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| <b>14. ABSTRACT</b><br>Ionizing radiation is a human carcinogen to which the military has increased risk of exposure. Radiation causes DNA damage and is a potent mutagen. Radiation also has epigenetic effects and may affect transgenerational cancer risk. Here we set out to determine if exposure to low dose radiation in utero leads to a change in DNA methylation in adult tissues and in ensuing unexposed generations, and if these changes correlate with increased cancer susceptibility. We report that a single dose of 0.5 Gy radiation at day 15 of embryogenesis increased lung cancer incidence in Balb/c mice and also resulted in reduced body weight. Examination of DNA methylation at 38 lung cancer relevant genes in normal lung tissue identified four that showed hypermethylation. We did not detect differential methylation between irradiated and control mice for these 38 genes. Examination of expression of 320 cancer related genes identified 12 that showed either an increase or decrease in expression in irradiated young adult mice relative to control mice. Seven genes also showed altered expression in normal lung from F3 mice. Thus, traces of the effects of a single dose of radiation during development persist into adulthood and may extend to subsequent generations. Five of the 12 genes that show altered expression in irradiated normal lung also showed altered expression in lung tumors and there was heterogeneity between individual lung tumors. We have generated an extensive tissue repository for future studies on the epigenetic and transgenerational effects of low dose radiation. |                         |                                |  |  |  |
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# Transgenerational Radiation Epigenetics

## Introduction

Ionizing radiation is a human carcinogen to which the military has increased risk of exposure (Mettler, 1996). Radiation causes DNA damage and is a potent mutagen. The dominant paradigm holds that the carcinogenic effects of radiation are due to direct mutagenesis of cancer genes such as tumor suppressors and oncogenes (Mullenders et al., 2009) (Little, 2000). However, a number of studies suggest radiation can have epigenetic effects and may affect transgenerational cancer risk as well. In 1990, Gardner reported an association between preconception irradiation and childhood leukemia and lymphoma (Gardner et al., 1990). In rodent studies, paternal exposure to radiation led to a large and significant increase in neoplasia in first and second generation offspring (Nomura, 1982; Vorobtsova et al., 1993; Tomatis, 1994). Exposure of cells to low dose radiation induces heritable genetic instability in both the irradiated cells and bystanders that were not irradiated (Koturbash et al., 2006). Exposure of male mice to radiation induced high frequency genetic instability in the unexposed offspring, implying an epigenetic mechanism (Baulch and Raabe, 2005; Dubrova et al., 1998; Koturbash et al., 2006; Barber et al., 2002; Koturbash et al., 2005). Indeed, offspring of parental mice that were exposed to ionizing radiation showed a loss of global cytosine methylation in DNA from thymus, implicating profound epigenetic dysregulation (Tawa et al., 1998; Pogribny et al., 2005), and increased cancer risk (Vorobtsova et al., 1993; Tomatis, 1994).

## Keywords

None

## Overall project summary

Our long term objective is to determine if epigenetic mechanisms underlying environmentally induced cancer and to determine if these effects can persist through succeeding unexposed generations. Our objective for this DOD Idea Award was to determine if an epigenetic signature of radiation exposure could be detected in normal lung tissue and irradiation induced lung tumors. We also sought to determine if radiation-triggered epigenetic alterations could be detected in subsequent unexposed generations. We hypothesized that exposure to ionizing radiation results in reproducible epigenetic changes, manifest as alterations in the pattern of DNA methylation and altered gene expression, and moreover that these changes are somatically heritable and will persist long after exposure. Further, some epigenetic alterations will be passed through the germline to subsequent unexposed generations. Identifying and characterizing these radiation-induced epigenetic changes will provide essential baseline data for further investigations on the mechanistic basis for the carcinogenic and transgenerational effects of radiation. It is also anticipated that these epigenetic signatures will be developed as biomarkers of exposure to be used for risk prediction.

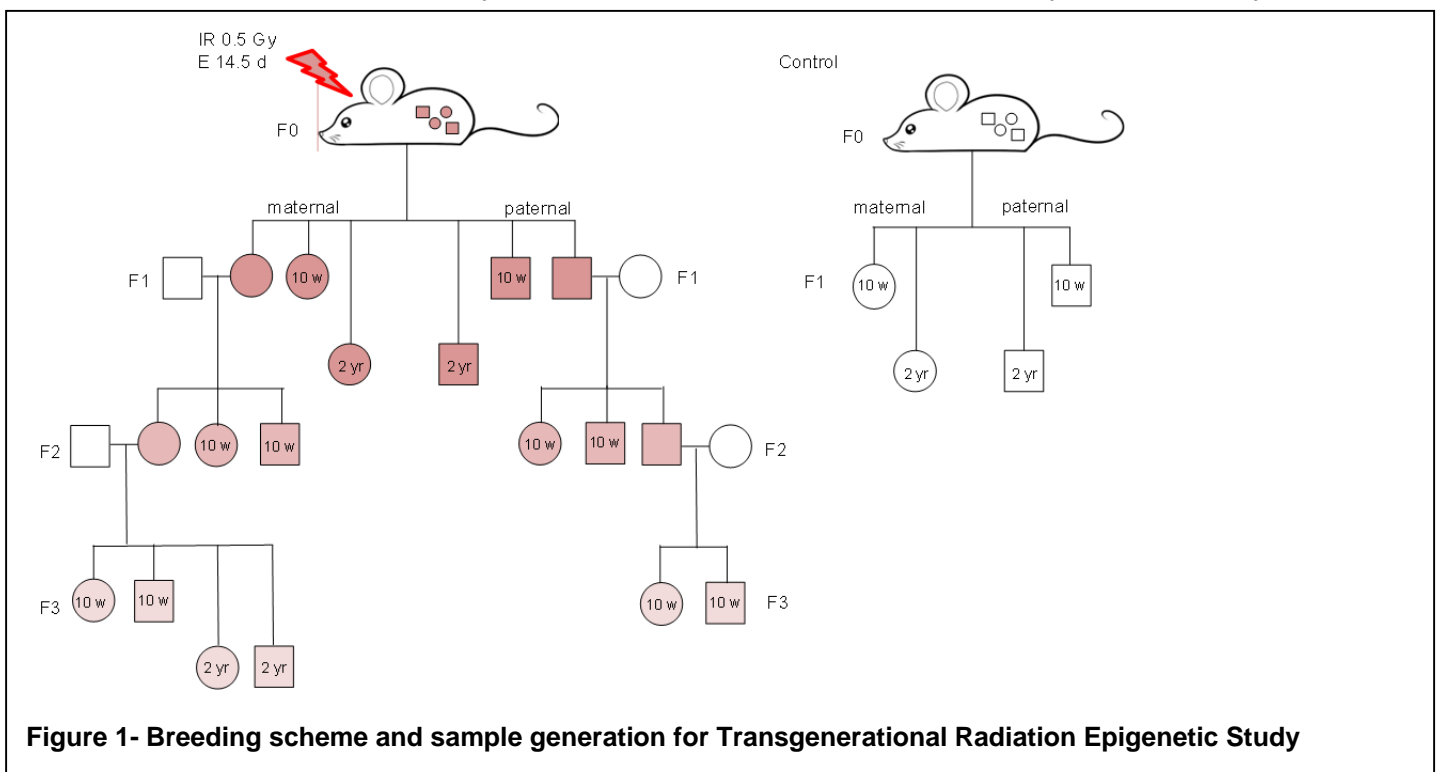
The specific objectives of this study were first to determine if *in utero* exposure to low dose irradiation results in a measureable change in DNA methylation and RNA expression in adult animals, second, to determine if irradiation led to an increase in cancer, particularly lung cancer, third, to determine if radiation induced lung tumors showed alterations in gene expression and finally to determine if traces of *in utero* irradiation could be detected in tissues from F2 and F3 generations.

## Key research accomplishments

Our first objective was to identify a strain of mouse that was susceptible to radiation induced tumorigenesis. We exposed eight to ten week old Balb/c mice to 4 doses of 1Gy irradiation (4 Gy or 400 rads total). Untreated Balb/c mice developed lung cancer at an incidence of 30% and the incidence increased in irradiated mice. Four Gy is a fairly high dose of radiation and to more closely mimic human exposures we selected a much

lower dose of 0.5 Gy (50 rads). We hypothesized that this dose would still be sufficient to elicit a measurable biological response, although at the outset we did not know if that would be the case. Breeder pairs were set up and female mice were checked daily for plugs. We exposed 63 plugged female Balb/c mice at E15 to a single dose of 0.5 Gy whole body ionizing radiation (Cesium<sup>137</sup>). In parallel, we set up control Balb/c breeder pairs of mice that were mock irradiated. The resulting irradiated and control F1 offspring were housed together under normal laboratory conditions.

Three cohorts of F1 mice of both sexes were generated (Figure 1). The first cohort was sacrificed at ten weeks of age for collection of normal tissue. We originally proposed to collect only male lung tissue for analysis. However, we expanded the project to include both males and females and collected tissue from lung, liver, kidney, spleen, testes, ovary, heart and plasma (Table 1). A piece of each tissue was taken for fixation in formalin and the rest of the tissue was snap frozen. Tissue samples (except for plasma) were put into beam capsules and frozen in liquid nitrogen. Blood for plasma was collected and put into EDTA tubes, allowed to sit then centrifuged to remove blood cells. The supernatant (plasma) was then moved into a cryotube and snap frozen. All frozen tissues are currently stored in a -80°C freezer as a biorepository for future analysis.



The second cohort was subjected to a long term tumor study. We originally proposed sacrificing mice for tumor analysis at 40 weeks of age. However when we examined a subset of mice at this time point, none had lung tumors so we extended the tumor study endpoint to 100 weeks of age. The absence of tumors at 40 weeks was likely due to the lower dose of irradiation provided. Mice were observed daily and sacrificed when they became moribund or showed signs of tumor development. Tumor and normal tissues were collected and stored in the biorepository as described above.

The third cohort of F1 mice was used to generate F2 mice by breeding with naïve unirradiated Balb/c mice. We originally proposed to study transmission only through the paternal line, but in order to determine if there were differences between paternal and maternal transmission, we added a maternal transmission experiment (Figure 1). This doubled the total number of experimental animals generated for this project. Two cohorts of F2 mice were generated. The first cohort was sacrificed at ten weeks of age for collection of normal tissue as described for F1 mice (Table 1). The second cohort of F2 mice was used to generate F3 mice by breeding

with naïve unirradiated Balb/c mice. Again, we maintained both paternal and maternal transmissions. Two cohorts of F3 mice were generated. The first cohort was sacrificed at ten weeks of age for collection of normal tissue as described for F1 and F2 mice. Although beyond the original scope of work, we decided to set up a second cohort of F3 mice for a long term tumor study to determine if grandparental exposure to radiation altered tumor susceptibility. Mice were observed daily and sacrificed when they became moribund or showed signs of tumor development. As for the F1 mice this study extends to over 100 weeks.

| Mice                   | Gender | #  | Liv | Lung | Kid | Spl | Ov/<br>Tes | Ht | Brain | Plasma | Lung<br>Tu | Other |
|------------------------|--------|----|-----|------|-----|-----|------------|----|-------|--------|------------|-------|
| IR F1<br>10 Week       | M      | 10 | 10  | 10   | 10  | 10  | 10         | 10 | 10    | 4      |            |       |
|                        | F      | 9  | 9   | 9    | 9   | 9   | 9          | 9  | 9     | 9      |            |       |
| Control F1<br>10 Week  | M      | 12 | 12  | 12   | 12  | 12  | 12         | 12 | 12    | 8      |            |       |
|                        | F      | 10 | 10  | 10   | 10  | 10  | 10         | 10 | 10    | 10     |            |       |
| IR<br>F1 Tumor         | M      | 26 | 20  | 26   | 26  | 20  | 26         | 26 | 20    | 20     | 7          | 13    |
|                        | F      | 31 | 30  | 31   | 31  | 31  | 29         | 30 | 26    | 27     | 4          | 9     |
| Control<br>F1 Tumor    | M      | 31 | 27  | 31   | 27  | 26  | 26         | 29 | 14    | 14     | 8          | 22    |
|                        | F      | 27 | 26  | 27   | 27  | 26  | 26         | 27 | 25    | 25     | 4          | 15    |
| F2 10W<br>Paternal     | M      | 23 | 23  | 23   | 23  | 23  | 23         | 23 | 23    | 23     |            |       |
|                        | F      | 20 | 20  | 20   | 20  | 20  | 20         | 20 | 20    | 20     |            |       |
| F3 10W<br>Paternal     | M      | 24 | 24  | 24   | 24  | 24  | 24         | 24 | 24    | 24     |            |       |
|                        | F      | 22 | 22  | 22   | 22  | 22  | 22         | 22 | 22    | 22     |            |       |
| F2 10W<br>Maternal     | M      | 25 | 25  | 25   | 25  | 25  | 25         | 25 | 25    | 25     |            |       |
|                        | F      | 27 | 27  | 27   | 27  | 27  | 27         | 27 | 27    | 27     |            |       |
| F3 10W<br>Maternal     | M      | 26 | 26  | 26   | 26  | 26  | 26         | 26 | 26    | 26     |            |       |
|                        | F      | 30 | 30  | 30   | 30  | 30  | 30         | 30 | 30    | 30     |            |       |
| F3 Tumor<br>Maternal * | M      | 25 | 12  | 22   | 20  | 20  | 20         | 7  | 4     | 4      | 4          | 19    |
|                        | F      | 22 |     |      |     |     |            |    |       |        |            |       |
| F3 Tumor<br>Maternal * | F      | 21 | 7   | 8    | 8   | 8   | 7          | 6  | 6     | 6      | 1          |       |
|                        | M      | 8  |     |      |     |     |            |    |       |        |            |       |

**Table 1-Tissue Repository from Transgenerational Radiation Epigenetics Study** \*-animals taken to date

In addition to the tissues collected from experimental and control mice of both sexes, through both maternal and paternal transmission, and from F1, F2, and F3 generations, we also collected samples from the breeders used to generate the study mice.

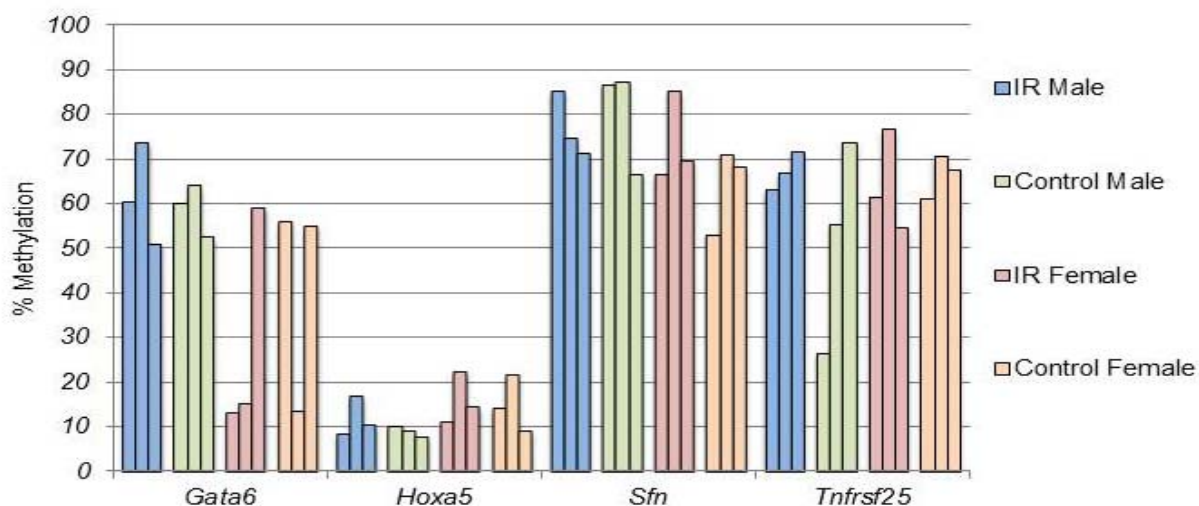
In summary we greatly expanded the number of tissues collected from each mouse, doubled the time point for tumor analysis to 100 weeks, doubled the number of mice in the transgenerational study to include maternal transmission, and added an additional F3 long term tumor study. This has generated an invaluable tissue bank for identifying epigenetic biomarkers of radiation exposure through genomic and transcriptomic profiling.

### Specific Aims

#### Specific Aim 1: Identify and validate epigenetic biomarkers of low dose radiation in normal lung tissue.

The bulk of DNA methylation marks are erased during gametogenesis and reprogrammed during early development (Morgan et al., 2005). We hypothesized that developing embryos will be highly susceptible to epigenetic alterations, including DNA methylation triggered by low-dose radiation, and some of these alterations will persist into adulthood. We initially propose to perform MeDIP (methylated DNA

immunoprecipitation) on whole genome promoter microarrays. However due to differences in commercial preparations of antibodies and extra steps of bisulfite conversion, this approach lacked sensitivity and reproducibility that was required for our analysis. We chose instead to use a Qiagen EpiTect Methy II PCR Array Mouse Lung Cancer Complete Panel (EAMM-8040Z). The EpiTect Methy II PCR Array system, using MethylScreen technology, relies on the differential cleavage of target sequences by two different restriction endonucleases whose activities require either the presence or absence of methylated cytosines in their respective recognition sequences. As real-time PCR quantifies the relative amount of DNA remaining after each enzyme digestion the methylation status of individual genes and the methylation profile across a gene panel are reliably and easily calculated. The use and analysis of both restriction digests, as well as their PCR amplification, allow the analysis of smaller, more heterogeneous samples. The EpiTect Methy II PCR Arrays allow the simultaneous analysis of DNA methylation of a panel of gene promoters on 96- or 384-well real-time PCR plates. 84 Genes were carefully selected from the following lung cancer relevant categories. Genes with differentially methylated promoters: *Apba1, Apc, Cadm1, Cdh1, Cdh13, Cdkn1a, Cdkn1c, Cdkn2a, Cdkn2b, Cxcl12, Cyp1b1, Fhit, Mlh1, Mthfr, Prdm2, Rarb, Rassf1, Rassf2, Sema3b, Slit2, Sfrp1, Tcf21*; Genes with Metastatic Potential: *Anxa5, Dlg2, Dusp6, Ercc1, Erbb3, Hgf, Hmnr, Irf4, Kit, Lck, Nf1, Slit2, Stat1, Stat2*; Genes Differentially Expressed Genes in Adenocarcinoma (AC) Versus Squamous Cell Carcinoma (SCC): Higher in AC than in SCC: *Dsg3, Krt5, Krt14, Sprr1a*. Higher in SCC than in AC: *Agr2, Nkx2-1*; Genes up-regulated in lung cancer: *Col10a1, Col11a1, Cp, Cxcl13, Grem1, Mki67, Mmp1a, Mmp12, Spp1, Thbs2, Top2a, Tox3*; Genes down-regulated in lung cancer: *Ager, Car4, Clic5, Fabp4, Gpm6a, Scgb1a1, Sftpc, Sostdc1, Wif1*; Genes involved in PI3K/AKT signaling: *Akt1, Bcl2, Cdh1, Cdkn2a, Egfr, Mapk1, Nfkb1, Trp53, Vegfa*; Apoptosis genes: *Anxa5, Apc, Bcl2, Birc5, Braf, Cadm1, Cdh1, Cdh13, Cdkn2a, Dlc1, Egfr, Erbb2, Erbb3, Hgf, Hras1, Kras, Lck, Mlh1, Mmp9, Nf1, Nfkb1, Ptgs2, Sema3b, Sfrp1, Stat1, Tert, Tgfb1, Tnf, Top2a, Trp53, Vegfa*; Cell cycle regulation genes: *Apc, Bcl2, Birc5, Cdkn1c, Cdkn2a, Cdkn2b, Egfr, Ptgs2, Rb1, Tgfb1, Tnf, Trp53*; Immune response genes: *Bcl2, Cadm1, Csf3, Cxcl12, Cxcl13, Irf4, Lck, Mlh1, Nfkb1, Pax5, Stat1, Stat2, Tgfb1, Tnf, Trp53, Vegfa*; and Extracellular matrix & cell adhesion genes: *Cadm1, Cdh1, Cdh13, Col10a1, Col11a1, Dsg3, Gpm6a, Hmnr, Krt14, Krt5, Mmp1a, Mmp2, Mmp12, Mmp9, Sftpc*.



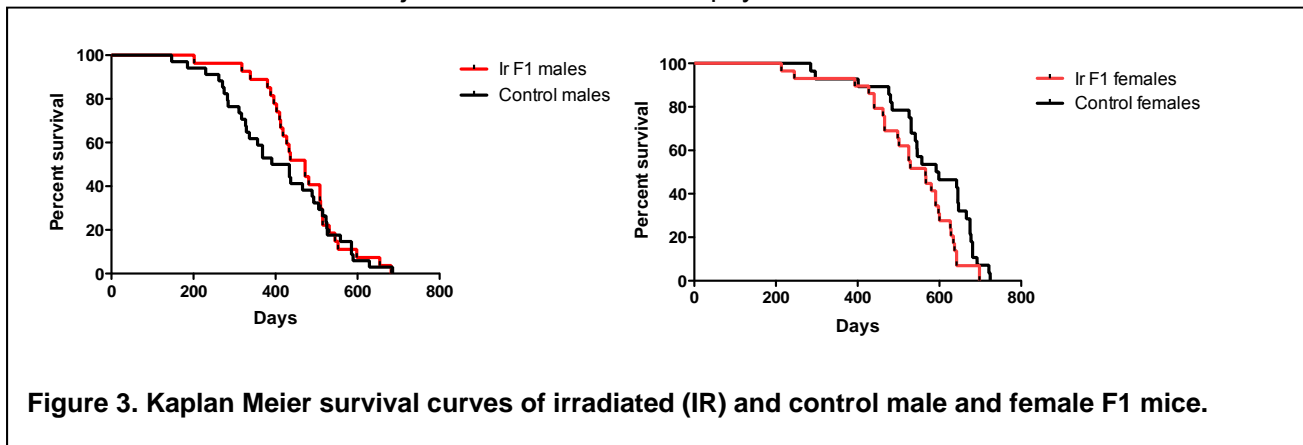
**Figure 2. DNA methylation levels for fur genes in normal lung tissue from control and irradiated F1 mice of both sexes.**

In an initial study we examined DNA methylation from lung samples from 10 week old F1 mice of both sexes.

Six irradiated and 6 control mice were analyzed for methylation on 84 genes found in lung cancer. We found that seven genes in this panel were hypermethylated in lung tissue from both irradiated and control mice at levels over 50% including Gata6, Stratifin (Sfn) and Tnfrsf25 (Figure 2).

It is interesting to note that Gata6 is important for lung epithelial development (Zhang et al., 2008) and along with Hoxa5 (Aubin et al., 1997) is involved in epithelial cell differentiation. Another gene Stratifin has been shown to have methylation changes in lung cancer (Shiba-Ishii and Noguchi, 2012). This could be indicative of why these Balb/c mice are susceptible to lung tumor development. In summary, four out of 84 genes examined showed DNA hypermethylation at the promoter and these levels were similar between irradiated and control lung tissue. Two of these genes are important in lung development and one in lung cancer and this could be related to the susceptibility of these mice to lung cancer. To further characterize the lungs, mRNA was isolated from normal lung tissue and lung tumors from both irradiated and control mice to measure expression levels by qPCR described in Aim 3.

**Specific Aim 2: Identify epigenetic alterations in radiation-induced lung tumors.** We hypothesized that a low dose of radiation given at a susceptible developmental window, at E14-E15 during embryogenesis, would enhance tumor development in the Balb/c strain of mouse. Should this be the case we also hypothesized that at least some of the carcinogenic effects of radiation could be due to epigenetic priming of normal tissue and some of these epigenetic changes will be found in tumors. In future, these could be developed as predictive biomarkers of neoplastic transformation. We generated two cohorts of F1 mice of both sexes, one irradiated at E15 while *in utero* and another mock irradiated. A small group was sacrificed at 40 weeks of age which was our initial time point, and no tumors were observed. This time point was based on our preliminary study using 4 Gy radiation. As we gave 1/8 less dose, or 0.5 Gy, we decided to age the mice and sacrificed them when they developed tumors or were moribund from other age related phenotypes. This study extended for 100 weeks. All mice were subjected to a full necropsy and tissues fixed or frozen for future analysis.



Irradiated female F1 mice (n=31) showed a significantly reduced survival relative to control female mice (n=27) ( $p=0.04$ ) (Figure 3). There was no significant difference in survival between the irradiated male (n=26) versus the non-irradiated male (n=31) mice.

In both the males and females, we found increases in lung tumor incidence in irradiated vs. non-irradiated mice (Figure 4, top). The incidence of tumors in irradiated females compared to control females increased from 17.2% to 29.0%, a nearly 50% increase. The incidence of tumors in irradiated male mice also increased from 33.3% to 42.3%. In both the irradiated and non-irradiated groups the incidence of tumors was higher in the male mice (Figure 4, top next page).



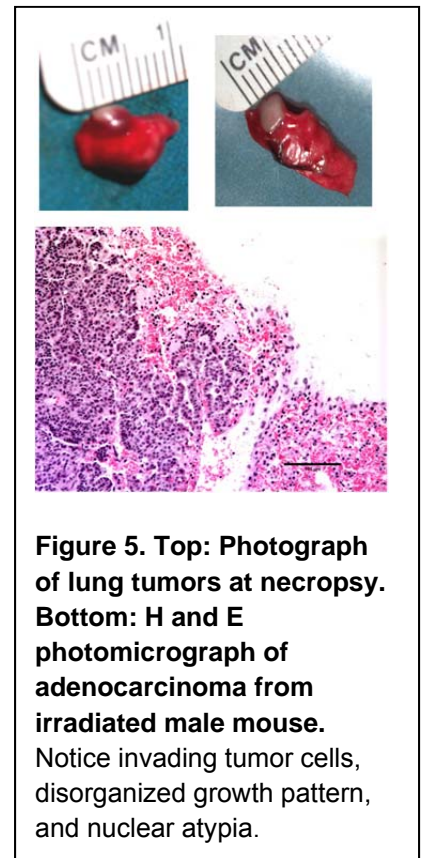
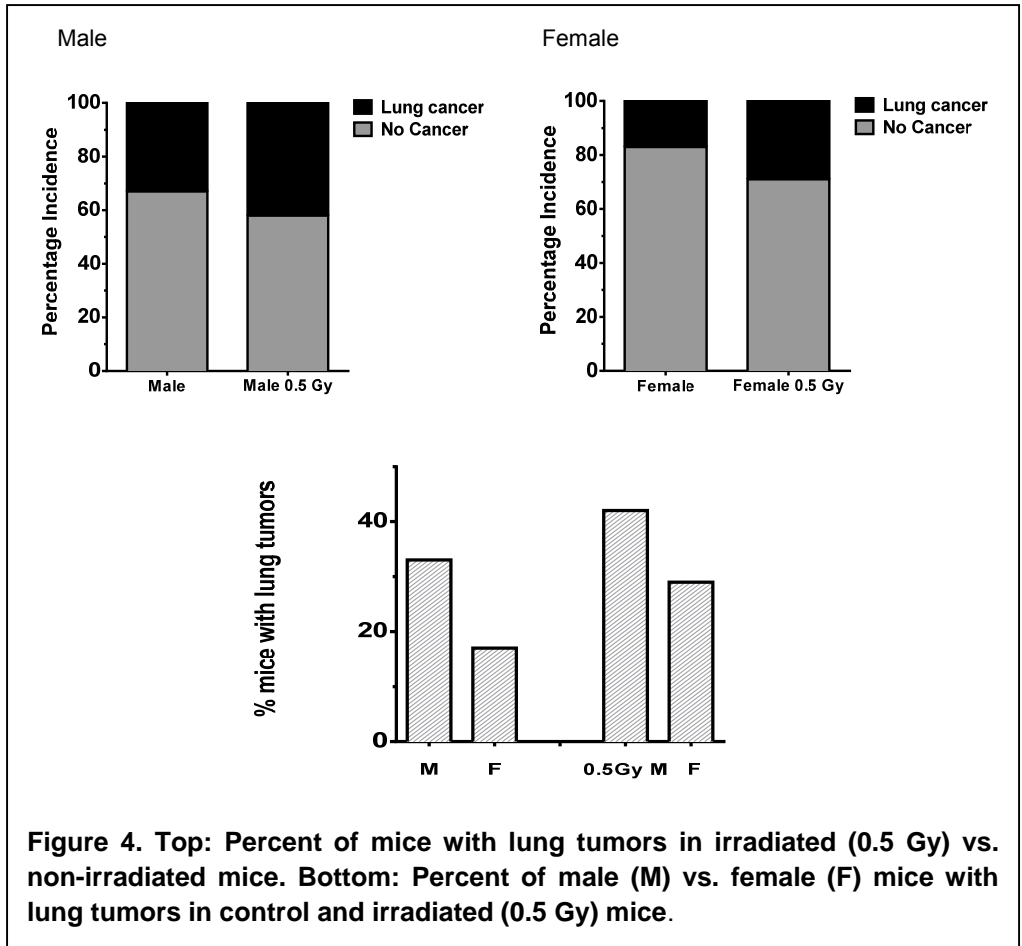
In addition, irradiation increased the size of tumors (Figure 5). In the non-irradiated male mice the average diameter of the lung tumors was  $3.9 \pm 2.5$  mm compared to  $4.4 \pm 4.7$  mm in the irradiated mice. The largest tumor (20mm) was found in the irradiated group. In the non-irradiated females the average size of the tumors was  $2.1 \pm 1.7$  mm compared to  $4.6 \pm 3.2$  mm in the irradiated group. The largest tumor was also found in the irradiated cohort (12mm).

Histopathological analysis of lung tumors in both the F1 irradiated and control male mice showed a similar spectrum of tumor pathology with 54% confirming as adenocarcinomas and 46% as adenomas (Figure 5). In the females 3 of 4 (75%) of the tumors analyzed in the control group were adenocarcinomas while 5 out of 6 (83%) of the F1 irradiated tumors were confirmed as adenocarcinomas.

In conclusion, exposure to 0.5 Gy radiation *in utero* increased the incidence and average size of lung tumors in male and especially female mice.

**Specific Aim 3: Identify epigenetic changes in unexposed mice derived from irradiated parents or grandparents.** It is suspected that epigenetic dysregulation may underlie transgenerational effects caused by environmental factors, such as radiation. Here we sought to identify such transgenerational epigenetic effects by comparing gene expression in lung tumors and normal tissue from F2 and F3 offspring derived from mice that were irradiated or non-irradiated.

All mice were weighed at ten weeks of age. Ten week old F1 irradiated mice of both sexes showed a 10% reduction in median body weight (Table 2 and Figure 6) relative to control mice. The F2 and F3 experimental male mice showed no difference in body weight relative to control mice. However, the body weights of the F2 and to a slight degree, F3 experimental female mice were reduced relative to control mice (Table 2 and Figure 6). Thus a single low dose of radiation during a susceptible developmental window leads to reduced body weight in young adult mice. Even more interesting, this effect persists in the next generation: daughters



of exposed females also showed reduced body weight.

To examine gene expression in lung tissue and lung tumors we used qPCR to quantitate levels of mRNA between irradiated and control tissues. We focused on genes that have been shown to be altered in cancer and employed a Bio-rad custom plate with primers for 320 cancer related genes based on their PrimePCR 384 human cancer gene plate. These panels were designed using the National Library of Science Data Base for

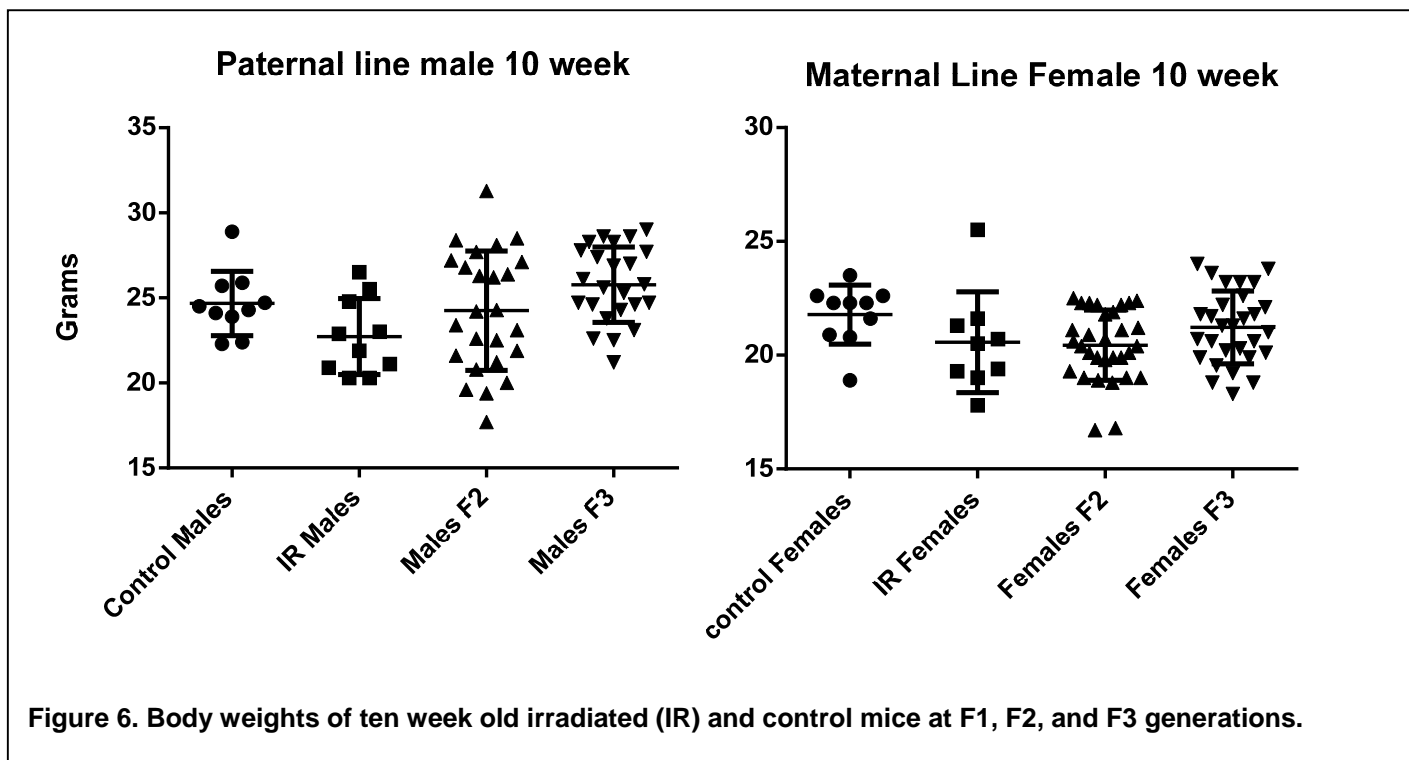


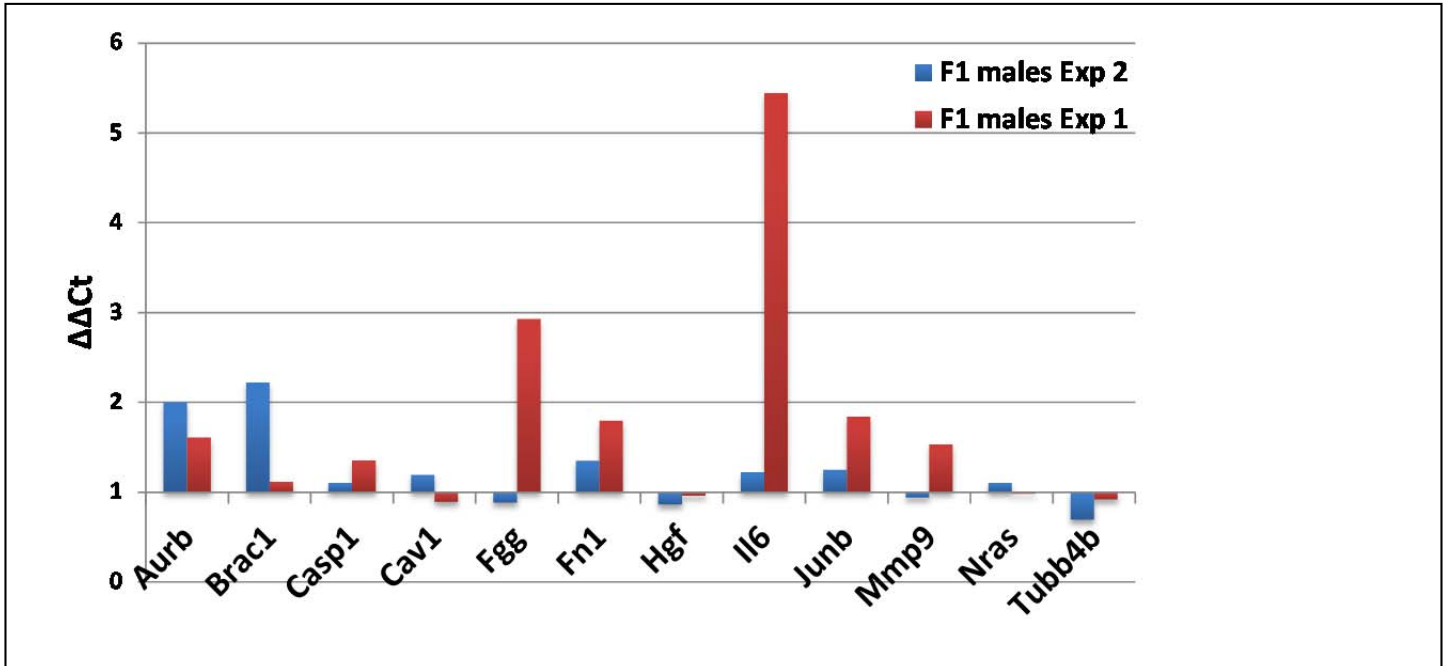
Figure 6. Body weights of ten week old irradiated (IR) and control mice at F1, F2, and F3 generations.

differentially expressed genes associated with cancer using validated PCR primers to these genes and SYBR Green as the

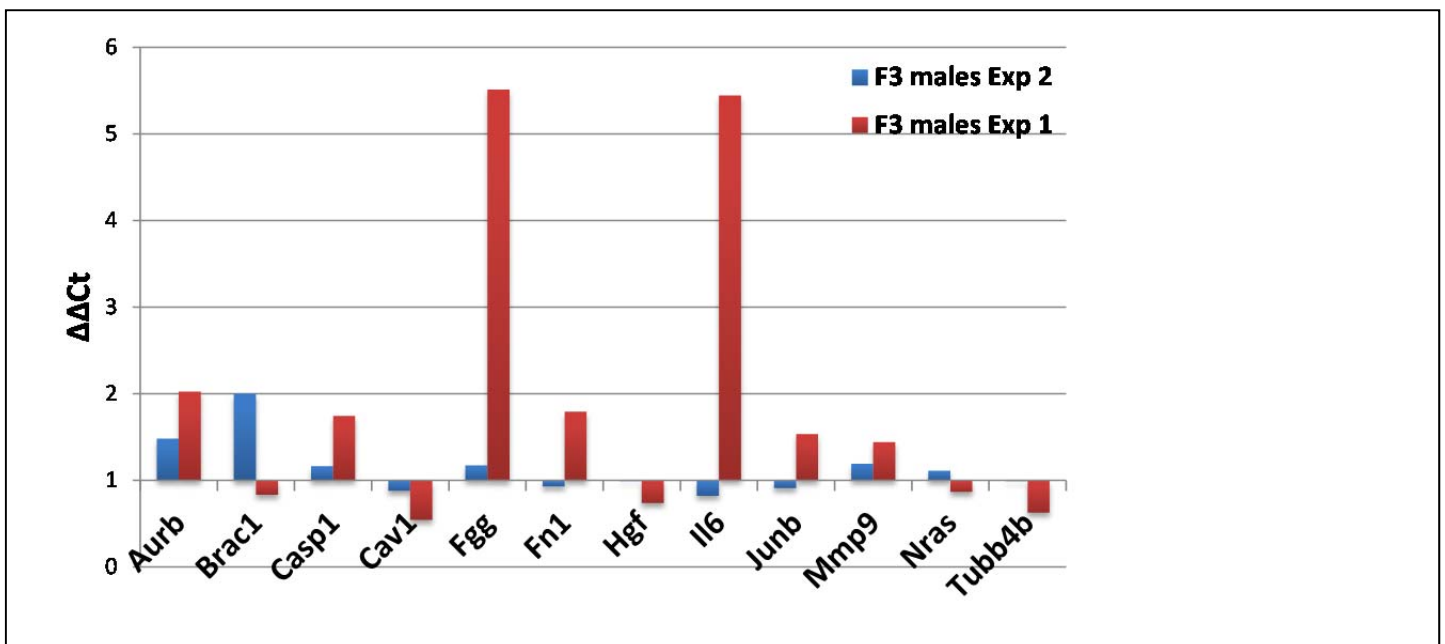
|                 | Control | F1    | F2    | F3    |
|-----------------|---------|-------|-------|-------|
| Males, mean     | 24.67   | 22.72 | 24.25 | 25.77 |
| Males, median   | 24.4    | 22.4  | 24.2  | 25.7  |
| Females, mean   | 21.78   | 20.57 | 20.42 | 21.22 |
| Females, median | 22.3    | 20.5  | 20.4  | 21.3  |

Table 2-Average weight in grams of mice at 10 weeks

readout. In a preliminary experiment (Exp 1), we isolated RNA from the lungs of two irradiated F1 10 week old male and female mice, two control F1 male and female mice and two F3 10 week old males and one lung tumor. We used the comparative CT method to compare mRNA levels between irradiated and control samples. After first calculating the Ct value using Beta 2 microglobulin (B2M) as reference gene, we subtracted the control sample values from the irradiated sample values. Out of the 320 genes examined, we identified 12 genes with expression levels that were either increased or decreased in irradiated samples relative to control samples and that also showed differences in samples from the F3 mice. We then designed a second Bio-rad custom plate (Exp 2) to validate these 12 genes with additional samples. For the second set of experiments, we isolated RNA from the lungs of eight control and eight irradiated F1 ten week old male mice, 11 F3 ten week male mice and one lung tumor (Figure 7).



**Figure 7. mRNA levels in irradiated relative to control lung tissue from ten week old F1 males.** Red bars show results of the first discovery experiment (n=2) and blue bars show results from second validation experiment with more samples (n= 8). Results are mean values that show differences between irradiated and control mice.



**Figure 8. mRNA levels in irradiated relative to control lung tissue from ten week old F3 males.** Red bars show results of the first discovery experiment (n=2) and blue bars show results from second validation experiment with more samples (n= 8). Results are mean values that show differences between irradiated and control mice. Seven genes validated between experiments

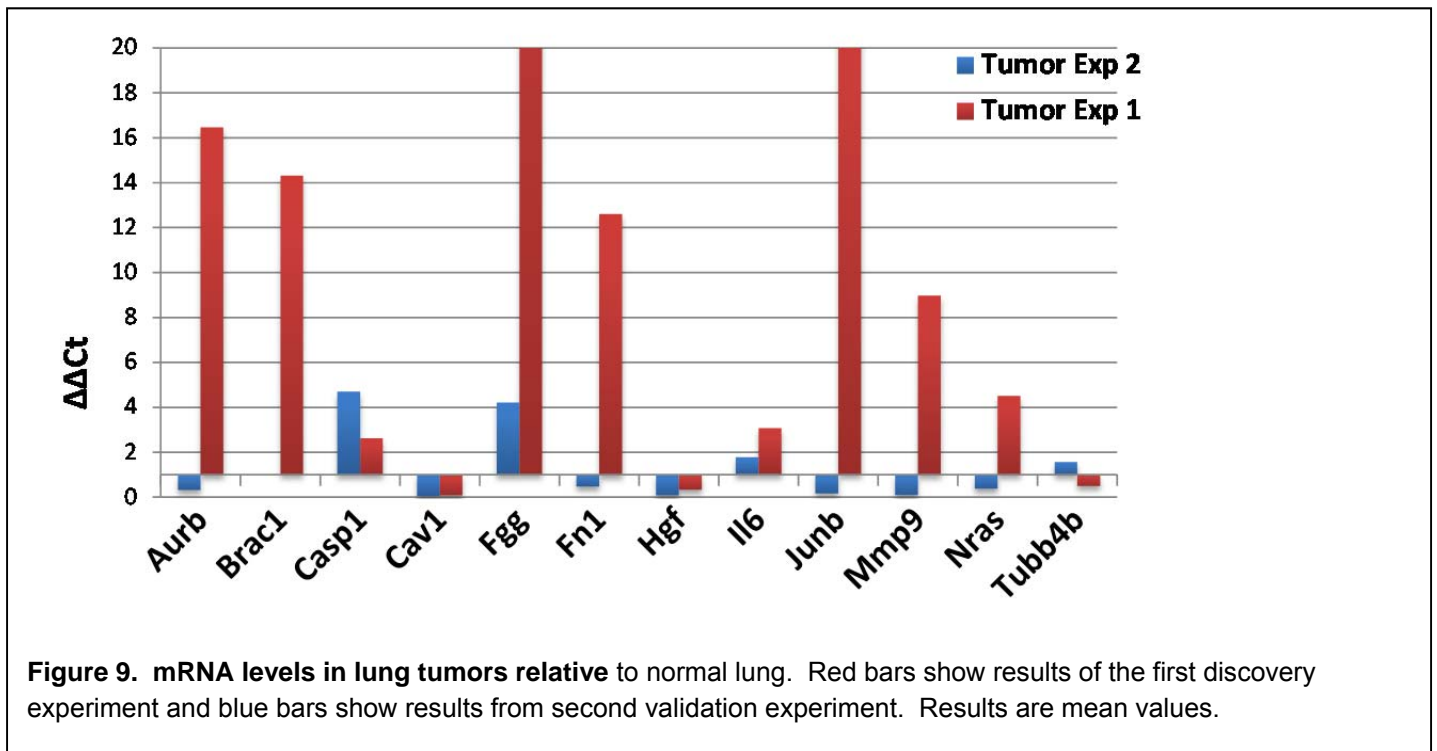
Eight of the twelve genes tested in the F1 generation male mice had increased or decreased mRNA expression in irradiated sample in both qPCR experiments: Aurb, Brac1, Casp1, Fn1, Il6 and Junb had higher levels of mRNA compared to non-irradiated controls and Hgf and Tubb4b had reduced levels.

In the F3 generation male mice, seven of the genes showed the same relative expression pattern in both qPCR experiments, Aurb, Casp1, Fgg, Mmp9 were up, while Cav1, Hgf, and Tubb4b were down (Figure 8).

Comparing the F1 data with the F3 data we found four genes with altered expression levels in irradiated compared to control mice that continued through to the F3 generation. Aurb and Casp1 had higher levels of mRNA expression while Hgf and Tubb4b had decreased expression.

In the lung tumors, five genes out of the 12 validated showed a similar pattern of expression in both tumors. Casp1, Il6 and Fgg had higher levels of mRNA while Cav1 and Hgf were reduced (Figure 9).

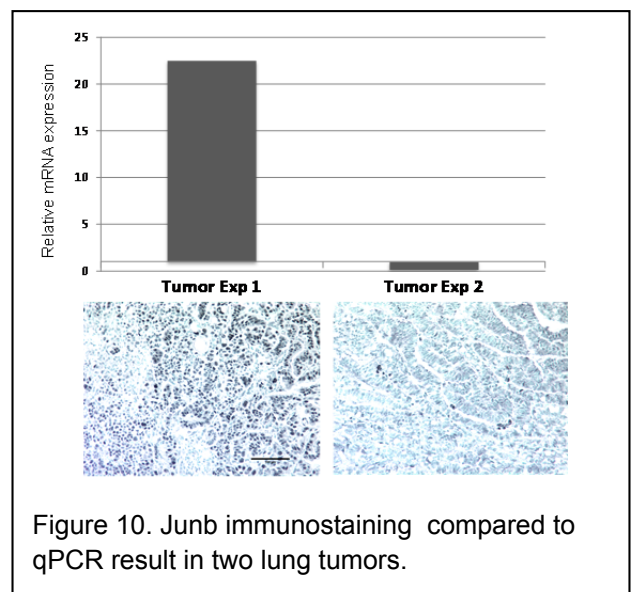
Tumor one was from a female mouse and tumor two was from a male mouse. Sample one was an adenocarcinoma while sample two was an adenoma. These differences between samples point to epigenetic heterogeneity that underlies radiation induced lung tumor development.

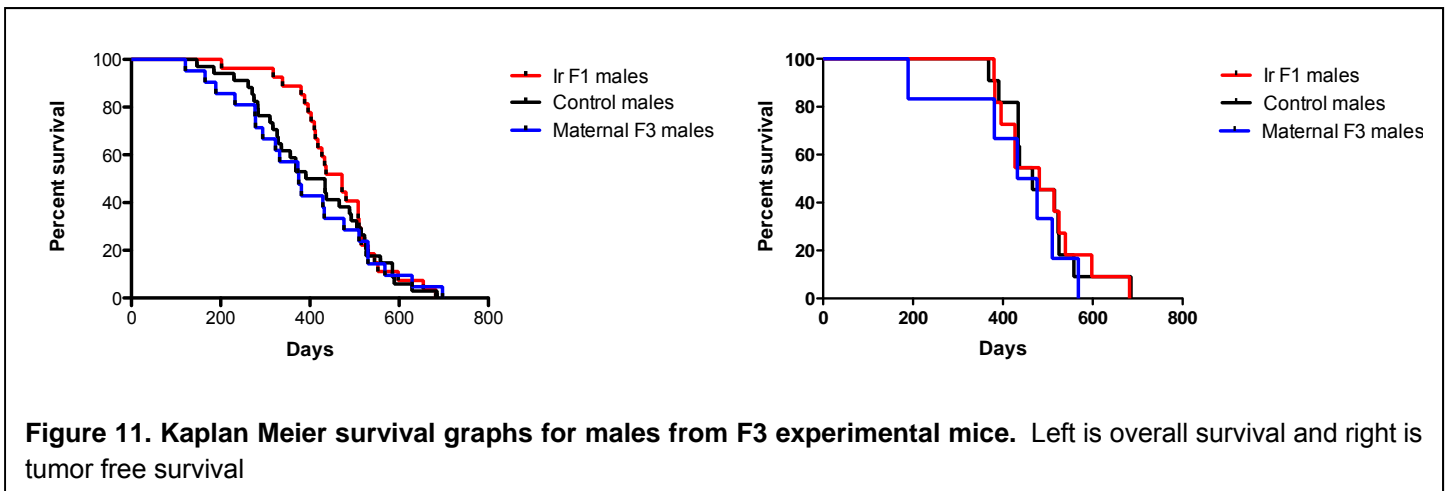


Reduced Caveolin-1(Cav1) gene expression has been reported in both human and mouse lung carcinoma cell lines. Upregulation of Cav1 has also been reported to be associated with increased metastasis (Senetta et al., 2013). The Par4 locus has been linked to lung tumor susceptibility in mice (Manenti et al., 1997) and one of the genes located in this region is Cav1.

Expression of hepatocyte growth factor (Hgf) and its receptor c-Met in lung tumors is also associated with lung cancer (Ma et al., 2007). High expression has been shown to be a negative prognostic indicator in lung cancer (Siegfried et al., 1998). Cav1 and c-Met the receptor for Hgf have been shown to crosstalk in hepatocellular carcinoma. In our study we show reduced mRNA expression of both Cav1 and Hgf.

Fibrinogen Gamma Chain (Fgg) is one of three genes that produce separate polypeptide chains that make up Fibrinogen. In lung carcinoma A549 cells blocking FGG with siRNA lead to decreased growth.





One example of a gene that showed heterogeneity in expression in the tumors is *Junb*. While it showed increased RNA expression in one lung tumor sample it showed decreased expression in a second tumor sample. Immunocytochemistry analysis on tumor tissue revealed that *Junb* protein expression in the matched tumor samples confirmed the qPCR result (Figure 10). One tumor had abundant nuclear staining for *JunB* and the other was only barely detectable.

The qPCR experiments identified a number of genes that increased or decreased in irradiated F1 samples compared to controls, only to have reversed expression pattern in samples from the F3 generation. To determine if the molecular profile was associated with an altered lung cancer incidence in F3 mice, we set up an additional cohort of F3 mice (25 male and 21 female F3 mice from the maternal line) and observed them long term for tumor development. Although this study was not in our original experimental plan, we wanted to determine if tumorigenic effects of radiation persisted to the third generation. At this time we are still collecting tissue from these mice. Most of the tissue from the males have been collected and a few from the females (see Table 1). The survival graph for the males this far is not significantly different from controls (Figure 11).

### Publications, abstracts, and presentations

None at this time

### Inventions, patents, and licenses

None at this time

### Reportable Outcomes

A single dose of 0.5 Gy radiation at day 15 of embryogenesis increased lung cancer incidence in Balb/c mice.

A single dose of 0.5 Gy radiation at day 15 of embryogenesis resulted in reduced body weight gain in young adult mice in the daughters of exposed female mice.

Examination of DNA methylation at 38 lung cancer relevant genes in normal lung tissue identified four that showed hypermethylation. Two, *Gata 6* and *Hoxa5* are involved in lung development and *Stratifin* in lung cancer which could explain the susceptibility of these mice to lung cancer. We did not detect differential methylation between irradiated and control mice for these 38 genes.

Examination of expression of 320 cancer related genes in normal lung tissue by RTPCR identified 12 that showed either an increase or decrease in expression in irradiated young adult mice relative to control mice. Seven genes also showed altered expression in normal lung from F3 mice. Thus traces of the effects of a

single dose of radiation during development persist into adulthood and may extend to subsequent generations. The phenotypic or disease consequences, if any, remain to be characterized. Five of the 12 genes that show altered expression in irradiated normal lung also showed altered expression in lung tumors and there was heterogeneity between individual lung tumors.

### **Other achievements**

We have generated an extensive bio-repository of major organs from experimental (irradiated) and control mice of both sexes, through both maternal and paternal transmission, and from F1, F2, and F3 generations. We also collected samples from the breeders used to generate the study mice. These samples including plasma, will be available for in depth analysis for epigenetic signatures of early radiation exposure and for examination of genetic or epigenetically inherited alterations. The reduced cost and increased accuracy of quantitative genome wide DNA methylation analysis makes this a promising approach to search for epigenetic biomarkers of radiation exposure.

### **Unexpected developments**

In early 2012 our mouse room was put on quarantine due to pinworm contamination that arose from another investigator's mice. Although our mice never tested positive, all mice were put on chow containing Fenbendazole from May 14 to Aug 1, 2012. During this time no live mice were allowed to leave the room. We do not anticipate any adverse outcomes from the diet but as no studies have been done on methylation changes due to Fenbendazole we cannot completely rule it out. Since both controls and irradiated groups were on the diet this should negate any effect on our results.

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## Appendices

None