

**BIOLOGICAL AND GENETIC COMPARISON OF X-RAY AND  
GAMMA RAY RADIATION EFFECTS**

by

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**Bachelor of Arts Honors Thesis**

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## **ABSTRACT**

Cesium-137 gamma irradiators have been commonly used in hospitals and research facilities for more than 70 years. Although they remain effective, the U.S. government is currently providing incentives for facilities to replace Cesium irradiators with x-ray irradiators. This is being done to minimize bioterrorism risk and maximize the security of isotope-based radiation. Although there is significant information in the literature regarding the potential differences in gamma and x-ray radiation, at the same dose levels, some differences remain poorly characterized.

In this study MC38 (murine colon adenocarcinoma) and B16F10 (murine melanoma) cellular responses to equivalent gamma and x-ray radiation doses were examined. Colony forming assays were performed and survival fractions compared. The results indicate MC38 cells are more sensitive to radiation than B16F10 cells. Additionally, MC38 cells are more susceptible to gamma radiation than x-ray radiation at any given dose, whereas the opposite is true for B16F10 cells. Thus, there were potentially important comparative differences (gamma vs x-ray) in cell survival. Western blot protein analysis and NanoString mRNA analysis were completed for gamma radiation of B16F10 cells to examine the mechanisms of cell death. Changes in protein and mRNA expression were mainly relatively small. Importantly, for research purposes, it appears genes associated with the apoptosis pathway will increase similarly, with dose, for both types of radiation.

Due to the COVID-19 pandemic, this research could not be completed as planned on the Dartmouth campus. The project required the physical irradiation of cancer cells. Without access to the Dartmouth irradiators, approximately one half of the planned

experiments could not be completed. Therefore, some data was calculated using previously existing information. The studies will be completed by returning laboratory personnel.

## **ACKNOWLEDGEMENTS**

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I would like to thank Professor Jack Hoopes for welcoming me into his lab my junior year and providing me with an opportunity to conduct my thesis research. Thank you also to Robert Wagner, Kayla Duval, and Margaret Crary-Burney for teaching me several of the skills I needed in the course of this project. Thank you for helping guide me through the process. Thank you to Kenneth Orndorff for operating the Cesium irradiator for me.

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## TABLE OF CONTENTS

Abstract.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
List of Figures.....	v
List of Tables.....	v
1. Introduction.....	1
1.1 Background.....	1
1.2 Thesis Purpose.....	5
2. Materials and Methods.....	6
2.1 Cell Culture.....	6
2.2 Irradiators.....	6
2.3 Assessment of Plating Efficiency.....	7
2.4 Colony Forming Assays.....	7
2.5 RNA Analysis.....	7
2.6 Protein Analysis.....	8
3. Results.....	9
3.1 Survival Fractions.....	9
3.2 Genetic Expression.....	10
3.3 Protein Expression.....	15
4. Discussion.....	20
4.1 Survival Fractions.....	20
4.2 RNA (Genetic) and Protein Expression.....	21

5. Conclusion.....	23
References.....	24

**LIST OF FIGURES**

Figure 1: Surviving fractions relative to a control of untreated cells as a function of dose, type of irradiation, and cell type based on colony forming assays.....	10
Figure 2: Overview of differences in mRNA expression of B16F10 cells 24 hours post gamma irradiation relative to untreated cells.....	11
Figure 3: Linear fold change of mRNA expression 24 hours post gamma radiation treatment of B16F10 cells relative to untreated control cells.....	13
Figure 4: Volcano plot indicating changes in mRNA expression of apoptosis regulatory genes 24 hours after receiving 18 Gy of gamma radiation relative to untreated control cells.....	14
Figure 5: Western blot protein expression levels of B16F10 cells 24 hours post gamma irradiation.....	16
Figure 6: Linear fold change of protein expression relative to control and normalized relative to $\beta$ -actin in B16F10 cells 24 hours post gamma irradiation.....	17

**LIST OF TABLES**

Table 1: Summary of gene function and observed changes in expression.....	15
Table 2: Summary of protein function and observed changes in expression.....	19

# 1. INTRODUCTION

## 1.1 Background

In the Energy Policy Act of 2005 (section 651), the U.S. Congress requested the U.S. Nuclear Regulatory Commission (NRC) “submit to Congress the results of a study by the National Academy of Sciences about industrial, research, and commercial uses for radiation sources” and establish “the Task Force on Radiation Source Protection and Security to evaluate and report to Congress and the President on the security of radiation sources in the United States from potential terrorist threats, sabotage, theft, or use of a radiation source in a radiological dispersal device” [1]. The 2008 report by the Committee on Radiation Source Use and Replacement examined Category 1 and 2 radioactive sources and determined that over 99% of high security risk radioactive sources in the U.S. are americium-241, cesium-137, cobalt-60, and iridium-192 sources. The greatest threat is posed by cesium-137 in cesium chloride (CsCl) sources due to its prevalence in high population density areas and its solubility and dispersibility as a pressed powder [2].

The committee recommended replacement of CsCl sources by discontinuing licensing of new CsCl irradiator sources, implementing incentives to decommission sources already in use, and prohibiting the export of these sources except for disposal at a licensed facility [2]. The committee recommended x-ray irradiators as a replacement for the current cesium irradiators [2]. To aid in the disposal of these cesium sources, the U.S. Department of Energy’s National Nuclear Security Administration began the Cesium Irradiator Replacement Project (CIRP) in 2015. CIRP incentivizes replacement of cesium irradiators with x-ray irradiators by fully funding the removal and disposal of the cesium

irradiator using the government's Off-Site Source Recovery Project and reimbursing about half of the price of the x-ray irradiator [3]. At an estimated rate of 70 irradiators a year, the CIRP projects disposal of all high activity cesium sources will be complete by 2027 [3].

In commenting on the 2008 report by the Committee on Radiation Source Use and Replacement, the Advisory Committee on the Medical Use of Isotopes (ACMUI) reported concern about the recommendation to replace cesium irradiators with x-ray irradiators given the efficacy of such alternatives was not established [4]. Little research has been undertaken to examine significant differences between gamma irradiation provided by Cs-137 sources and x-ray irradiators, and characterization of the differences between irradiator types is incomplete. Pacific Northwest National Laboratory, who is aiding the Defense Nuclear Nonproliferation Office of Radiological Security to implement the CIRP project, is funding research at institutions exchanging cesium irradiators for x-ray irradiators (or other alternatives) in order to better understand the transition process and changes in irradiation protocols required by the transition to a new technology [5].

Irradiators are used for a wide variety of clinical and research applications including, but not limited to, irradiating blood to prevent transfusion-associated graft-versus-host disease, irradiating mice for research in bone-marrow transplant studies, and irradiating cells or animals for cancer treatment [6]–[8]. Several factors contribute to cellular responses induced by radiation. There are many factors that affect the ability of radiation to induce cell death, including the radiation dose, the dose rate, and the cell type[9]. Classically, radiation is delivered in a fractionated form, with multiple treatments



of a dose given over several days or weeks, such as 30 doses of 2 Gy (30 x 2 Gy), or 3 doses of 8 Gy (3 x 8 Gy). These fractionation schemes also factor into radiation's ability to induce cell death. These factors can lead to biological differences, depending on the cell type, altering the overall effectiveness of a given dose. The variation in radiations effectiveness across cell types is known as the relative biological effectiveness (RBE) [10], [11].

Ionizing radiation is used as a cancer therapy to induce cell death and prevent proliferation within a tumor. The heightened proliferation of some cancers is thought to rely on the suppression of apoptosis signals (programmed cell death) [6]. However, radiation induced cell injury can trigger cell death mechanisms, including apoptosis. Ionizing radiation can cause both direct and indirect damage; indirect damage occurs through the formation of reactive oxygen species. These reactive oxygen species can oxidize proteins and lipids as well as damaging DNA [12]. Radiation induced DNA damage has been most closely studied and is considered the most important factor in cell responses to radiation [13]. Cellular enzymes can metabolize reactive oxygen species to minimize damage, including superoxide dismutases, peroxiredoxins, and glutathione peroxidases [13]. However, sufficient damage can trigger apoptosis or other forms of cell death.

Ionizing radiation induced DNA damage can involve alteration of the nucleotide bases and sugars, cross-link formation, DNA clustering, and single and double stranded breaks [13]. This DNA damage triggers a cell cycle checkpoint response. The checkpoint response pauses the cell cycle, either allowing time to repair the DNA damage before continuing, or initiating cell death pathways if there is great enough damage [13].

Ionizing radiation can also target lipids and proteins. Lipids are damaged either directly or, more frequently, indirectly. The interaction of reactive oxygen species with the lipid bilayer causes lipid peroxidation, particularly of polyunsaturated fatty acids, which leads to an increase in membrane permeability and thereby disrupts transmembrane processes and ion gradients [13]. Proteins affected by ionizing radiation can be subjected to reducing or oxidizing post-translational modifications which can alter the structure, function, and regulation of the protein and its enzymatic activity. Protein modification, due to the effect of ionizing radiation, results in pathway signaling changes and may explain some of the cellular responses to ionizing radiation [13].

Some published studies indicate gamma and x-ray radiation are not exactly equivalent at the same dose. Bone marrow transplant research in mice has suggested, in this case, the RBE of x-rays is 20-30% higher than that of gamma radiation, meaning a lower overall x-ray dose is required for the same effect [10]. In a study on blood irradiation using gamma and x-ray radiation, small changes in red blood cell permeability were observed. Additionally, there was a slight elevation of free plasma hemoglobin at 25 Gy x-ray radiation relative to gamma radiation and slight decrease of extracellular potassium levels at 35 Gy for x-ray irradiation when compared to 35 Gy gamma radiation. While it was concluded these differences were unlikely to be clinically relevant, they nevertheless suggest cells may respond differently to gamma and x-ray irradiation [7]. A study comparing the effect of 320 kV x-rays to Cs-137 gamma rays on bone marrow and splenocyte cells in C.B-17 mice determined the x-rays were more effective at destroying bone marrow cells and less effective destroying splenocytes than the gamma rays [11]. Other studies examining bone marrow irradiation have also

indicated differences in leukocyte, myeloid, and B cell reconstitution. Specifically, x-rays radiation resulted in less reconstitution of leukocytes and B cells but increased myeloid reconstitution. Additionally, there was reduced survival of mice after x-ray radiation due to a lack of CD45 as a result of bone marrow depletion [14]. Additional studies have indicated the two radiation methods are functionally equivalent at the same dose for insect sterilization, mitotic inactivation of mouse embryonic fibroblasts, and fibroblast survival *in vitro* and thus require no change in protocol [14]. The differing biological effects of gamma and x-ray irradiation highlighted by these previous studies indicate a cell type dependent, complex relationship between type of irradiation and survival rate.

## **1.2 Thesis Purpose**

The purpose of this project is to examine differences in the effects of gamma and x-ray irradiation in accordance with the PNNL statement of work. Several methods are used in combination to better understand the effects of each radiation dose on the cancer cells. *In vitro* comparison of the survival fraction provides information about relative cell kill at each dose. Examination of protein and RNA expression after radiation treatment provides further insight into the mechanisms of cell death and repair occurring as a result. Studying the ways in which the survival fraction, protein expression, and RNA expression differ provides insights into the ways gamma and x-ray irradiation of cells differ. By extension, this research provides insight into how the process of transferring from cesium-137 irradiators to x-ray irradiators will require changes to research and treatment protocols.

## **2. MATERIALS AND METHODS**

### **2.1 Cell Culture**

Two cancer cell lines were used for the in vitro radiation studies, B16F10 (murine melanoma) and MC38 (murine colon adenocarcinoma). B16F10 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA), and MC38 cells were obtained from Professor Sentman's Lab at Dartmouth.

B16 cells were cultured in RPMI (Mediatech, Inc., Manassas, VA) with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 1% of a penicillin-streptomycin solution (10,000 units/mL penicillin and 10,000 µg/mL streptomycin, HyClone Laboratories, Inc., Logan, UT). Cells were plated at a density of  $1.25 \times 10^5$  cells/mL on standard tissue culture dishes and incubated at 37°C and 5% CO<sub>2</sub>.

MC38 cells were cultured in DMEM high (Mediatech, Inc., Manassas, VA) with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT) and 1% of a penicillin-streptomycin solution (10,000 units/mL penicillin and 10,000 µg/mL streptomycin, HyClone Laboratories, Inc., Logan, UT). Cells were plated at a density of  $1.5 \times 10^6$  cells/mL on standard tissue culture dishes and incubated at 37°C and 5% CO<sub>2</sub>.

### **2.2 Irradiators**

Delivery of two different radiation types, gamma rays and x-rays, required different types of irradiators. Gamma rays were generated from an 8,000 Curie JL Shepard Cesium-137 irradiator. Cesium gamma rays have an energy of 662 keV. X-rays were delivered from a 320 kV irradiator (XRAD 320) manufactured by Precision X-Ray, North Branford, CT. The dose rate for both types of radiation was 10 Gy per minute.

### **2.3 Assessment of Plating Efficiency**

Cells were plated at a density of 100 cells per cell culture plate. The number of cells attached to the plate were counted the following day. Plating efficiency was calculated as the percent of cells that attached relative to the number of cells plated.

### **2.4 Colony Forming Assays**

Cells were plated at a density of 100 cells/well in a 12-well cell culture plate. Twenty-four hours later cells received 0 Gy, 0.5 Gy, 1 Gy, 2 Gy, 4 Gy, 6 Gy, 8 Gy, or 10 Gy. Culture media was changed four days following irradiation. Eight days following irradiation, cells were fixed using 100% ethanol at room temperature for 10 minutes. Cells were then stained for 10 minutes with Methylene Blue (Sigma Aldrich, St. Louis, MO). Quantifiable colonies contained at least 50 cells. Colonies were counted, and the total number of colonies from each experimental group were compared to an unirradiated control to determine percent survival for each radiation dose.

### **2.5 RNA Analysis**

The day prior to irradiation, cells were plated at a density of 150,000 cells/well. Cells then received 0 Gy, 2 Gy, 6 Gy, or 18 Gy. Twenty-four hours post irradiation, cells were lysed, and the RNA purified. Purification of RNA was carried out using, and in accordance with, the instructions for the Qiagen RNeasy Mini Kit (Qiagen, Venlo, Netherlands). A GeneQuant II spectrophotometer from Pharmacia Biotech (Piscataway, NJ) was used to establish the amount and purity of RNA in order to normalize the amount

of RNA across samples. For each sample, 50ng of RNA were used for NanoString RNA expression quantification and analysis. Over 700 genes were analyzed using the NanoString PanCancer Pathways Panel (murine). Quantification of expression levels was completed using the NanoString nCounter Analysis System (NanoString Technologies, Seattle, WA) according to their instructions. The samples were normalized with respect to the expression of housekeeping genes. The normalization factor was determined with the geometric mean. Samples with fewer than 20 mRNA counts were not included in the analysis. The nSolver Analysis (v. 4.0) and Advanced Analysis Software were used to examine the collected data.

## **2.6 Protein Analysis**

Cells were plated at a density of 150,000 cells/well the day prior to irradiation. The experimental groups received 0 Gy, 2 Gy, 8 Gy, or 18 Gy. Twenty-four hours following irradiation, cells were lysed using a lysate buffer. The lysate buffer consists of 10ml of 40mM HEPES buffer, a complete mini tablet, and 100 $\mu$ l of orthovanadate. Cell lysate was then stored at -20°C. In order to normalize the protein concentration across samples a Bradford assay was used. Prepared, normalized samples were stored at -20°C.

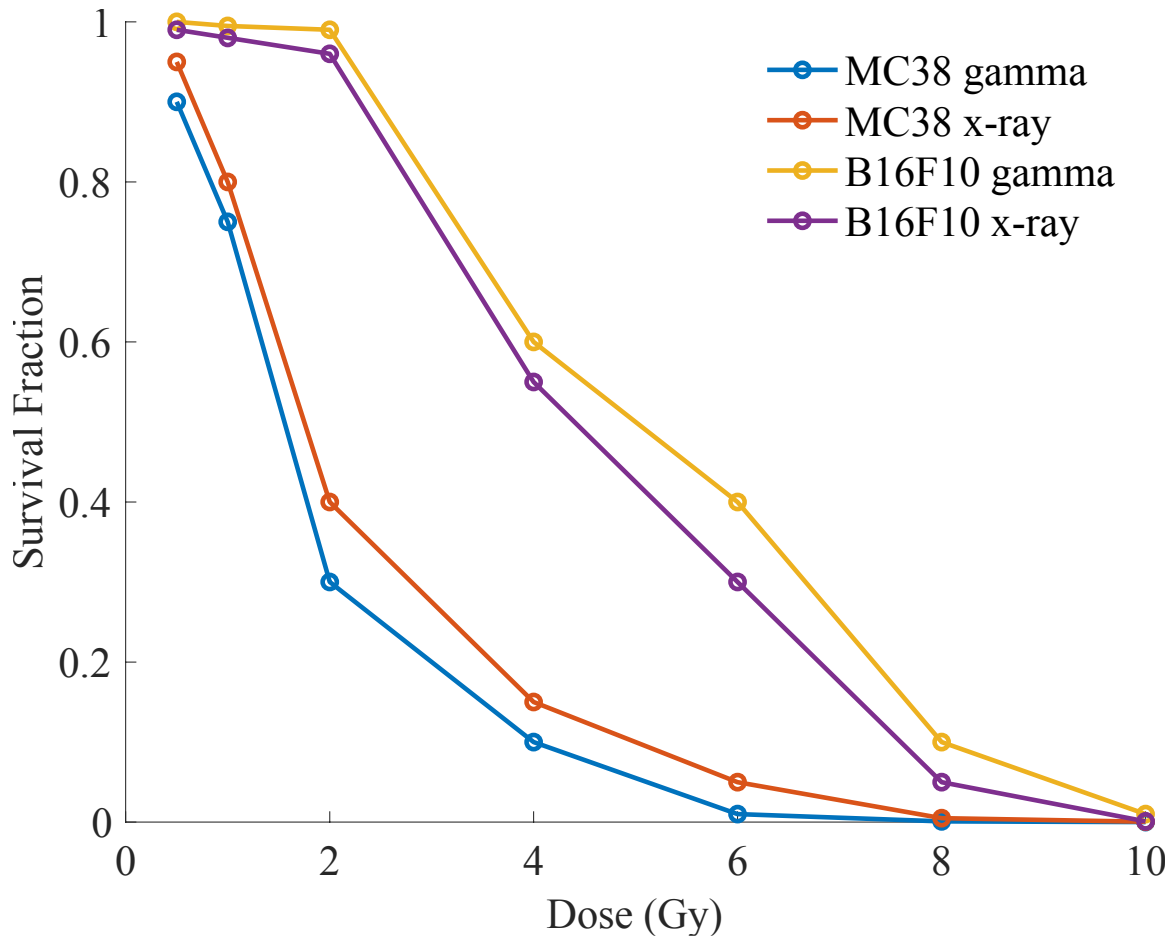
A traditional Western blot protocol using SDS-PAGE was used. Briefly, 12% polyacrylamide gels were used to separate proteins (run at 85V). Proteins were then transferred to a nitrocellulose membrane (run at 70V for 90 minutes in cold water). The membrane was incubated with the blocking solution (bovine serum albumin (BSA) with Tris-Buffer Saline/Tweet (TBST)) for 30 minutes. The membrane was then incubated with primary antibody for at least 12 hours at 4°C. The membrane was subsequently

thoroughly washed in TBST before being incubated at room temperature with secondary antibodies conjugated with horseradish peroxidase for one hour. Visualization of the proteins was achieved with enhanced chemiluminescence in a G:Box Syngene (from New England Biogroup, Atkinson, NH). Protein expression was quantified through densitometry analysis in ImageJ and normalized with respect to the expression of  $\beta$ -actin, the chosen loading control.

### **3. RESULTS**

#### **3.1 Survival Fractions**

Our results, using of the colony forming assay, suggest that the same dose of gamma and x-radiation demonstrate similar, but not identical cytotoxicity profiles in two different murine cancer cell lines. Overall, the colon cancer cells (MC38) were more radiosensitive than the melanoma cells (B16) for both gamma rays and x-rays. With respect to radiosensitivity and radiation type, MC38 cells were more sensitive to gamma irradiation while B16F10 cells were more sensitive to x-rays, at all doses used (Figure 1).



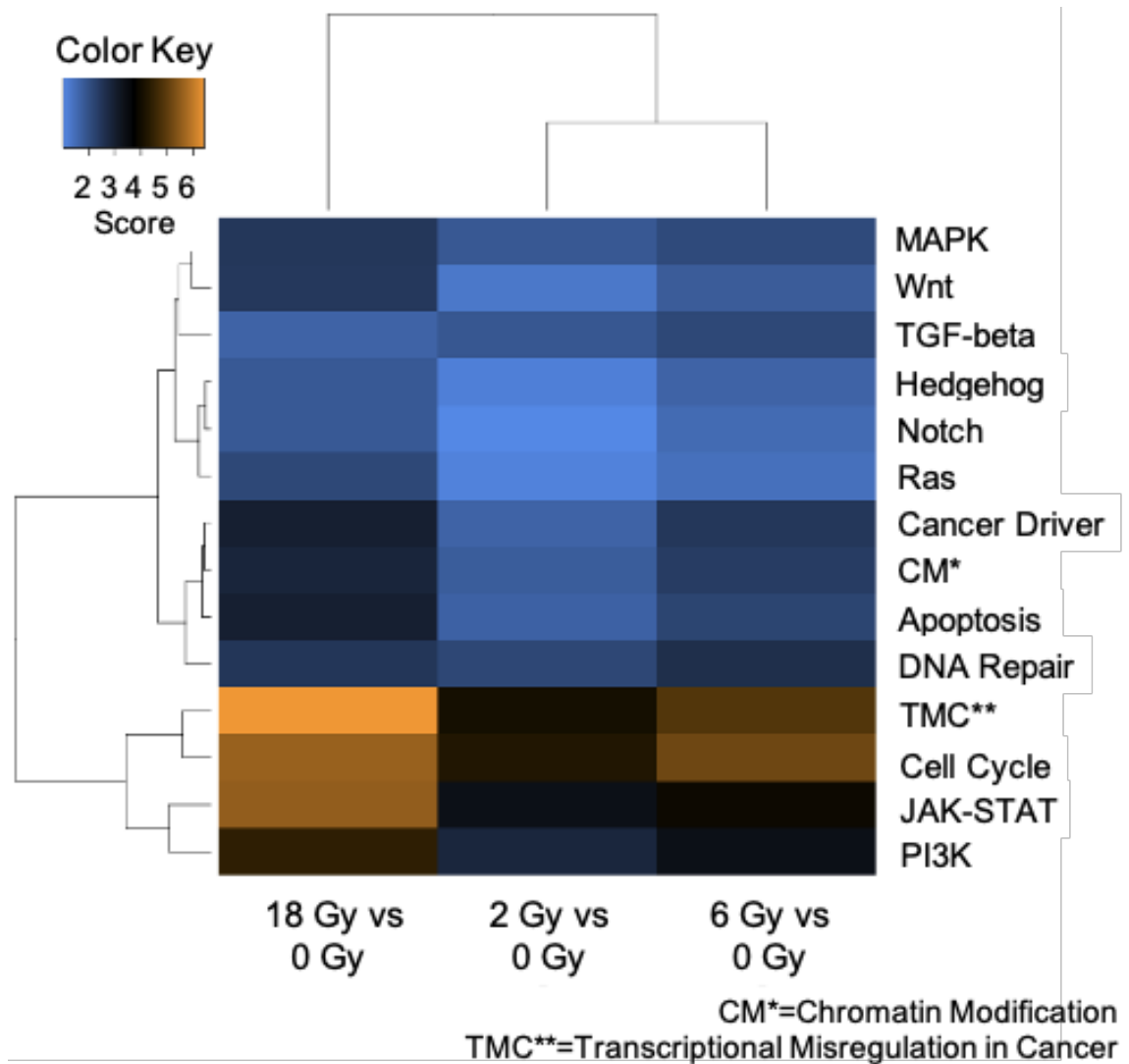
**Figure 1:** Surviving fractions relative to a control of untreated cells as a function of dose, type of irradiation, and cell type based on colony forming assays. MC38 cells are more susceptible to gamma radiation while B16F10 cells are more susceptible to x-ray radiation at any dose. This figure was created using data collected in the laboratory and extrapolating based on the data provided in a study by Scott et al [11].

### 3.2 Genetic Expression

The genetic expression of irradiated cells was only conducted in the B16F10 cell line using Cesium gamma irradiation, twenty-four hours following irradiation. The most significant changes in genetic expression (mRNA expression) following irradiation, relative to control, were in genes associated with cell cycle and transcriptional misregulation pathways. Although we expected the genes associated with apoptosis to



change in a dose dependent manner relative to control, we saw minimal change at any dose (Figure 2).



*Figure 2: Overview of differences in mRNA expression of B16F10 cells 24 hours post gamma irradiation relative to untreated cells. Transcriptional Misregulation in Cancer genes including Cdkn1a (p21), a cell cycle regulatory gene, and AKT1, a pro-survival gene, show the greatest dose dependent response in expression. Apoptotic genes (eg. pro-apoptotic genes Tnfrsf10b, Bax, and Caspase 3 and anti-apoptotic genes BCL2 and AKT1) show relatively little change in expression as a function of the gamma dose administered.*

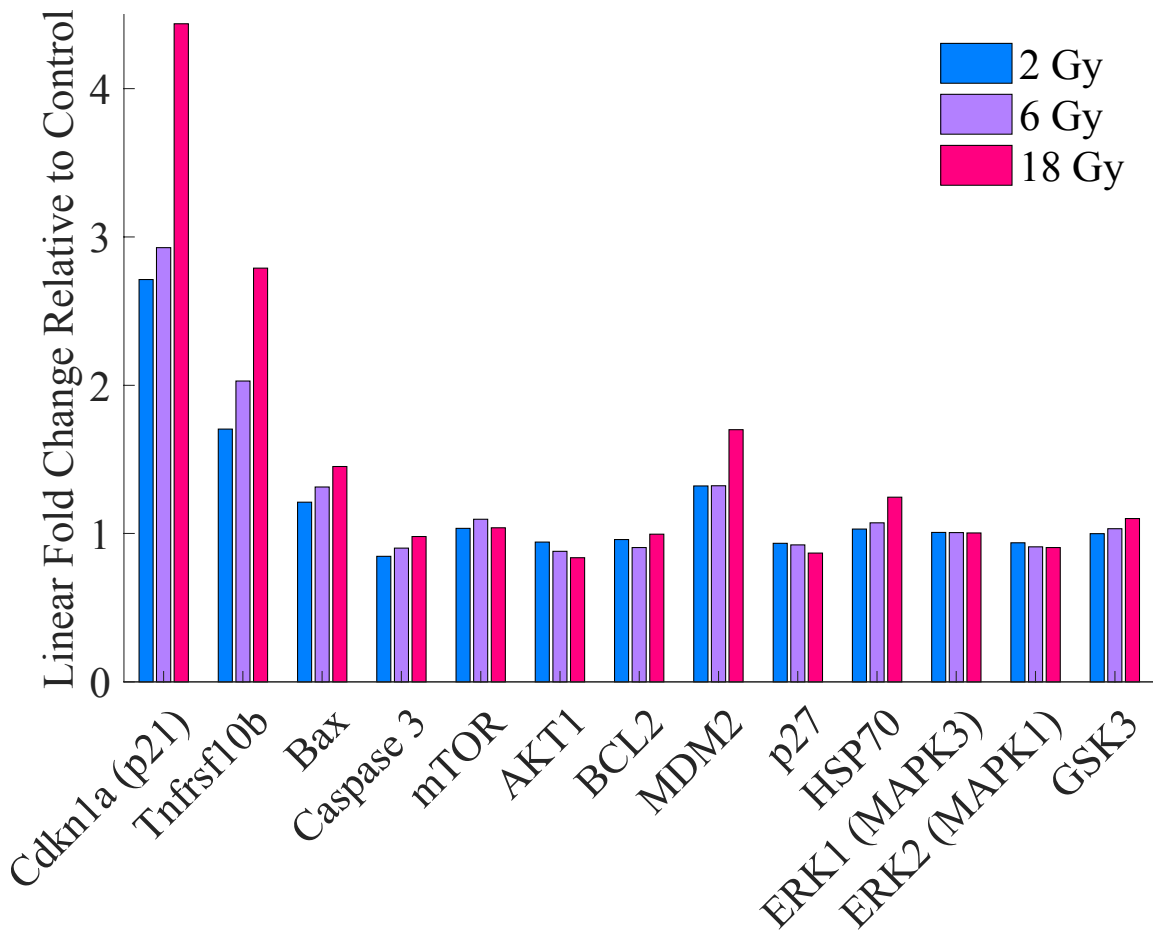
The most differentially expressed gene at all radiation doses, relative to control, was Cdkn1a. Cdkn1a, also known as p21, is a cell cycle regulatory gene that is in the transcriptional misregulation pathway [15]. Its mRNA expression increased with increasing dose (Figure 3). The expression of p27, a key regulator of cell proliferation,

was decreased slightly relative to control at all radiation doses [16]. GSK3, a regulator gene associated glycogen metabolism and protein translation for cell cycle regulation, cell proliferation and apoptosis, showed essentially no change relative to control at 2 Gy but increased slightly relative to control with increasing radiation dose, 6 and 18 Gy [17], [18].

TNFRsf10b expression, associated with intracellular based cytotoxicity, increased at all radiation doses relative to control and had the most significant expression difference of any apoptotic gene at any dose examined [19]. Bax, a pro-apoptotic regulator [20], mRNA expression increased significantly relative to control at all doses with increased expression as the radiation dose increased. Caspase 3 is an apoptotic protein promoting DNA fragmentation and reducing immunogenicity of cell death [6], [20]–[22]. Caspase 3 mRNA expression decreased significantly (a log<sub>2</sub> fold decrease of 0.24) at a low dose of radiation (2 Gy). As the radiation dose increased, genetic expression of caspase 3 increased back to unirradiated levels. BCL2 and mTOR, both pro-survival apoptotic regulators, showed almost no difference in genetic expression relative to control at any dose [6], [20]–[24].

Akt1 expression, associated with increased survival, decreased slightly with increasing radiation dose [6], [12], [17], [23], [25]. At a dose of 18 Gy, Mdm2, involved in the inhibition of p53 and associated with increased survival, mRNA expression exceeded a 1.5-fold change relative to control [24], [26]. Mdm2 expression increased with increasing radiation dose. HSP70 mRNA, an apoptotic inhibitor involved in immunogenic cell death, showed little change relative to control at 2 Gy but its expression increased slightly with increasing dosage [20]–[22], [27]. ERK1/2 (MAPK3

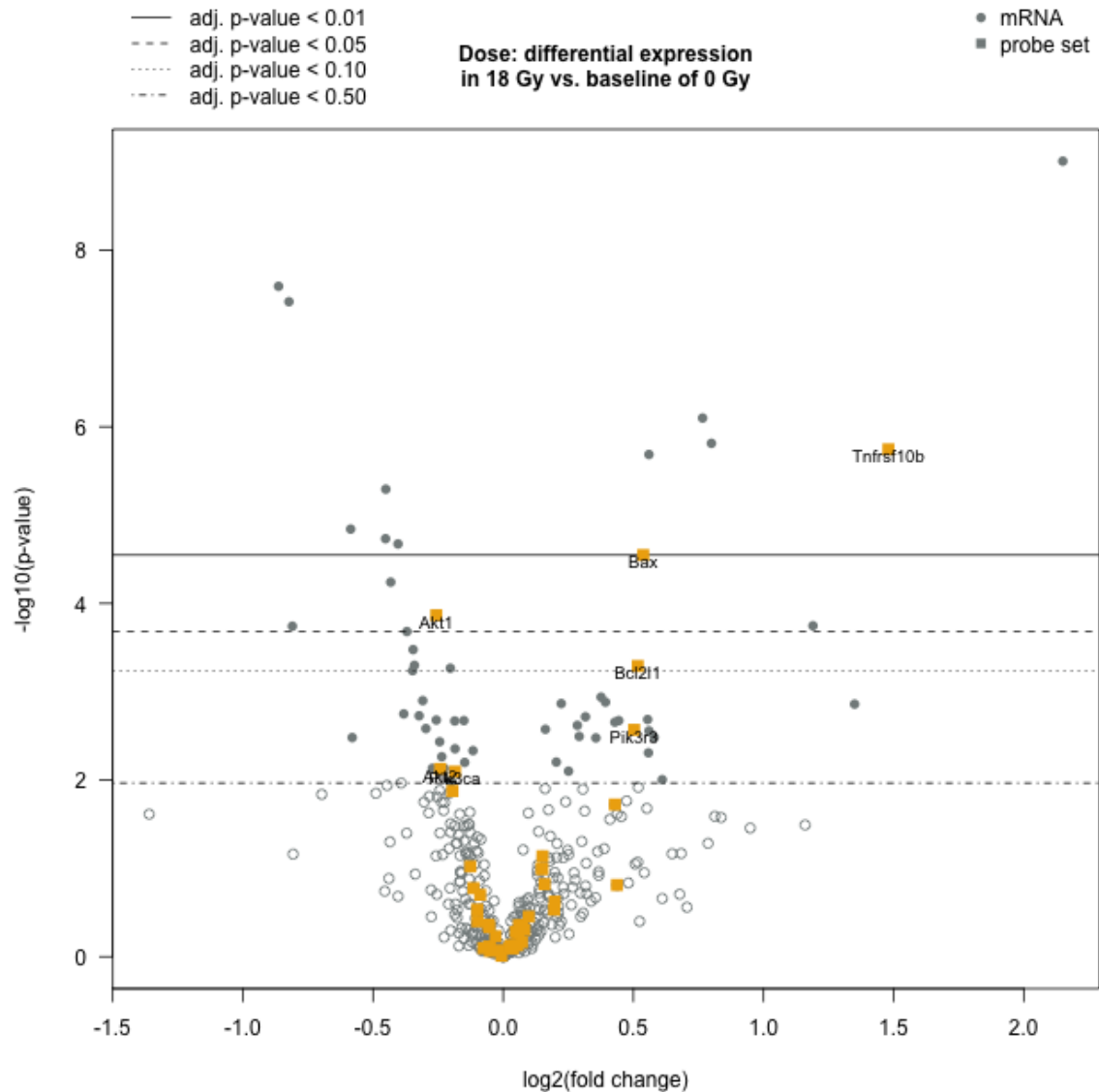
and MAPK1) genetic expression, involved in immunogenic cell death, remained at the same level as control over the range of 2-18 Gy doses [21], [24]. A more comprehensive summary of the functional changes associated with the expression of the genes discussed is shown in Table 1.



**Figure 3: Linear fold change of mRNA expression 24 hours post gamma radiation treatment of B16F10 cells relative to untreated control cells.** Most genes show relatively little change in expression relative to control. However, Cdkn1a (p21), a cell cycle regulatory gene, Tnfrsf10b, which encodes a death receptor involved in extrinsic apoptosis, and Mdm2 (at 18 Gy), a pro-survival p53 inhibitor, exceeded a 1.5-fold change in expression relative to control.

Figure 4 is a volcano plot that demonstrates the difference in mRNA expression of individual genes 24 hours after receiving 18 Gy. All genes examined are shown in grey except those involved in apoptosis, which are shown in yellow. The y-axis represents statistical significance with more significant differences in expression at the top. The

relative difference in gene expression, at that dose, relative to control is measured on the x-axis with larger differences toward the lateral edges of the plot. For apoptotic genes, only changes in expression of TNFRsf10b and Bax at 18 Gy were statistically significant at an adjusted p-value of 0.05. The change in expression of AKT1, at 18 Gy, was statistically significant at an adjusted p-value of 0.10.



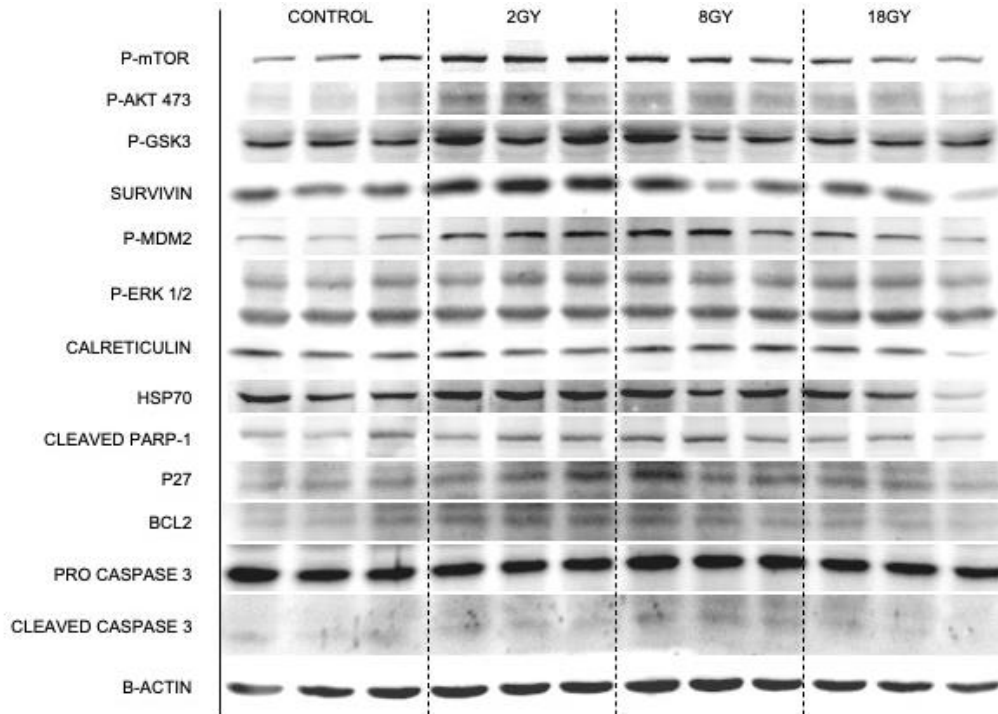
**Figure 4: Volcano plot indicating changes in mRNA expression of apoptosis regulatory genes 24 hours after receiving 18 Gy of gamma radiation relative to untreated control cells.** Apoptosis genes are shown in yellow while other genes tested are in grey. The amount by which expression levels changed is indicated on the x-axis: larger changes in expression fall to the lateral edges. The statistical significance of the change in expression is shown on the y-axis with more statistically significant differences toward the top of the figure. The apoptosis genes with the most statistically significant (with an adjusted p value of less than 0.05) change in genetic expression relative to control at a dose of 18 Gy are TNFRsf10b, which is pro-apoptotic, Bax, also a pro-apoptotic gene, and Akt1, a pro-survival gene.

Table 1: Summary of gene function and observed changes in expression

Gene	Pathways(s) and Function	Change in Expression
Cdkn1a (p21)	Required for p53-mediated repression of transcription	Large increase
Tnfrsf10b	Encodes protein death receptor 5, extrinsic apoptosis, anti-survival	Increase
Bax	anti-survival, promotes cytochrome c release, intrinsic apoptosis	Increase
Caspase 3	apoptosis, lysosome dependent cell death, leads to DNA fragmentation and reduces immunogenicity	Slight decrease
mTOR	autophagy, apoptosis, pro-survival	Steady
AKT1 (Protein Kinase B)	autophagy, apoptosis, metabolism, pro-survival	Decrease
BCL2	intrinsic apoptosis, lysosome-dependent cell death, senescence, inhibits cytochrome c release (pro-survival)	Steady
p-MDM2	cell cycle, p53 inhibitor, pro-survival	Increase
p27	cell cycle arrest	Steady
HSP70	apoptosis inhibitor, promotes phagocytosis by dendritic cells (immunogenic cell death)	Slight increase
p-ERK1 (MAPK3)	immunogenic cell death	Steady
p-ERK 2 (MAPK1)	pro-survival in apoptosis, promotes immunogenic cell death	Steady
p-GSK3	metabolism, promotes glycogen synthesis	Slight increase

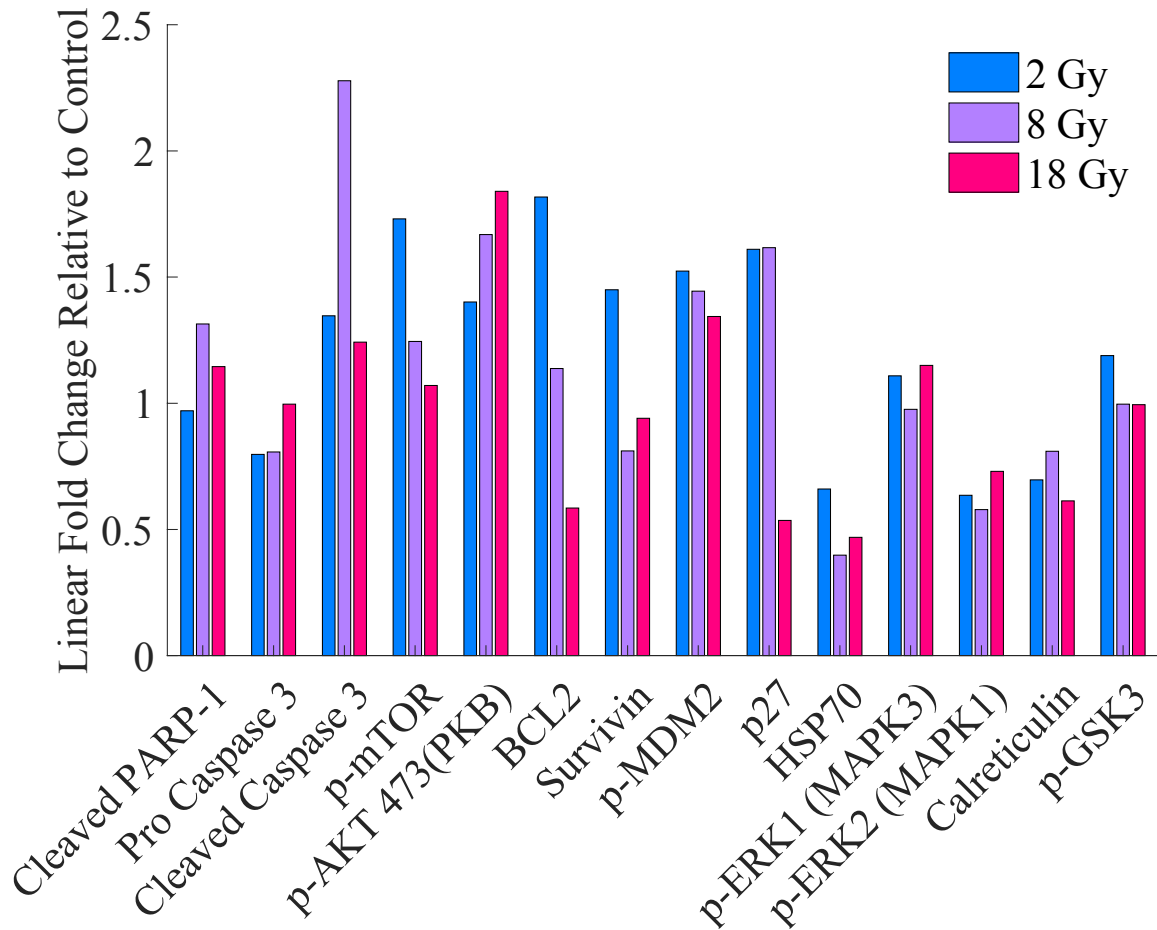
### 3.3 Protein Expression

The data presented in this section was collected from gamma irradiation of B16F10 cells. Several proteins involved in regulation of cell survival and death were examined. Table 2 provides a summary of the proteins and the changes in their expression.



**Figure 5: Western blot protein expression levels of B16F10 cells 24 hours post gamma irradiation.** The proteins shown are involved in metabolic activity regulation (p-GSK3), cell cycle regulation (p27), pro-survival proteins (p-mTOR, p-AKT 473 also known as protein kinase B, BCL2, survivin, and p-MDM2), pro-apoptotic proteins (cleaved PARP-1 and the inactive pro-caspase 3 as well as the active form cleaved caspase 3), and pro-immunogenic cell death proteins (HSP70, p-ERK1 also known as MAPK3, p-ERK2 also known as MAPK1, and calreticulin). B-actin was chosen to be the loading control. Surprisingly, cleaved caspase 3 shows little protein expression at any dose.

The translation of genetic expression to protein production does not always track as closely as one might predict. For irradiation of cells the challenges include dose, cells type and assay timing. In our study protein changes that tracked with genetic changes included: an increase p-GSK3, a metabolic protein promoting glycogen synthesis, expression at 2 Gy, but no change relative to control after 8 or 18 Gy exposure [17], [18]. The relative amount of p27, promoting cell cycle arrest, increased relative to control at radiation doses of 2 and 8 Gy and reduced levels relative to control at 18 Gy [16].



**Figure 6: Linear fold change of protein expression relative to control and normalized relative to  $\beta$ -actin in B16F10 cells 24 hours post gamma irradiation.** Protein expression showed more change in expression relative to control than the mRNA. Proteins that exceeded a 1.5- or 0.66-fold change are pro-apoptotic protein cleaved caspase 3, pro-survival proteins p-mTOR, p-AKT 473 (Protein Kinase B), BCL2, and p-MDM2, cell cycle regulatory protein p27, and pro-immunogenic cell death proteins HSP70, p-ERK2 (MAPK1), and calreticulin.

Cleaved PARP-1 expression, involved in apoptosis and DNA damage repair, had little change relative to control at a 2 Gy dose but increased at 8 Gy and dropped slightly from its 8 Gy expression level at a dose of 18 Gy [6], [21], [28], [29]. Pro-caspase 3 (the inactive form of caspase 3) protein expression decreased slightly relative to control at 2 Gy and 8 Gy doses and returned to the same levels as control at 18 Gy. Cleaved caspase 3 is a pro-apoptotic protein promoting DNA fragmentation and reducing immunogenicity of cell death [6], [20]–[22]. Contrary to expectation, the cleaved caspase 3 bands from the western blot did not show much expression (see Figure 5). It is possible an error occurred

in the process of cutting the membrane or staining with the antibodies. Thus, the data for cleaved caspase 3 is potentially unreliable, although it is possible there was simply not very much cleaved caspase 3 expression. However, the data collected suggests a slight increase in expression at 2 Gy and 18 Gy with a larger increase at 6 Gy.

p-AKT 473 expression, a pro-survival protein also known as protein kinase B, increased with increasing radiation dose and passes a 1.5-fold change threshold at 6 Gy [6], [12], [17], [23], [25]. p-mTOR expression, pro-survival and anti-apoptotic, decreased with increasing dosage with an initial fold change greater than 1.5 at 2 Gy and returning to baseline by 18 Gy doses [22]–[24]. BCL2 is involved in intrinsic apoptosis [6], [20], [21]. BCL2 expression levels were elevated above 1.5-fold change in expression at 2 Gy, a decrease to roughly pre-radiation expression levels at 6 Gy and show reduced expression (below a 0.66-fold change) at 18 Gy. The pro-survival protein survivin expression shows an increase at 2 Gy and a slight decrease relative to control at 8 and 18 Gy [6], [27]. p-MDM2, a pro-survival p53 inhibitor, shows increased expression of roughly 1.5-fold relative to control at 2 Gy and demonstrates a slight decrease in expression with continuing increase in radiation dosage [24], [26].

Although the genetic expression was significant, following irradiation, for the thermal regulatory and immunostimulatory gene HSP70, protein expression was minimal except at 8 Gy [20]–[22], [27]. p-ERK1, also known as MAPK3 and involved in immunogenic cell death, expression remained at roughly the same level as the control at all doses [21]. p-ERK2, also known as MAPK1, an anti-apoptotic and pro-immunogenic cell death protein, expression was decreased relative to control at all doses and below 0.66-fold change at 2 and 8 Gy [21], [24]. Somewhat disappointingly, the potent



immunostimulatory protein calreticulin showed little expression change at all radiation doses [6], [21], [22].

Table 2: Summary of protein function and observed changes in expression

Protein	Pathways(s) and Function	Change in Expression
Cleaved PARP-1	DNA damage repair, apoptosis, parthanatos, product of caspase 3 or 7	Increase at 8 Gy, slight decrease by 18 Gy
Pro caspase 3	inactive form of caspase 3	Slight initial decrease, return to normal levels at 18 Gy
Cleaved caspase 3	apoptosis, lysosome dependent cell death, leads to DNA fragmentation and reduces immunogenicity	Increase, especially at 8 Gy
p-mTOR	autophagy, apoptosis, pro-survival	Initial increase and steady decrease
p-AKT 473 (Protein Kinase B)	autophagy, apoptosis, metabolism, pro-survival	Increase
BCL2	intrinsic apoptosis, lysosome-dependent cell death, senescence, inhibits cytochrome c release (pro-survival)	Initial increase and steady decrease
Survivin	pro-survival, inhibits apoptotic proteins	Initial increase, quick return to normal levels
p-MDM2	cell cycle, p53 inhibitor, pro-survival	Initial increase and steady decrease
p27	cell cycle arrest	Increased at 2Gy and 8 Gy, decreased at 18 Gy
HSP70	apoptosis inhibitor, promotes phagocytosis by dendritic cells (immunogenic cell death)	Decrease
p-ERK1 (MAPK3)	immunogenic cell death	Steady
p-ERK 2 (MAPK1)	pro-survival in apoptosis, promotes immunogenic cell death	Decreased, steady
Calreticulin	immunogenic cell death, promotes phagocytosis	Decreased, slight increase at 8 Gy
p-GSK3	metabolism, promotes glycogen synthesis	Initial increase, quick return to normal levels

## **4. DISCUSSION**

### **4.1 Survival Fractions**

Overall this study demonstrates a relatively consistent and similar genetic, proteomic and cytotoxic response in two murine cancer cell lines following gamma and x-ray radiation, at the same dose. It was notable that B16F10 murine melanoma cells were slightly more sensitive to x-rays than to gamma rays, at the same dose, meaning the RBE of x-rays relative to gamma rays for B16F10 cells is less than 1. The MC38 murine colon cancer cells were slightly more sensitive to gamma rays than x-rays, at the same dose, meaning an RBE of greater than 1 for x-rays relative to gamma rays. For neither cell line was the difference in RBE especially pronounced. This result is unsurprising given that both techniques provide photons, though the energy of the photons differ (662 keV for gamma radiation and up to 320 keV for x-ray radiation). Moreover, this finding is in agreement with the results of the study performed by Scott et. al. examining survival at different doses in bone marrow and splenocytes of mice [11]. However, the results are complicated by comparison with other studies; the functional differences between gamma and x-ray radiation at a given dose have been shown in other publications to be significant in some cases and insignificant in others [7], [10], [11], [14]. In order to determine what factors contribute to the different responses of some cells to gamma and x-ray radiation but not others at a given dose, further research examining the characteristics of the cells affected versus cells unaffected is necessary. This information could then be used to predict whether or not alterations to protocols written for cesium irradiators are necessary when transferring to an x-ray irradiator.

## 4.2 RNA (Genetic) and Protein Expression

mRNA and protein expression of B16F10 cells 24 hours after gamma irradiation was examined in this study. The cell cycle regulatory gene *cdkn1a* (also known as p21) showed increased mRNA expression relative to control. Cell cycle regulatory gene p27 generally showed little change in mRNA expression but showed increased protein expression, especially at low radiation doses. The metabolic activity of the cells, as measured via GSK3 expression, also likely increased as a result of radiation exposure. The expression of the pro-apoptotic genes and proteins TNFRSF10b, BAX, cleaved PARP-1, caspase 3, and mTOR generally showed increased expression, growing with increasing dosage. This data suggests an increase in apoptotic cell death with increasing doses of radiation treatment. However, apoptotic death potentially peaks at a dose lower than 18 Gy as the protein expression showed a decrease in expression of pro-apoptotic proteins at 18 Gy relative to the 8 Gy levels. Considered together, the expression of the pro-survival, anti-apoptotic proteins (AKT, BCL2, Survivin, and p-MDM2) is increased at low doses of radiation and decreases with increasing dosage as the cells sustain more damage from ionizing radiation. The pro-immunogenic cell death proteins (HSP70, p-ERK1/2, and calreticulin) were generally decreased relative to control after treatment with gamma radiation, though the mRNA expression was generally at the same level as control at all doses.

Overall, most of the genes and proteins examined in this study showed little change in expression relative to control at any dose. mRNA expression after gamma irradiation showed the most effect on cell cycle regulatory genes, though expression of genes regulating apoptosis also demonstrated a dose-related effect. *Cdkn1a*, a cell cycle

regulatory gene associated with apoptosis, had the largest and most significant difference in expression of any gene assessed. In general, gamma radiation resulted in limited changes in the mRNA expression of apoptotic regulatory genes. The overall mRNA expression trends show an increase in pro-survival and pro-apoptotic genes with increasing radiation dose, and little or no change in genes linked to immunogenic cell death. The protein expression changes, as examined by western blot analysis, show slightly greater variation in expression than the mRNA, though the overall protein expression changes of the apoptotic, immunogenic cell death, cell cycle, and pro-survival proteins studied was also generally limited. The data demonstrates a slight increase of pro-survival proteins especially at lower radiation doses, a slight increase in pro-apoptotic and anti-apoptotic proteins at 2 Gy doses, a continuing increase in pro-apoptotic proteins and a decrease in anti-apoptotic proteins at 6 Gy, and a decrease in both pro-apoptotic and anti-apoptotic proteins at 18 Gy. These trends suggest apoptosis provides an important role in preventing further tumor cell proliferation after receiving gamma radiation, but apoptosis is not a sufficient explanation for the decrease in cell proliferation.

It seems likely there will be slight differences in protein and mRNA expression when using x-ray irradiation given that there was a difference in the survival fractions at a given dose in this study, and with other blood irradiation and bone marrow studies demonstrating slight differences [7], [11], [14]. Unfortunately, there is not enough published information about these differences to determine if there will be a significant effect without conducting a study such as the one outlined above.

## 5. CONCLUSION

Although we demonstrated relative minor differences in the genetic, protein and cytotoxic effects of x-ray and gamma ray irradiation, in two cancer cell lines, these differences could prove clinically significant for higher doses and fractionated treatments. Expansion of the experiments demonstrated in this thesis are an important next step in the quest to understand and quantify the differences (and clinical relevance of those differences) between the two types of irradiation. The methods and types of analysis utilized provide a meaningful framework for future studies. Such *in vitro* experiments will be very important for gamma and x-ray radiation dose conversions as gamma irradiators are gradually eliminated from biomedical use worldwide. Accurate conversion information is especially important for researchers with decades worth of gamma radiation data who no longer have access to that radiation source. *In vivo* experiments examining not only the mechanisms and pathways of cell death, but also tumor and normal tissue responses, following x-ray and gamma ray irradiation will also be very important.

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