxygen species, and alterations in the pattern of DNA methylation. The central hypothesis of this NSCOR is that this broader stress response amplifies the carcinogenic risk from a primary DNA damage event. Preliminary studies suggest that a small noncoding RNA, microRNA-21 (miR-21) plays a key role in coordinating the HZE particle-associated stress response. Center investigators will use genetic, epigenetic, and biochemical approaches to address the role of miR-21 dependent and independent stress responses in HZE particle-induced lung cancer. There are four projects: Determine whether the lung cancer suppressor, Gprc5a, protects against HZE particle-induced lung carcinogenesis, and whether miR-21 overexpression blunts this protective effect. Determine whether HZE-particle radiation exposure results in hyper-reliance on error-prone DNA repair pathways, whether miR21 mediates this effect, and whether dysregulation of DNA repair contributes to HZE particle-induced lung carcinogenesis, and the role of miR-21 in this process. Determine the nature of the HZE-particle radiation-induced alterations in DNA methylation patterns, whether these alterations contribute to lung carcinogenesis, and the role of miR-21 in this process.
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Lung cancer is the most common fatal cancer among men and women worldwide. Lung cancer is believed to be one of the major risks of HZE-particle exposure, although quantitative and mechanistic understanding of this risk is lacking. The EU-GHSU NSCOR will address this important knowledge gap. In addition, our NSCOR team will answer the question concerning whether and how different qualities (LET) of radiation affects lung tumorigenesis. These results will not only provide important information that will aid in the facilitation of the NASA Mars project, but will also provide the public with useful information concerning lung carcinogenesis and the benefits of cancer prevention.
 Radiation: this proposal includes two major categories: Animal and cell experiments. We have performed several types of studies, which are described as follows: Cell studies: We carried out several experiments using human lung epithelial cells and cells over-expressing miR-21. We are currently preparing mouse lung epithelial cells from miR-21 knock-in or Gprc5a-/- mice. Exposure of those cells to X-ray or HZE- particles is planned for the next year of support. Animal studies: We have irradiated 1120 mice and plan to finish additional miR-21-/- mice exposure in 2013. X-ray exposure was performed at the Department of Radiation Oncology, Emory University and the HZE-particle exposure was performed at NASA Space Radiation Laboratory (INSRL), Brookhaven National Laboratory (BNL), We originally planned to sacrifice the mice at 8 months after irradiation; due to the fact that no significant tumorigenesis at this time point was observed from 3 irradiated miR-21 mice, we extend the observing period from 8 months to 1.5 year to secure obtaining the results of IR-induced tumorigenesis. During 2013, we will obtain some results of tumorigenesis from the irradiated mice. We expect to obtain the entire tumorigenesis is described as follows: Project 1: The main purpose of this project is to identify the targets of miR-21 and their regulation and effects on miR-21 on the generation of radiation-induced reactive oxygen species (ROS), the results have been published (Cancer Res, In Press, 2012). We found that miR-21 could directly target SOD3 and indirectly target SOD2 to enhance HZE particle-induced ROS and transformation in the human lung epithelial cells. In addition, we collaborate with Dr. Dynan to have found that the increased level of miR-21 and EGPR is correlated with the increased frequency of radiation-induced tumorigenesis, and also provides an additional explanation concerning why radiation-induced towa of the screess relative risk of HZE-particle radiation in lang e
To investigate mechanism, unexposed, 0.3 Gy, and 1.0 Gy groups were profiled in triplicate. Of some ~20,000 genes,

only 234 mRNAs were significantly altered at 7 d post-irradiation. The gene set highly enriched for genes that participate in the senescence associated secretory phenotype (SASP). Our results showed the top 15 genes, of which 5 are part of the SASP and two are related. Results were surprising as SASP was initially defined in fibroblasts (not the epithelial tumor cells used here) and with high doses of gamma radiation (not the moderate doses of Fe used here). However, our reading of the literature suggests there may be a common mechanism, which is the presence of unrepaired DNA damage, possibly including telomere-associated repair foci, and consequent oxidative stress. These are testable hypotheses that we will address in the coming year.

Specifically, we hope to soon extend results to a second, non-transformed epithelial cell line (initial results were in tumor cells). We also suspect that our cultures may contain a mixture of population of responder and nonresponder cells, particularly after 7-14 days continuous passage, and we therefore hope to perform studies at the single-cell level. Specifically we hope to correlate unrepaired damage, SASP, and mutagenic NHEJ at the single-cell level.

Project 3: DNA damage inflicted by radiation or chemotherapeutic drugs induces a cellular stress response by still undefined mechanisms and consequences for cell survival and genomic stability. The goals for Project 3 are to determine the nature of the HZE-particle induced stress response and the resulting DNA damage and genomic instability that contributes to HZE-particle induced lung carcinogenesis and the role of miR-21 in this process. In this second year we have measured reactive oxygen species (ROS), DNA damage, and genomic instability in response to low and high LET radiation (1Gy X-rays or Fe particles). We have identified an acute increase in ROS within the first 30-60 minutes following exposure, which is mediated by a cellular NADPH oxidase (NOX) and induces oxidative damage on the DNA bases, while mediating signaling necessary for efficient double strand break repair. Between 4 and 6 hours following radiation exposure, cellular ROS levels increase again, but this time the induction is dependent on a mitochondrial function of respiratory chain activity. We found that ROS produced by this pathway promotes the formation of micronuclei during the first cell division following exposure to radiation. ROS levels remain elevated for up to two weeks in the progeny of surviving cells, co-existing with biomarkers of cellular senescence and genomic instability. Importantly, Fe particle irradiation induced a more robust senescence-like response compared to low-LET X-rays. Mir-21 expression reduced the expression of some of the phenotypes associated with senescence as well as genomic instability. For the next year of support we will complete these studies and publish these distinct ROS responses and effects on genomic instability. We will focus next on the mechanism involved in the induction of the response and molecular targets of ROS mediating genomic instability.

Project 4: The primary goal of this project is to define the epigenetic determinants of HZE radiation exposure induced lung carcinogenesis and the extent to which this is mediated by miR-21. Based on preliminary results showing that exposure of liver cells to a single dose of high (Fe) vs. low (gamma) LET ionizing radiation induced stable alterations in DNA methylation that could be observed weeks after the initial exposure, we proposed that there may be an epigenetic "memory" of high LET radiation exposure wherein alterations in DNA methylation resulting from acute radiation exposure and local DNA damage have the potential to become 'fixed' if they are subsequently replicated, leading to permanent changes in DNA methylation and new gene expression programs. To test this hypothesis, immortalized human bronchial epithelial cells (3KT) were exposed to varying doses (0.3, 1.0 Gy) and sources (Si, Fe) of high LET radiation at the Brookhaven National Laboratory facility. Samples were collected from a fraction of the exposed population after 48hrs and the remaining cells maintained in continuous culture for an additional 50 population doublings (~4 months) with weekly collection for genomic DNA, RNA, and cellular protein. Unexposed cultures underwent the same handling procedures and were maintained in parallel. The methylation status of > 485,000 CpG residues across the human genome was analyzed using the Illumina Infinium Human Methylation 450K platform. We compared acute DNA methylation patterns (eg. 48 hr after exposure) with that observed 2 weeks, 3 weeks and 2 months after high LET radiation exposure. An analytical pipeline was developed to identify statistically significant changes in DNA methylation associated with dose, source or time after exposure. A mixed-effects model was applied using an in-house tool ('CpG assoc') wherein Beta values (methylation level) were treated as the outcome (dependent variable). with various co-variates considered including dose, time elapsed, chip position and a random effect for chip number. We considered the Fe and Si exposed cohorts separately in the analyses. Significance was assessed by the Holm, and Benjamini-Hochberg methods, and permutation analyses were incorporated to test for robustness of the results.

Our results indicate that the most significant association is with time; more than 100,000 CpG sites underwent a significant drift in methylation over time in culture, independently of radiation exposure. Nevertheless, high LET radiation exposure led to alterations in DNA methylation at a subset of these sites, with both hyper and hypomethylation events observed. In particular, we identified 124 CpG sites for which a change in methylation was significantly associated with Fe radiation dose (FDR<0.05). Interestingly, in all cases the radiation-induced methylation changes were observed at baseline (48h after exposure) and persisted over time, exhibiting the same intrinsic direction and rate of epigenetic drift as observed at the same site in control cells. These data suggest that a single exposure to high LET radiation may 'reset' baseline methylation levels at a subset of sites that is then acted on by an age-dependent mechanism that is cell type and site intrinsic. This has important implications in that it implies that a single exposure to 1.0 Gy Fe hLET irradiation led to a permanent change in methylation that approximates 30-40 days in cell culture, effectively accelerating aging-related DNA methylation changes. There also appears to be an influence of radiation quality (ion source/dose rate/energy) in that there were no significant methylation changes associated with Si ion exposure (0 CpG sites associated with Si dose at FDR<0.05) at the same dose and over the same time frame.

In related studies, we have begun to characterize the epigenetic modulators that may be targets of miR-21. Given the methylation changes observed and the proposed relationship between radiation exposure and miR-21 levels, we determined the impact of miR-21 on DNMT1 levels. MiR target prediction algorithms suggest that the 3' UTR of DNMT1 might be affected by miR-21*. We tested this directly in two models. First, we examined DNMT1 protein and mRNA levels in NL-20 cells stably overexpressing miR-21 compared to a vector only control, and second, in a transient transfection system using miR-21 si-RNA mimics. While a known target of miR-21 PDCD4, was consistently downregulated in response to miR-21 overexpression, DNMT1 was unaffected. Further analysis revealed that DNMT1 sequence may be targeted by miR-21* strand. Few studies have investigated the role of miR-21* despite growing evidence of the primary miR-21 transcript (which would give rise to both miR-21 and miR21*) being upregulated by ionizing radiation and other cellular stresses. We are currently testing the role of miR-21* in the radiation response using miR mimics and anti-miRs. Our goal over the next year will be to validate the methylation changes observed and to determine the potential consequences of such changes on gene expression. We are also in the process of repeating the radiation exposures/ extended time frame experiments to determine the reproducibility of our DNA methylation findings. Planned are a direct repeat with exposure of 3KT bronchial epithelial cells at the Brookhaven facility in October 2012 (0.1, 0.3, 1.0 Gy Fe and Si) followed by extended culture; again taking both an acute measurements and

Task Progress:

	extended time course after exposure to validate our above findings. As one component of these studies we will also be testing the possibility that these epigenetic changes underlie a persistent inflammatory state by testing for cytokine profiles in the culture medium over the same time course. Pending successful replication, our goal is for publication of these finding in early 2013.
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