Gene Expression Pathways Activated and Repair of DNA of Double Strand Breaks (DSBs) Following Low Dose Ionizing Radiation in Human Bronchial Epithelial Cells (HBECs) With and Without Defined Premalignant Oncogenic Changes.

Michael Story [2,7], Yang Xie[2,5], A. Asaithamby[7], Lianghao Ding[2,7], Seongmi Park[7] **,** Chin-Rang Yang[2], Mitsuo Sato[1,2], Amit Das[1,2], Luc Girard[1,2,4], Xian-Jin Xie[2,5], Michael Peyton[1,2], Boning Gao[1], Oliver Delgado[6], David Chen[2,7], Jerry Shay[2,6], John D. Minna [1,2,3,4]
[1]Hamon Center for Therapeutic Oncology Research, [2]Simmons Cancer Center, and Departments of [3]Internal Medicine, [4]Pharmacology, [5]Clinical Sciences, [6]Cell Biology, and [7]Radiation Oncology, University of Texas Southwestern Medical Center, 6000 Harry Hines Blvd, Dallas, TX 75390-8593. john.minna@utsouthwestern.edu

We want to determine the effect of low dose ionizing radiation on the molecular progression of lung epithelial cells towards lung cancer. Our endpoints are focused on specific molecular and cellular changes known to be associated with lung cancer development and quantitating those changes as a function of low dose ionizing radiation. We use immortalized human bronchial epithelial cells (HBECs) developed from >40 individuals (including those from current, former, and never smokers, males, females, persons with and without lung cancer), and also those we have constructed as special isogenic variants with defined oncogenic changes (e.g. KRASV12, EGFR, p53) found in lung cancer. These studies are even more important given the recent interest and NCI National Lung Cancer Screening Trial (NLST, of 55,000 persons) to test repeated chest CT scans for early lung cancer diagnosis. If this trial indicates the use CT scans for screening the general population there will be a large increase in repeated low dose radiation exposure to lungs of individuals potentially at very high risk of developing lung cancer (current and former smokers).
Gene Expression Studies: To determine the acute and long-term effects at the gene expression level of low dose radiation we treated HBECs and their isogenic derivatives with 2, 10, 25, 50 100, 200 and 300 cGy exposures from 1-24h post-IR. The HBECs were collected at various time points following irradiation (0, 4, 12, 24 hr, and 3 months), RNA extracted and gene expression measured using Illumina Whole Genome microarrays (~20,000 unique genes). All of the data are being integrated into a model to provide quantitative risk assessment for developing lung cancer after low dose γ irradiation. We have used Bayesian network (BN) analysis to identify genes differentially regulated that also display expression relationships with one another, combined with signal transduction pathway analysis for biological context. Expression comparisons were determined by comparing the difference in gene expression at 0.10 Gy at all times to the unirradiated controls, while the 1.0, 2.0 and 3.0 Gy samples were grouped together and compared to unirradiated controls. These differences, low dose, 0.10 Gy, vs. high dose, -1-3 Gy, were then used to build networks of inter-related genes using a Bayesian Network (BN) approach. By this analysis, there are several “hub genes (such as IL-8) with many connections with other genes, in particular. The genes identified by BN analysis were imported into Ingenuity Pathways software in order to develop a biological context for these BN relationships. From the 172 genes identified by BN, 137 could be mapped to a gene ID and 123 were eligible for network analysis. Interestingly, only 2 major networks were identified, cell cycle and cellular movement, and cell death. As examples, chemokines, cadherins, cyclin dependent kinases are represented. Cell cycle checkpoints at each phase of the cell cycle are represented. The second network is Cell Death. Here, the major death pathway represented is apoptosis with Casp8, TNFSF10 and IL8 as critical biological nodes. We learned that by combining ANOVA to select differentially expressed genes with Bayesian analysis to build numerical relationships between genes followed by biological interpretation we were able to identify 2 major biological pathways that are down-regulated at low dose radiation exposure when compared to high dose exposures (> =1.0 Gy). These genes are unique in that they were identified as differentially regulated when compared to controls and then differentially regulated when compared against a high dose set of samples. Identification of both the Bayesian networks and biological networks allows for the identification of

genes critical to radiation response pathways that can be tested or evaluated in different genetic backgrounds. **Testing Repair of DNA DSBs in HBECs After Low-Dose γ Radiation.** DNA double-strand breaks (DSBs) lead to cell death, senescence, genomic instability, and carcinogenesis if not repaired in an accurate and timely manner. Although biological effects of low levels of radiation are not clear, the risks of low-dose radiation are of societal importance in relation to issues as varied as screening tests for cancer, occupational radiation exposure, and manned space exploration. The Aim of this Project is to directly monitor induction and repair of single DSBs in living human lung epithelial cells (HBECs) using tumor suppressor p53 binding protein 1 fused to an enhanced green fluorescent protein (EGFP-53BP1) expression construction in as a surrogate marker to monitor foci formation. To visualize induction and repair of low-dose ionizing radiation (IR) induced DSBs, in single living mammalian cells, we established a stable HBEC cell line that expresses 53BP1 fused with EGFP. We first characterized the subnuclear localization of EGFP-53BP1 in live 2D and 3D cultures and found a distinct pattern of EGFP-53BP1 distribution within the nucleus. In some cells YFP-53BP1 showed pan nuclear distribution. In a subset of cells it was concentrated in one to more bright speckles and in another group of cells there were several foci. To get quantitative information on the distribution of YFP-53BP1 in these stable cells, we counted about 300-500 live cells and found that before radiation on average there are ~0.5 and ~0.6 EGFP-53BP1 foci per cell in 2D and 3D cultures, respectively Further, when we exposed these cells to graded low doses of IR we found that exogenous expression of EGFP-53BP1 does not interfere with the sensitivity of these cells to γ-irradiation. Thus, we can use EGFP-53BP1 expressing HBECs for monitoring induction and repair of DNA DSBs after low-dose IR exposure. In order to get precise quantitative information on the IR induced EGFP-53BP1 foci, live cell images were acquired prior to DNA damage and these cells were monitored after irradiation with 5, 10, 25, 50 and 100 mGy of γ-rays. The EGFP-53BP1 foci were counted 30 min after irradiation and these foci numbers were subtracted from the pre-irradiated time point. The number of EGFP-53BP1 foci formation showed a linear dependence on dose over the range of 5-100 mGy. Our live cell imaging data, provide evidence that the number of DNA DSB induced by γ-irradiation increases linearly with increasing dose. Although the number of γH2AX foci are commonly used to reflect the number of DSBs, some reports indicate that the number of such foci may not represent true DSBs (Bouquet *Cell Cycle* **5**(10)**:** 1116-1122, 2006). In addition, the number of background γH2AX foci are relatively greater in exponentially growing cells than in confluent cells (Banath *Cancer Res* **64**(19)**:** 7144-7149, 2004) and may hinder the quantification of DSBs after one to ten cGy of γ-radiation. To circumvent this problem, we utilized live cell imaging approach to monitor EGFP-53BP1 foci dissolution kinetics in cells irradiated with 10, 50 and 100 mGy of γ-rays. To get accurate quantitative data on the low-dose γ-radiation induced EGFP-53BP1 foci dissolution kinetics, live cell images were acquired prior to DNA damage and these cells were monitored continuously for eight hours following γ-irradiation. Since the formation of spontaneous EGFP-53BP1 foci in mock-irradiated controls increases with time and to avoid any inter-experimental errors in calculating the kinetics of γ-rays induced EGFP-53BP1 foci disappearance, we subtracted IR induced foci number from the mock-treated controls at each time point and used this number to calculate the DSB repair kinetics. We found that DNA DSBs induced by 10, 50 and 100 mGy are repaired efficiently. Almost all the DSBs induced by 10 and 50 mGy of γ-rays were repaired by eight hours. On the other hand few percent of DSBs remained eight hours after 100 mGy of IR. Nevertheless, DSBs induced by low-dose radiation appear repaired and can be studied using live cell imaging technique we have developed.
Conclusions: First, we found defined mRNA signatures (which are involved in two major and related pathways) associated with low dose radiation of HBECs. Second, our results suggest that DNA DSBs induced by low-dose IR are repaired in HBECs. Further, these results support the current model of risk assessment based on the assumption that cellular responses to DSBs are equally efficient at low and high doses. This study also validated a new sensitive means to directly study the induction and repair of radiation-induced DSBs and similar approach can be employed for both 3D as well as for cells grown in tissues culture.