NON-TARGETED EFFECTS OF IONIZING RADIATION IN FISH CELL LINES

NON-TARGETED EFFECTS OF IONIZING RADIATION IN FISH CELL LINES

By

ALICIA O'NEILL, B.Sc.

A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Master of Science

McMaster University

MASTER OF SCIENCE (2006) McMaster University

(Biological Sciences) Hamilton, Ontario

TITLE: Non-targeted Effects of Ionizing Radiation in Fish Cell Lines

AUTHOR: Alicia O'Neill, B.Sc. (Brock University)

SUPERVISOR: Dr. C. Mothersill

NUMBER OF PAGES: x, 113

ABSTRACT

This study is one of the first to examine non-targeted effects of radiation in fish cell lines, with the aim of identifying a reliable reporter system for evaluating radiation damage in fish. The ability of the fish cell lines to clone was determined as the clonogenic assay was a major end point used to measure survival. A direct survival curve was completed for all cell lines that were deemed clonogenic using a cobalt-60 γ-radiation source. Non-targeted effects of radiation were evaluated by conducting bystander experiments on all fish cell lines. Delayed Cell Death (DCD) experiments were completed on the fish cell line that showed evidence of a cell death associated bystander effect as these phenomena may be linked. Four of the eight cell lines were found to be clonogenic. The cell line, RTG-2, was found to be the most radiosensitive at lower doses. All of the clonogenic cell lines, with the exception of RTG-2 cells, generally showed increased Plating Efficiency (P.E.) when Irradiated Cell Conditioned Media (ICCM) was tested on unirradiated autologous cells. ICCM from the clonogenic and non-clonogenic cells was also tested on the mammalian cell line. This resulted in increased cell survival, with the exception of the RTS-pBk+ ($p<0.001$), RTS-34st ($p<0.01$) and ZEB 2J ($p<0.05$) cell lines. Since RTG-2 showed the most prominent cell killing bystander effect, DCD experiments were performed on this cell line. DCD was found in the progeny of irradiated parental cells at all doses tested. Cell kinetics also showed the generation of possible DCD. The results show that both bystander signal production and cellular responses vary depending on the cell line and that DCD and bystander effects are tentatively linked through genomic instability. The RTG-2 cell line

may be a suitable model for a reliable reporter system to aid in determining the non-targeted effects of radiation in fish in the environment.

ACKNOWLEDGEMENTS

I would like to thank Dr. Carmel Mothersill, whose guidance, teachings and kindness made this thesis achievable. Thank-you to Dr. Michael Kilemade, who taught me all I know about cell culture and Dr. Colin Seymour and Dr. Andrew Rainbow, who helped me with interpretation of results and general encouragement.

To all the friends I have made throughout this seemingly never-ending trek, you made this journey bearable – and with a few laughs to boot.

To my family, thank-you so much for being there for me on my good days and bad; everyone of you have helped me through these past couple of years in one way or another; thank-you for your encouragement and never wavering support.

I dedicate this thesis to my loving husband, Chris, who always pushed me to keep going even when I wanted to quit. Without his love and support this would not have been possible.

Table of Contents

	Page
Title Page	i
Descriptive Note	ii
Abstract	iii
Acknowledgements	v
Table of Contents	vi
List of Tables and Figures	ix

Chapter 1: INTRODUCTION

1.0 Introduction	2
1.1 What is radiation?	3
1.2 How is radiation measured?	5
1.3 Sources of Radiation	5
1.4 Discussion of Non-targeted Effects of Radiation	7
1.4.1 Linear No-Threshold (LNT) Hypothesis	8
1.4.2 Adaptive Response	10
1.4.3 Low dose hyper-radiosensitivity	11
1.4.4 Hormesis	14
1.4.5 Bystander Effects	17
1.4.6 Genomic Instability	20
1.4.7 Genomic Instability and Bystander Effects	22
1.4.8 Summary of Non-targeted Effects	25
1.5 Non-targeted Effects of Radiation on Non-human Biota	25
1.6 Objectives	29

Chapter 2: BYSTANDER EFFECTS

2.1 MATERIALS AND METHODS	
2.1.1 Cell Lines	33
2.1.2 Cell Culture	36
2.1.3 Growth Curve	37
2.1.4 Clonogenic Assay Technique	38
2.1.5 Irradiation	39

2.1.6 Survival Curves 40

2.1.7 Clonogenic Fish Cell Lines 40

2.1.8 Non-cloning Fish Cell Lines 42

2.1.9 Statistical Analysis 43

2.2 RESULTS

2.2.1 Growth Curve 45

2.2.2 Clonogenic Assay 45

2.2.3 Survival Curve 47

2.2.4 Clonogenic Cell Lines 50

2.2.5 Non-clonogenic Fish Cell Lines 55

2.3 DISCUSSION

2.3 Discussion 60

Chapter 3: LETHAL MUTATION EXPERIMENT

3.1 MATERIALS AND METHODS

3.1.1 Cell Line 67

3.1.2 Cell Culture 67

3.1.3 Irradiation 67

3.1.4 Lethal Mutation/ Delayed Cell Death Technique 67

3.1.5 Cell Kinetics Post Irradiation 68

3.1.6 Calculation of Delayed Cell Death Frequency 69

3.1.7 Statistical Analysis 69

3.2 RESULTS

3.2.1 Lethal Mutation 71

3.2.2 Cell Kinetics 71

3.3 DISCUSSION

3.3 Discussion 78

Chapter 4: BYSTANDER AND LETHAL MUTATION EXPERIMENTS: GENERAL CONCLUSIONS

4.1 General Conclusions 84

4.2 Future Directions 87

REFERENCES 90

APPENDICES 105

List of Tables and Figures:

Figure 1. Direct and indirect ionizing radiation 4

Figure 2. Sources of radiation on the earth 6

Figure 3. Depiction of linear no threshold hypothesis (LNT) 6

Figure 4. Dose-response for cell killing using ionizing radiation 12

Figure 5. U-shaped dose-response curves 16

Figure 6. Possible model for expression of the bystander effect 19

Figure 7. Growth Curves of all cell lines

(a) Clonogenic cell lines 46

(b) Non clonogenic cell lines 46

Figure 8. Survival curves of all clonogenic cell lines 49

Figure 9. Bystander results for HPV-G mammalian cell line 49

Figure 10. Bystander experiment results for all clonogenic fish cell lines

(a) RTG-2 51

(b) ZEB 2J 51

(c) CHSE-214 52

(d) RT-Gill W1 52

Figure 11. Clonogenic cell lines that received irradiated media containing no cells 54

Figure 12. Bystander experiment results for all non-clonogenic fish cell lines

(a) RTS-pBk+ 56

(b) RTS-34st 56

(c) PBLE 57

(d) EPC 57

Figure 13. Initial Survival Fraction (ISF) and Progeny Survival Fraction (PSF) of RTG-2 cells 72

Figure 14. (a). Uncorrected (directly irradiated) and Corrected (progeny of directly irradiated) growth curves over a period of 40 days 73

(b) Growth curves of uncorrected data for the exponential phase 73

(c) Growth curves of corrected data for the exponential phase 74

Figure 15. Cell kinetics of the progeny of irradiated cells during the growth of cells plated over forty days 76

Figure 16. Comparison of PSF for delayed cell death experiment with last progenic count in cell kinetics 76

Table I. Doubling Times of all cell lines 48

Table II. Plating efficiencies of all fish and mammalian cell lines 48

Table III. Plating efficiencies of 0Gy sham directly irradiated and recipients from clonogenic fish cell lines 54

Table IV. Slopes of uncorrected and corrected values derived from the exponential phase of figure 14 72

CHAPTER 1

INTRODUCTION

1.0 Introduction:

There has been newfound interest on the non-targeted effects of radiation on cells, which cannot be attributed to direct DNA damage (Reviews: Morgan, 2003; Lorimore and Wright, 2003; Little and Morgan, 2003; Prise *et al.*, 2003; Mothersill and Seymour, 2004a; 2004b). Essentially, cells that have not been exposed to radiation act as if they have been, through signals received from irradiated cells. This phenomenon has been termed the bystander effect (Mothersill and Seymour, 1997; Prise *et al.*, 2002; Schettino *et al.*, 2003). The signal itself that produces this bystander effect is yet to be identified, however it is known to cause sister chromatid exchanges (Nagasawa and Little, 1992), chromosomal aberrations (Lorimore and Wright, 1998), micronucleus induction (Azzam et al., 2001), initiation of apoptosis (Lyng *et al.*, 2002), cell death (Mothersill and Seymour, 1997) and genomic instability (Kadhim et al., 1992). The signal causes a cascade of events in un-hit cells resulting in the above end points (Limoli *et al.*, 1997; Lyng *et al.*, 2002). The Irradiated Cell Conditioned Medium (ICCM) also appears to induce an elevation in intracellular levels of reactive oxygen species (ROS) (Lyng *et al.*, 2002), which can be a precursor to cell death (Lorimore and Wright, 2003; Little and Morgan, 2003). In addition to this, Delayed Cell Death (DCD), also known as lethal mutations, may be linked to the bystander effect, and this is a phenomenon implicated in non-targeted effects. DCD is a reduction in survival of the progeny of cells that have not been irradiated, but have been produced from irradiated parental cells (Seymour *et al.*, 1986; Lorimore *et al.*, 1998; Seymour and Mothersill, 2000). There has been increased interest in these non-targeted effects of radiation because of their

importance following low doses and their possible relevance for radiation protection of genomic instability (Dowling *et al.*, 2005; Lyng *et al.*, 2002; Mothersill *et al.*, 1998; Lorimore *et al*, 1998).

These non-targeted effects usually predominate at low doses (Mothersill and Seymour, 2002; Schettino *et al.*, 2003; Ponnaiya *et al.*, 2004a). Therefore, they are of concern in radiation protection, although the recent BEIR VII report stated that it was too early to assess relevance for risk from low dose exposure. Most of these studies are targeted at mammalian systems, but in view of the recent interest in developing a framework for radiation protection of non-human species (Hinton *et al.*, 2004), this work aims to investigate non-targeted effects in fish cells.

1.1 What is radiation?

There are different forms of radiation, including ionizing and non-ionizing radiation. Non-ionizing radiation has enough energy to move atoms in a molecule, but not enough to remove electrons. Ionizing radiation refers to a high energy of radiation and can cause damage to tissue by displacing electrons from molecules to produce potentially destructive ions (BEIR, 2006). It is present in either the X- or gamma- (γ-) ray or particle form. Ionizing radiation can cause damage because its energy is greater than the ionizing potential of matter (it is greater than 24.6 eV). This form of radiation can interact either directly or indirectly with matter. Indirect radiation interaction consists of a process in which the initial energy is deposited in the cell and a charged particle is released (Figure 1) (BEIR, 2006). The released particle continues to deposit

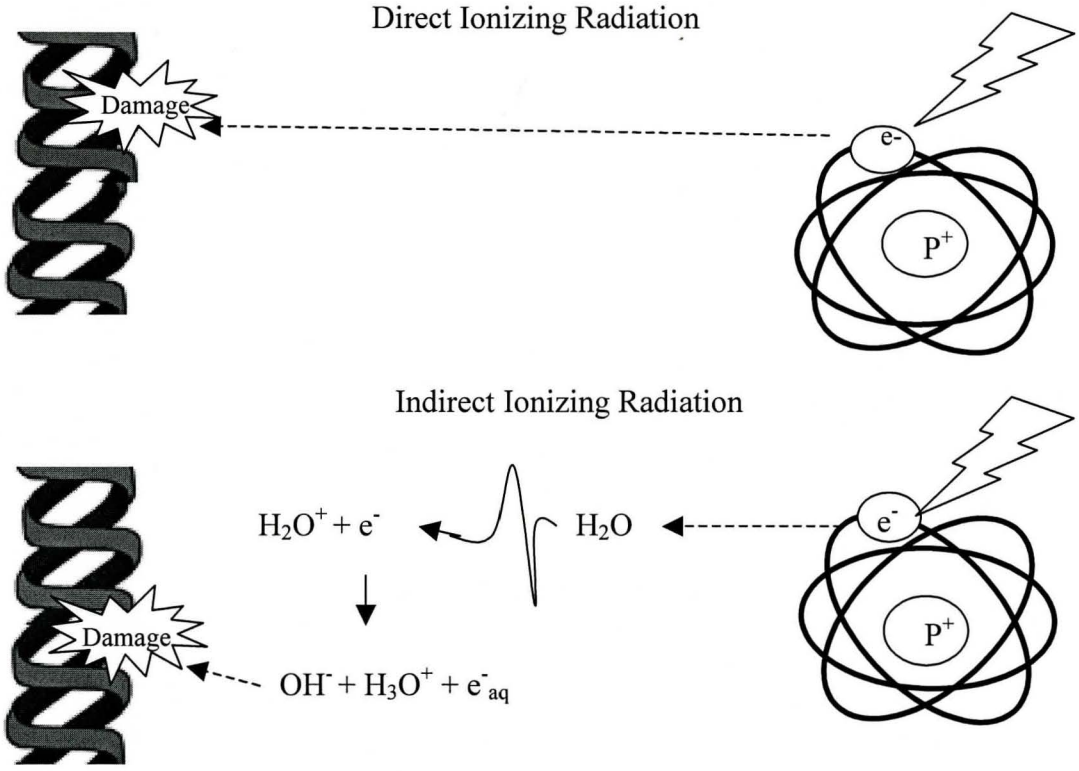


Figure 1. Direct and indirect ionizing radiation. Direct: Charged particles deposit energy into the medium between directly ionized charged particles and electrons, potentially leading to damage. Indirect: Radiation hits an electron, which interacts with water that can produce damage in the DNA of the cell. Derived from Morgan (2003).

energy to the medium through direct interactions with other targets, such as water. Direct ionizing radiation consists of charged particles that deposit energy into the medium through direct interactions between the directly ionizing charged particle and electrons of the atoms in the medium. Controlling the amount and rate of exposure to both direct and indirect forms of ionizing radiation is necessary in order to prevent potential damage (Hall, 2000).

1.2 How radiation is measured?

Radiation can be measured in various units. Radiation dose is measured using units of either sieverts (Sv) or Grays (Gy) (joule/kg). Most of the reports concentrating on humans, with respect to radiation, use Sv, but in this study, most references to radiation dose will be measured using Grays. The Sievert (Sv) is a corrected unit of absorbed dose (Gy), which is weighted, to account for the relative effectiveness of the particle or ray to cause damage. This was developed because not all sources of ionizing radiation are equally damaging at the same doses. For instance, one α-particle is at least 10 times more damaging than one γ-ray (BEIR, 2006).

1.3 Sources of Radiation:

Ionizing radiation comes from many sources, most of which are naturally occurring (Figure 2). For instance, radon and its decay products, which are the highest natural sources of radiation, are present in radon gas form (BEIR, 2006). Radon gas, which is undetectable to the human senses, emanates from the earth and releases both high and low LET (Linear Energy Transfer) radiation. The average estimated annual background radiation dose due to natural sources is 2.4mSv (BEIR, 2006). Cosmic rays,

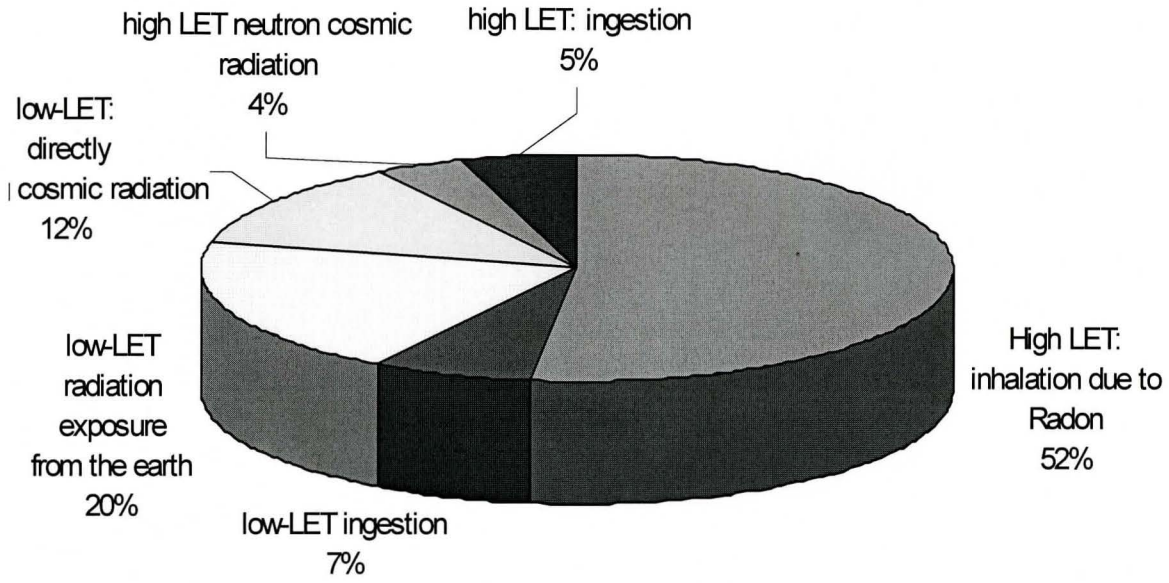


Figure 2. Sources of natural radiation on the earth. LET is linear energy transfer. (BEIR, 2006)

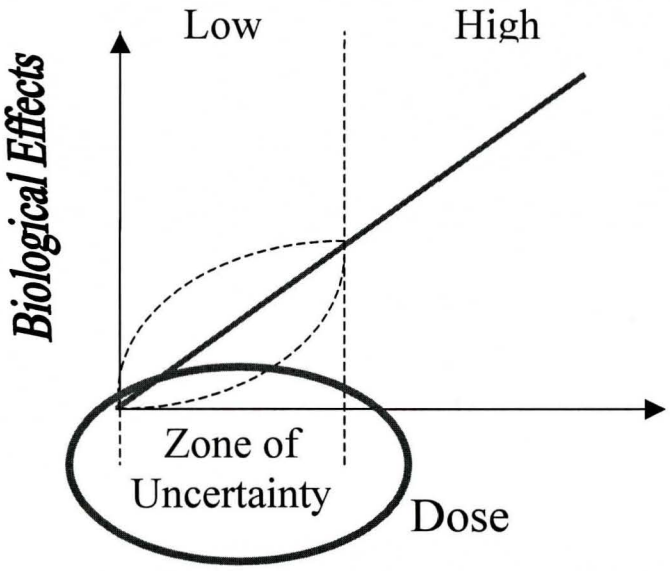


Figure 3. Depiction of linear no threshold hypothesis (LNT). Biological effects were thought to increase linearly with increasing dose, but recent studies have come to question this theory, as at low doses, there have been reports of either increased or decreased biological effects that do not follow the predicted linear threshold. Adapted from Brooks, 2005; Mothersill *et al.*, 2002.

and internal emissions are next in order of the highest sources of naturally occurring background radiation. Cosmic rays come from particles that travel through space; the sun and exploding stars are one of these sources of these particles (BEIR, 2006). Internal emissions are due to the presence of naturally occurring uranium and thorium radioisotopes found in food and water that are ingested.

Natural radiation makes up 82% of all background radiation, leaving 18% man-made background radiation (BEIR, 2006). Sources of man-made radiation include nuclear medicine, consumer products, occupational radiation, fallout, nuclear fuel cycles and medical x-rays. Of these, medical x-rays make up the largest portion of man-made background radiation. Cobalt is another source that humans use to generate γ radiation (man-made radiation) and is commonly used in radiation therapy devices. The studies conducted for this project all used cobalt-60 (^{60}Co) as the energy source of γ radiation.

1.4 Discussion of Non-targeted Effects of Radiation:

In recent years, low dose, non-targeted effects have proven to be of importance in numerous studies that have found non-linear dose response relationships that could not have been predicted by extrapolation from higher doses. Both *in vivo* and *in vitro* bystander (then known as clastogenic) effects were reported as early as the 1950s, beginning with a report on children whose spleens were irradiated for treatment of leukemia. Soon after, changes in the sternal bone marrow were found; suggesting clastogenic factors were secreted into the blood of individuals (Parsons *et al.*, 1954). In 1968, there were reports of persistent genetic damage causing factors found in the serum of patients given radiotherapy (Goh and Sumner, 1968; Hollowell and Littlefield, 1968),

which could cause sister chromatid exchanges (SCE) in cells receiving the serum. In addition, studies were conducted by Poncy *et al.* (1980), who found that rats exposed to α-particle-emitting radon, which primarily affects lung tissue, showed both acute and delayed increases in SCE in their bone marrow cells. This suggests that SCE-inducing factors may have moved from the lung to the bone marrow where damage occurred. Despite these findings, many researchers did not focus on these non-targeted effects of ionizing radiation but remained loyal to the conventional linear no-threshold (LNT) paradigm.

1.4.1 Linear No-Threshold (LNT) Hypothesis:

The LNT hypothesis is based on the assumption that all biological radiation effects are proportional to dose and dose rate, in other words, that the dose-effect relationship is linear, even at low doses (Figure 3) (ICRP, 1991). This suggests that every dose, including the smallest dose delivered is capable of producing a biological effect (UNSCEAR, 1958). This model has been followed for many years and has led to the principle of reducing human exposures to 'As Low As Reasonably Achievable' (ALARA) levels of radiation, restricting the public dose limit to 1mSv/y above the background dose. Many people believe there is insufficient evidence to support the LNT hypothesis in extrapolating the health effects of low levels of radiation (UNSCEAR, 1958; NAS/NRC, 1959; ICRP, 1966). The advisory committee on the Biological Effects of Ionizing Radiation (BEIR) provided risk estimates for cancer at low doses based on a linear extrapolation from cancer mortality data at high doses in Japanese bomb survivors (NAS/NRC, 1972). These estimates use the LNT hypothesis to

determine low dose risk. More recently, evidence has been found to disprove the LNT hypothesis. Responses elicited in cells that are hit by radiation, such as cell killing, mutations, chromosomal aberrations and changes in gene expression may not be linearly dependent on dose. Such occurrences were seen in a study conducted by Yin *et al.* (2003). Low doses of radiation have been found to elicit repair signals *in vivo* in the brains of mice. These signals that included changes in the expression of genes involved in stress response, cell cycle control and DNA synthesis and repair were produced by low doses (0.1Gy) and were mechanistically and significantly different than those seen at 2Gy (Yin *et al.*, 2003). Radiation at high doses elicits responses such as cell killing, cell proliferation, and alteration of the cell phenotype from disruption of the extracellular matrix. This suggests that there are different mechanisms involved after low doses compared to high doses, making predictions of effects through extrapolation from high dose effects, unreliable.

In addition to the findings that the LNT hypothesis is challenged by numerous studies, the risk assessments derived for acceptable levels of radiation exposure, that were calculated based on the LNT model were developed only for humans (ICRP, 1977; 1991; Hinton, 2004). In 1991, the International Commission on Radiological Protection (ICRP) stated that the radiation dose limits set forth to protect humans would ensure the protection of all other species. It was not until recently that radiation risk assessments were incorporating consideration of non-human biota (ICRP, 2003). The ICRP has since established that with an increase in environmental hazards as well as an increase in interest in the safety of animals, there needs to be radiation dose limits put in place to

protect biota and ecosystems (ICRP, 2003). There are four reasons used to justify this: 1) The LNT has not been applied pristine habitats or marine habitats, where no humans are present. 2) It does not consider ecosystems, only individuals. 3) Rare or endangered species are also not considered. 4) This LNT hypothesis does not consider multiple stressors on populations, only radiation in isolation.

As more studies emerge, it is evident that the dose-response linear relationship, as enshrined by the LNT hypothesis, does not hold true due to such factors as induction of an adaptive response, hyper-radiosensitivity, hormesis, bystander effects and induction of genomic instability in the low dose region.

1.4.2 Adaptive Response:

The adaptive response can be defined as an effect in cells that are primed with a small dose of radiation, which are then resistant to a subsequent large challenge dose (Olivieri *et al.*, 1984). Such responses have been seen *in vivo* and *in vitro* and have been observed using various end points such as chromosomal aberrations, mutation induction, radiosensitivity and DNA repair (Cai and Liu, 1990; Yonezawa *et al.*, 1996; Wolff, 1998; Cai, 1999; Gajendiran *et al.*, 2001; Cramers *et al.*, 2005). Adaptive response experiments show a large amount of variability, both inter- and intra-individual, across a number of species. Studies have found adaptive responses compared to the controls as low as 6% increased survival (Zhang, 1995) and as high as 59% (Shadley and Wiencke, 1989). This variability may be due to such factors as dose rate (Shadley and Wiencke, 1989), genetic variation among individuals (Vijayalaxmi et al., 1995), experimental conditions (Bosi and Olivieri, 1989) and time between doses (Sasaki, 1995). Synergistic

responses, or an increase in damage following a challenging dose when cells are irradiated have been found in lymphocytes (Hain *et al.*, 1992).

Several studies have found adaptive responses in animals. In a study conducted by Yonezawa *et al.* (1996), it was found that mice exposed to 0.05Gy of X-rays two months before a second, higher dose resulted in significant increases in survival that was not sex-dependent. Ulsh *et al.* (2004) studied fibroblasts from three species of ungulates that were irradiated at doses ranging from 1-100mGy, followed by a 4Gy-challenging dose. Adaptive responses were seen as measured by micronucleus formation at all priming doses (Ulsh *et al.*, 2004). The LNT model does not take into account any of these studies. In addition to adaptive responses, non-targeted effects of radiation include low dose hyper-radiosensitivity.

1.4.3 Low dose hyper-radiosensitivity:

It is accepted that mammalian cells can induce some repair and control of DNA injury, but the idea that cells may increase the activation of repair processes after ionizing radiation was not universally acknowledged (Joiner, 1994). Low dose hyper-radiosensitivity is seen when cells die from low single doses (<0.5Gy) of ionizing radiation that cannot be predicted from data observed for the response of the cell line at higher doses. A decrease in cell survival or increased radiosensitivity is succeeded by the occurrence of relative resistance (Increased RadioResistance, IRR) to cell killing at doses greater than 0.5Gy (Figure 4) (Joiner *et al.*, 2001). This suggests that the processes involved in the induction of repair systems at low doses differ from processes involved at higher doses. Evidence of this effect was seen in 1963, when Eriksson measured

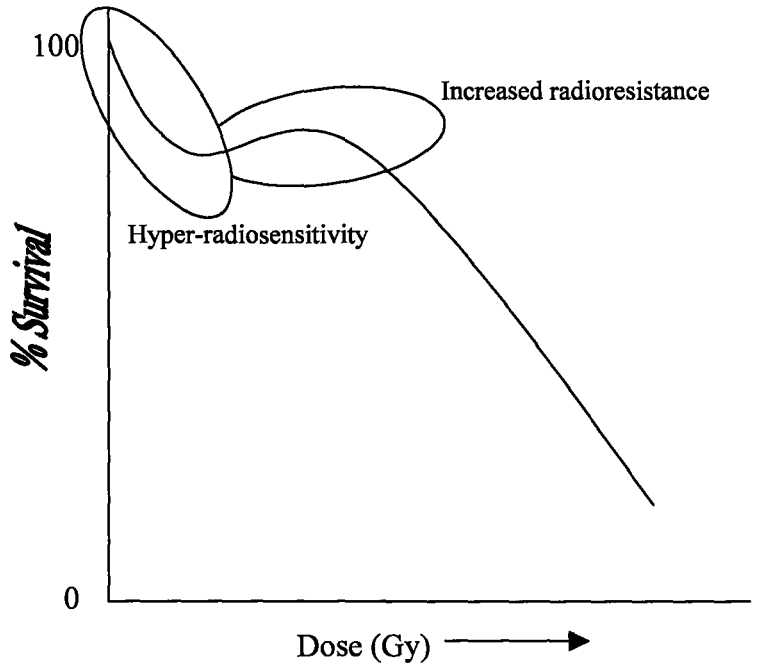


Figure 4. Dose-response for cell killing using ionizing radiation. Low dose hyper-radiosensitivity is recorded at low dose followed by increased radioresistance (IRR) at higher doses. Derived from Prise *et al.*, 2005.

mutation induction and survival in pollen grains after acute low-dose γ-ray exposures to ionizing radiation. Earlier still, budding yeast was found to display a similar low-dose hyper-radiosensitive pattern (Beam *et al.*, 1954). More recently, this phenomenon has been characterized in human cell lines using different radiation qualities and biological end points and has been found to have a common link of being present at low doses of ionizing radiation, independent of the form of radiation employed (Marples, 2004). It is hypothesized that hyper-radiosensitivity is the automatic survival response in cells to injury due to radiation at very low doses (Joiner et al., 2001). Thus, IRR was considered to be the result of an enhanced survival outcome after the activation of a damage response mechanism. Studies performed by Marples and Joiner (1995) found that the hypersensitivity was abolished in V79 Chinese hamster cells that were pre-treated with doses of either 20cGy or 100cGy. These priming doses eradicated any hyper-radiosensitivity in subsequent exposed cells, but a 5cGy-priming dose had no effect (Marples and Joiner, 1995). This suggests that low-dose hyper-radiosensitivity can be overcome if cells are 'primed' or 'adapted' to repair DNA damage by pre-treatment with small but significant doses of ionizing radiation. This phenomenon is also thought to be cell cycle-dependent. Studies have found that irradiation of cells in the G2 phase of the cell cycle resulted in significantly greater low-dose hyper-radiosensitivity/IRR (Marples *et al.*, 2003; Short *et al.*, 2003). A checkpoint exists in the cell cycle between G2 and M (mitosis) phases in order to prevent damaged cells from prematurely entering mitosis. Activation of this checkpoint seems to be a threshold as it is dose independent (1-10Gy) and the system is activated after any dose irrespective of the level of damage. At

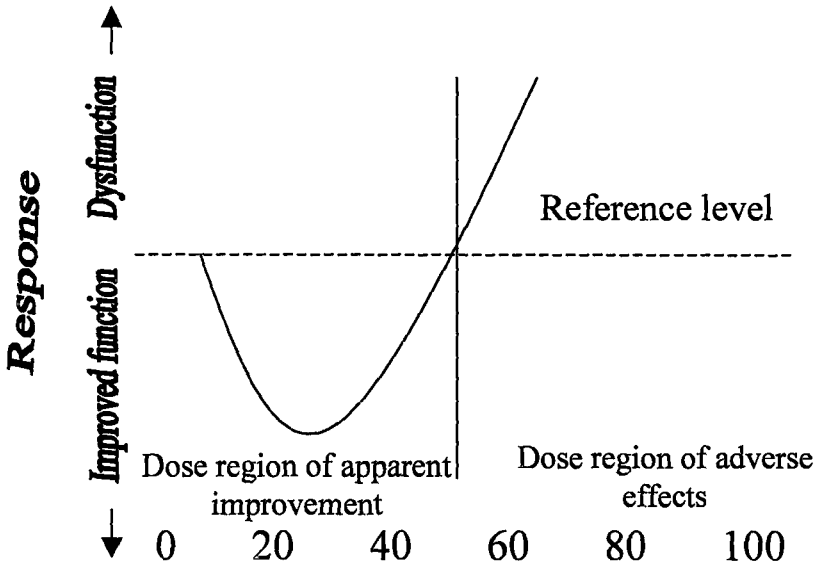
extremely low doses, this checkpoint is not activated, confirming the presence of a threshold. It was found that a threshold dose of ~40cGy was necessary in order to activate this checkpoint repair system (Marples *et al.*, 2003). This suggests, as has been shown in previous studies, that at doses below 40cGy, a hypersensitive response is seen without the induction of repair processes (Marples *et al.*, 2003). These findings add to the increasing evidence that suggests the LNT hypothesis is not correct at low doses. In addition to these findings of non-targeted effects of radiation, another effect is found at low doses, where cells actually show an increase in survival to these low doses of radiation. This is termed hormesis.

1.4.4 Hormesis:

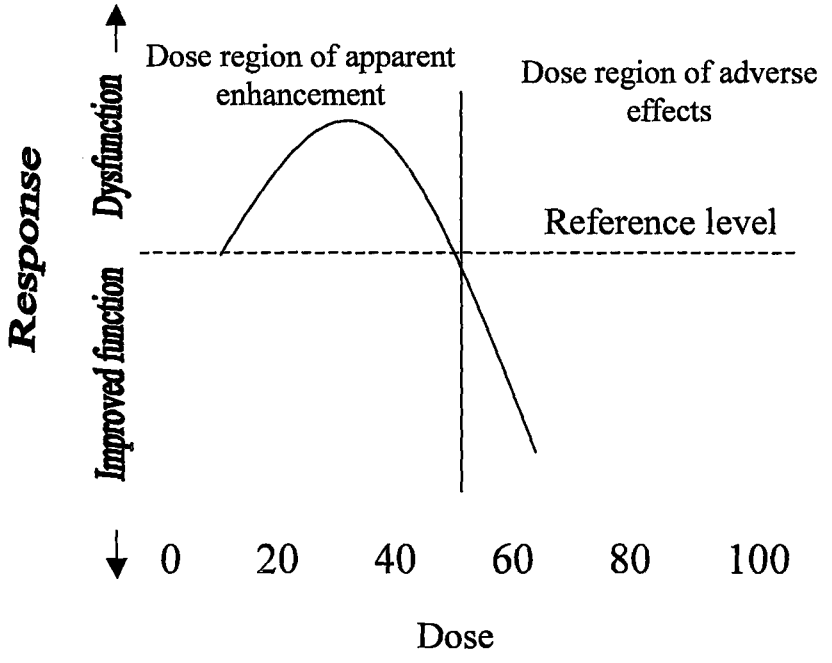
Hormesis is defined as stimulatory effects of low doses of substances that are toxic at high doses (Southam and Erhlich, 1943). These can include arsenic, ethanol, antibiotics and heavy metals as well as radiation (Luckey, 1982; Calabrese *et al.*, 1987). In small doses, exposure may actually have beneficial effects on an organism whereas larger doses are harmful. However, hormesis may have adverse effects when considering such instances as increased growth in cancer cells.

Acute low doses and low dose rates of radiation have been shown to produce this radiation hormesis effect (Calabrese and Howe, 1976; Luckey, 1980). All life on earth is constantly exposed to background radiation and therefore some form of adaptation to these background doses must have occurred for the ongoing survival of biota (Calabrese and Baldwin, 1999; Parsons, 2003). Such inducible adaptive response mechanisms include stress protein responses, DNA repair mechanisms and cell and tissue repair

mechanisms (Hart and Frome, 1996). A combination of the induction of these adaptive mechanisms and exposure to external stressors determines the shape of the dose-response curve for hormesis (Calabrese and Baldwin, 1999). Adaptive responses may be affected by the amount of dose of the stressor. For example, high doses of stressors may make repair mechanisms, at least temporarily, non-functional (Calabrese and Baldwin, 1999). Most biological systems operate within an optimal zone of responsiveness, usually at low doses that is maintained by a series of repair mechanisms. Calabrese and Baldwin (1999) suggest a different concept that neither reflects the linear nor the non-linear threshold hypotheses, but rather suggests that the hypothesis for low-level stressors should reflect the capacity for systems to adapt to the effects of physical and chemical stressors over a broad range of doses. Thus, the shape of the dose-response curve is either an inverted U-shape or U-shaped, depending on the biological end-point measured (Figure 5) (Southam and Erhlich, 1943; Luckey, 1980; 1991; Stebbing, 1981; Calabrese and Baldwin, 1999). For example, for end points such as longevity, growth and fecundity, the dose-response results in a stimulatory response at low doses compared to controls (inverted U-shape). Whereas end points such as mutations, cancer or disease incidence compared to controls results in a diminished effect; a U-shaped dose-response curve is the result. At low doses of stress, organisms are thought to overcompensate repair activities to ensure full recovery of homeostasis (Calabrese and Baldwin, 1999). During this overcompensation phase, the organism would eliminate the low-level stress-induced damage, however, as the dose increased, it would reach a level at which the capacity to overcompensate is overwhelmed and toxicity would result. Evidence of hormesis has been provided in



(a)



(b)

Figure 5. U-shaped dose-response curves. (a) U-shaped dose-response curve showing response relative to a control or reference value. This figure includes a region of improvement or reduction in dysfunction and a region of adverse effects with increasing dose. (b) Inverted U-shaped curve showing a region of enhancement or an increase above the normal level of function. This figure includes a region of adverse effects with increasing dose. Derived from Calabrese and Baldwin, 1999.

numerous studies dating back as far as 1943, where the term hormesis was first described (Southam and Erhlich, 1943; Luckey, 1980; 1991; Stebbing, 1981; Calabrese and Baldwin, 1999). Hormesis is dependent on a number of factors including different experimental conditions, species and stage of individual development, response measured, and stressor type (such as radiation), dose and dose rate (Luckey, 1982). In addition to hormesis, bystander effects have been found to occur at low doses that cannot be accommodated within the LNT hypothesis.

1.4.5 Bystander Effects:

Bystander effects are effects in cells that were not irradiated but have received signals from directly irradiated cells. These unirradiated cells can act as if they have been irradiated: by dying or showing signs of genetic instability. First documented reports of what are now considered to be the bystander effect were as early as 1921 (Murphy *et al.*, 1921) but have since received renewed attention due to a greater interest in the biological effects and mechanisms of low doses of radiation on the environment (Zhou *et al.*, 2002; Curtis *et al.*, 2004; Avila *et al.*, 2004) along with new technological developments that allow for greater detection of genome and epigenome changes, such as the microbeam and microarray technology (Amundson *et al.*, 2001; Stern and Zon, 2003). The renewed interest in the bystander effect began with a paper by Nagasawa and Little (1992), which reported that when low doses of α-particles were deposited into a cell culture, such that all cells were not hit, responses occurred in a statistically greater number of cells than could have been hit. The endpoint used to measure this bystander effect was sister chromatid exchanges (SCE). It was found that even though less than 1%

of the Chinese hamster ovary cells received an α-particle deposition, over 30% of the cells contained 61-80% SCE compared with approximately 5% of the controls containing 61-80% SCE (Nagasawa and Little, 1992). Deshpande *et al.* (1996) found a similar result using α-particle irradiation in normal human lung fibroblasts. Shortly after, Mothersill and Seymour (1997) found that filtered medium harvested from γ-irradiated cells could cause cell death in cells that had never been previously exposed to radiation. In addition to cell death, several studies have found that carcinogenesis and cell mutation can also occur in these unirradiated cell populations (Zhou *et al.*, 2000; Sawant *et al.*, 2001; Nagasawa and Little, 2002). Figure 6 illustrates the possible effects that occur in a cell during an ionizing radiation event (Mothersill *et al.*, 2002; Morgan and Sowa, 2005).

Bystander effects are now known to occur via two mechanisms: Cells can communicate either through secreted soluble factors, such as ROS (Narayanan *et al.*, 1997), nitric oxide (NO) (Matsumoto *et al.*, 2001; Shao *et al.*, 2002; Sonveaux *et al.*, 2002) and long-lived cell line dependent transforming growth factor β1 (TGF-β1) (Iyer and Lehnert, 2000), that can transmit signals to unexposed cells (Mothersill and Seymour, 1997; Prise *et al.*, 2002; Morgan and Sowa, 2005; Schettino *et al.*, 2005) or through gap junction intercellular communication (GJIC) (Nagasawa and Little, 1992; Belyakov *et al.*, 2001; Shao *et al.*, 2003; Ponnaiya *et al.*, 2004b). Gap junctions were found to be important in molecular events leading to the modulation of p53 and p21 gene expression in non-irradiated bystander cells in a human fibroblast cell line that was exposed to low doses of radiation. These changes were significantly decreased when GJIC was inhibited by lindane, a membrane toxin (Azzam *et al.*, 1998; 2000; 2001).

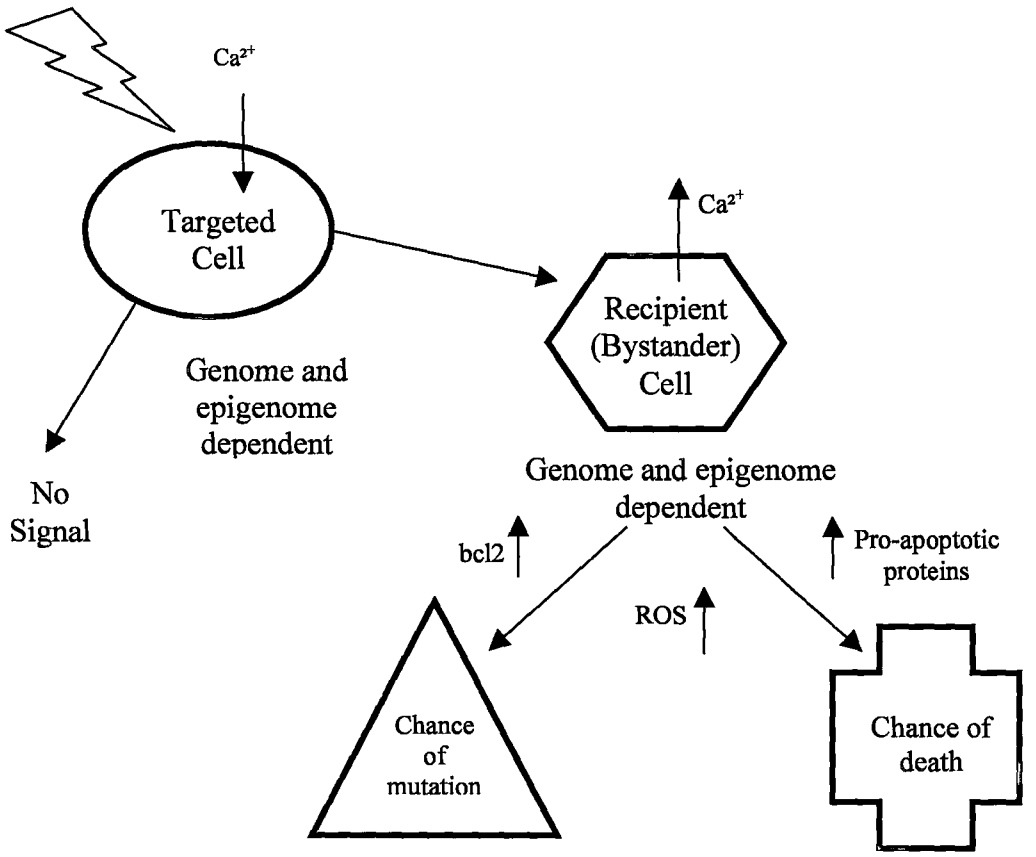


Figure 6. Possible model for expression of the bystander effect. Bystander effects are biological responses in cells that have received signals from irradiated cells but have not been irradiated themselves. The targeted or irradiated cell either creates no signal or a signal is created, along with an influx of calcium. If produced, secreted factors either result in an increased chance of mutation (increase in bcl2 proteins) or an increased chance of death (increased pro-apoptotic proteins), in both cases, there are reports of increased reactive oxygen species (ROS) and an increase in calcium. Both targeted cell and recipient or bystander cell are genome dependent. Gap junction intercellular communication (GJIC) can also create the same result, however, this occurs only when the target cell and the recipient cell are in contact (Not shown). Mothersill *et al.*, 2002.

The signal itself that produces the bystander effect is yet to be defined, however it is known that it causes sister chromatid exchanges, chromosomal aberrations, changes in protein and gene expression, mutations, growth delay, micronucleus induction, initiation of apoptosis and genomic instability (Parsons *et al.*, 1954; Seymour and Mothersill, 2000; Azzam *et al.*, 2002; Little *et al.*, 2003; Maguire *et al.*, 2005; Mothersill *et al.*, 2002; 2006). The bystander effect also appears to dominate the low dose radiation response, whereas the effect seems to saturate at higher doses (Maguire *et al.*, 2005; Schettino *et al.*, 2005; Mothersill *et al.*, 2006).

Other non-targeted effects of radiation, besides the bystander effect have been well documented. These include delayed cell death (DCD) and/or lethal mutations, which can be seen through a decrease in survival compared to controls in the progeny of cells that have developed from directly irradiated cells. These effects are a manifestation of genomic instability (Mothersill and Seymour, 1997; Lyng *et al.*, 2002).

1.4.6 Genomic Instability:

Lethal mutations or delayed expression of cell death in surviving cell colonies post irradiation are one form of expression of an effect that has led to the development of the field of genomic instability (Seymour *et al.*, 1986; Alper *et al.*, 1988). Another form of expression of this instability is the delayed expression of non-clonal chromosome or chromatid damage in progeny, which was not present in the original unirradiated progeny (Kadhim *et al.*, 1992; Sabatier *et al.*, 1992; Marder and Morgan, 1993; Durante *et al.*, 1996). Previously, it was accepted that cells repaired their damage or died within a brief time following irradiation, making the survivors no different from the unirradiated

control population (Elkind and Whitmore, 1967). However, a study as early as 1956, found that some lethally irradiated cells were able to divide normally for several cycles before cell death occurred (Puck and Marcus, 1956). Instability in surviving progenitor cells, which appear normal and can divide but show delayed expression of damage, have several defining factors including: division failure in clonal progeny, chromosomal instability, which can result in non-clonal chromosomal mutations in clonal progeny, microsatellite instability and gene amplification (Tlsty *et al*, 1989; Branch *et al.*, 1993). Even though these phenomena are grouped under genomic instability, there is a specific difference between the clonal and non-clonal progeny. For the clonal progeny, a downstream 'mutator phenotype' event is preceeded by a clonal event that is induced by the radiation at the time of irradiation. Microsatellite instability and gene amplification may be radiation induced gene mutations that can lead to the production of unstable clones. These unstable clones can either proliferate successfully due to the possible development of a selective advantage, or clones may develop a disadvantage, leading to death. For example, mismatch repair defects can lead to flawed protein production and selection for cells, which conceal surface antigens and thus elude immune systems (Karran and Hampson, 1996). Also, mutations can lead to deregulation of p53 surveillance systems, which results in the evasion of apoptosis and formation of a mutator phenotype (Tlsty *et al.*, 1989; Loeb, 1991; Reznikoff *et al.*, 1994). The other forms of genomic instability refer to non-clonal events. These non-clonal events have been known to occur frequently following low dose exposure, eliminating the possibility that they are the result of specific radiation-induced direct mutations (Kadhim *et al.*,

1992; 1995; Seymour and Mothersill, 1992; Marder and Morgan, 1993; O'Reilly *et al.*, 1994a). This increases the probability that damage will manifest during the regular cellular divisions of the surviving cells (Mothersill *et al.*, 1998). This delayed death in distant progeny is relatively independent of dose and is not increased or decreased by cell generation time post irradiation for many population doublings (O'Reilly *et al.*, 1994a).

In the field of genomic instability, irradiated cells are known to have growth curves that deviate from the controls in terms of slope (Fox, 1985). This variation was found to be due to late death or division failure in the progeny of survivors in different cell lines and a primary cell culture (Mulgrew, 1988; Seymour and Mothersill, 1989; 1992; 0'Reilly *et al.*, 1994a) and not due to increased cell cycle time as was formerly thought (Nias *et al.*, 1965; Grote *et al.*, 1981). Both clonal and non-clonal instability can be grouped as epigenetic responses that allow damaged cells to survive initially and assists in the eventual production of an unstable phenotype (Clutton *et al.*, 1996; Mothersill and Seymour, 1997).

1.4.7 Genomic Instability and Bystander Effects:

Genomic instability may be related to bystander effects (Seymour and Mothersill, 1997; Prise *et al.*, 2002; Dowling *et al.*, 2005). Although it is known that both genomic instability and bystander effects mostly occur at low doses or dose rates and in many cases, the effects saturate with increasing dose, the mechanisms involved in these non-targeted effects are not fully understood (Prise *et al.*, 2002). If these effects are significant *in vitro* and occur *in vivo*, they may have significant consequences for estimating risk at low doses.

Several studies have shown that genomic instability can occur through a bystander mediated method (Lorimore *et al.*, 1998; Watson *et al.*, 2000; Lyng *et al.*, 2002; Morgan, 2003; Dowling *et al.*, 2005). An example of this occurring *in vivo* was seen in an accidental human exposure to acute high-dose total body neutron-gamma radiation (Chiba *et al.*, 2002). This accident took place in Tokaimura, Japan in 1999 where a 35-year old man was exposed to 5.4Gy of neutron- and 8.5-13Gy of γ- whole body radiation. The victim received a blood stem cell transplant from his twin sister. Days after the transplant, donor bone marrow cells displayed random chromatid breaks or genomic instability throughout, suggesting the presence of a bystander effect (Chiba *et al.*, 2002). *In vivo* work done by Watson *et al.* (2000) used irradiated mice and performed bone marrow transplantations with a mixture of irradiated and unirradiated cells, which could be identified because one set were male and the other female. Chromosomal instability was found in the progeny of cells of the non-irradiated bone-marrow cells. This is evidence of a bystander effect that resulted in genomic instability, suggesting that the phenomena are linked. Also, using cell lines, Lorimore *et al.* (1998) showed that chromosomal instability in unirradiated hemopoietic stem cells was still present in the progeny of these cells due to transfer of signals in irradiated to unirradiated cells. Lorimore *et al.* (1998) used a grid to shield some cells and lethally irradiated the unshielded cells. They found chromosome aberrations in the shielded cells and their progeny. Rapid calcium fluxes, loss of mitochondrial membrane potential and an increase in ROS have also been found in the recipient progenitor cells as well as delayed cell death and lethal mutations (Seymour and Mothersill, 1999; Lyng *et al.*, 2002). Thus,

apoptosis was triggered by a series of events initiated by ionizing radiation that resulted in signal(s) created that is seen in the progeny of irradiated cells for several generations. Direct damage due to radiation is thought to occur in the nucleus, but evidence exists to suggest extranuclear and extracellular targets may play key roles in the events that occur after radiation insult, which would be a key factor in linking delayed cell death and bystander effects (Alper, 1979; Lyng *et al.*, 2002).

In addition to the similar endpoints of radiation damage linking these two phemonema, there has been speculation that a similar mechanism is involved in both these phenomena. Studies show that, following irradiation, ROS is elevated. This can stimulate cytokine production, which in turn produces more free radicals, resulting in an unstable phenotype (Clutton *et al.*, 1996). It has also been found that intercellular signalling, production of cytokines and free radicals are all connected with inflammatory responses and have the ability to cause bystander-mediated and latent damage found in progeny, thus possibly linking these two phenomena (Lorimore and Wright, 2003; Morgan, 2003). Post-irradiation, the bystander effect seems to remain in the progeny of cells that have survived ionizing radiation. These delayed effects have been shown in a number of study organisms including both mammalian and fish cell lines using ionizing radiation and/or toxicants (Mothersill *et al.*, 1998; Seymour and Mothersill, 1999; Kilemade and Mothersill, 2003; Dowling *et al.*, 2005). However, more studies are needed to help understand the relationship between bystander effects and genomic instability in terms of the mechanisms involved.

1.4.8 Summary of Non-targeted Effects:

It is clear through numerous studies that non-targeted effects occurring after low dose radiation undermine the linear relationship between dose and effect. Mechanisms resulting in these effects need further study, however, it is suspected that bystander effects and genomic instability are connected and that bystander effects may actually be driving genomic instability through their ability to cause ROS (Clutton *et al.*, 1996; Lyng *et al.*, 2002). As these non-targeted phenomena are not linear with dose, and show the response(s) arise at low doses, non-targeted insults have the potential to impact radiation protection analyses, making the understanding of these phenomena critical.

1.5 Non-targeted Effects of Radiation on Non-Human Biota:

There are hundreds of papers studying bystander effects in vitro in mammalian cells and some concerning *in vivo* effects in mice, however, there are only a few which used lower vertebrates or invertebrates (Reviews: Little and Morgan, 2003; Lorimore *et al.*, 2003; Morgan, 2003; Mothersill and Seymour, 2001; 2004a). In addition, in the past most non-human studies in the radiobiology field used extremely high doses that are environmentally irrelevant (Altland *et al.*, 1951; Rackham and Woodhead, 1984; Chilmonczyk and Oui, 1988). Chilmonczyk and Oui (1988) exposed rainbow trout to doses of gamma-irradiation between 10-50Gy. Rackham and Woodhead (1984) exposed *Ameca splendens*, a fish that is now considered extinct in the wild, to chronic exposures of high levels of gamma-irradiation for as long as 244 days at a time.

While there are several ecological studies using fish, especially Medaka (*Oryzias latipes*), which have been used as models for human responses (Shima and Shimada,

1991; Knowles 1992; Karrow *et al.*, 1999; Loosli *et al.*, 2000; Mothersill *et al.*, 2006), little radiobiology assessing low dose radiation survival or bystander effects using fish or fish cell lines has been done (Lyng *et al.*, 2004). Fish are one of the most important aquatic animals both economically and ecologically. Thus, it is of interest to find a minimal acceptance level of radiation emissions (Olwell *et al.*, 2005). The constant release of radioactive materials into the environment, not only affects humans but also all flora and fauna in the ecosystems (ICRP, 2003). Recent concerns about environmental radiation effects on biota make this area of study of significant importance. What remains to be answered is if these ionizing radiation effects have any long-term impact on biota and whether regulators should consider them. It is important to consider whether environmental radiation effects take place due to non-targeted mechanisms and whether transgenerational effects occur.

Increasing evidence is available to suggest that radiosensitivity is species specific and that significant individual variation occurs (Woodhead, 1970; Mothersill *et al.*, 2001b; Hinton *et al.*, 2004; Lyng *et al.*, 2004). An example of species-specific sensitivity is represented in a study conducted by Mothersill *et al.* (2001b). It was found that explant tissue from hematopoietic tissue of the Dublin Bay prawn, *Nephrops norvegicus* displayed a bystander effect after very low doses of radiation, suggesting that these crustaceans are unusually sensitive to radiation, whereas terrestrial arthropods and some other invertebrates are considered to be radioresistant. As well, in a study conducted by O'Reilly and Mothersill (1997), it was found that the carp cell line, *Epithelioma papulosum cyprinid* (EPC), was more radiosensitive to UV than mammalian

cell lines used (O'Reilly and Mothersill, 1997), suggesting the need for a reliable system to monitor the effects of radiation in non-mammalian systems across different species. A recent study conducted by Mothersill *et al.* (2006) has found bystander effects in rainbow trout (*Oncorhynchus mykiss*) exposed to low levels of ionizing radiation *in vivo*. It was also found that the irradiated fish seemed to secrete signals into the water post irradiation that had an affect both on non-irradiated fish in the same tank as the irradiated fish and on the non-irradiated fish placed in the water previously occupied by the irradiated fish. This shows that signals that are induced in an irradiated fish can communicate information to other fish, leading to responses (Mothersill *et al.*, 2006). This study demonstrates that the environmental relevance of these non-targeted effects is real and the need to find a dependable system in order to monitor these non-targeted effects is pressing.

Bystander experiments using the Chinook Salmon Embryo (CHSE-214) and *Epithelioma papulosum cyprinid* (EPC) cell lines found that CHSE-214 but not EPC displayed a bystander effect at a dose of 0.5Gy (Dowling *et al.*, 2005). Dowling *et al.* (2005) also found that CHSE-214 displayed a lethal mutation using γ-irradiation, but the EPC cell line tested did not. A study conducted by O'Reilly and Mothersill (1997) found similar results using UV A and UV B irradiation with the same EPC cell line, which showed no lethal mutations. These studies suggest that different species do not all react to non-targeted effects of radiation similarly. A biomarker or reporter system that is able to determine these differences across different species would be extremely valuable.

Several attempts have been made to determine effects of radiation by using generic animals or sentinel species that act as, physiological or population indicators. They are held to be predictive of the responses of other species to the same or similar stressors at the same level of intensity (McCarty *et al.*, 2002). Those species defined as "sentinels" act as the test groups for examination of the effects of radiation. Data from experiments can then be used to predict responses in other species of similar function, size and behaviour (McCarty *et al.*, 2002). While better than nothing, this method is unreliable and is subject to inaccurate results due to the vast amounts of extrapolation across different species and life stages (McCarty *et al.*, 2002). There is not enough evidence in the literature to suggest that there are functional connections between species that would allow prediction of the responses of another species (McCarty *et al.*, 2002). Another way of determining radiation effects in animals is the use of biomarker or reporter systems. Biomarkers are response indicators. They are used as an indicator of change that can be induced by stressors, such as radiation, that elicit several changes at the molecular, cellular and physical level in exposed organisms and are an ideal method of employing this biomonitoring system (Durante, 2005). Biomarkers or reporter systems are well known, mostly for their role in toxicology in humans and non-human biota, and have only recently been defined in the radiation risk assessment group for animals, as there is extensive research that has already been conducted on humans for radiation risk (IAEA, 2002; ICRP, 2003; BEIR, 2006). A reporter system used as an end point would reveal significant damage in the species in question, providing a useful indicator of biological damage (Ulsh *et al.*, 2003). By determining a possible reporter

system in fish (beginning with cell lines), a more accurate and less invasive method may be utilized to help determine the amount of damage non-targeted effects of radiation can inflict in a non-mammalian species.

1.6 Objectives:

Indicators of radiation damage have traditionally been DNA or chromosomal damage, as these end points indicate DNA damage to an individual. This may not be useful after low doses as chromosomal damage may not be severe enough to have an impact on the visible health of an organism or a population of organisms but by inducing genomic instability, could predispose populations to above normal risk (Hinton *et al.*, 2004). Previous work by Mothersill *et al.* (2001a) used a mammalian cell line, HPV-G, derived from the human papillomavirus, as a standard 'reporter cell' enabling bystander effects to be compared across individuals and cell lines. As a step towards identifying a suitable reporter cell line for fish species and tissues in the environment, eight fish cell lines were randomly chosen to study based on their availability. Limited or no information about their radiation response was available.

Clonogenic survival was employed as the end point for all tests except the growth curves, in which cell number was monitored over an entire growth phase. Puck and Marcus (1956) first developed the clonogenic assay for mammalian systems, as there was no previous accurate method of measuring cell reproductive potential in non-bacterial cells. Cells that are able to grow post irradiation and continue to retain this characteristic and form colonies are labelled as survivors (Puck and Marcus, 1956; Elkind and Sutton, 1959). These survivors (the surviving fraction of cells after ionizing radiation) can be

defined by the ratio of plating efficiency (P.E.) for irradiated and unirradiated cell populations. Cells normally form visible colonies with a defined P.E. characteristic of that cell line. Any significant change in this P.E. after radiation insult would almost certainly be due to the stressing agent (Puck and Marcus, 1956). To standardize colony-forming ability, a surviving colony was defined as having 50 or more cells, i.e. having undergone 5-6 cell population doublings after cells were plated. Not all cell lines investigated in the current study were capable of forming colonies; these were labelled as non-clonal cell lines. Bystander experiments were performed on these, however, only the reporter mammalian cell line was used as the colony producing cell line.

Growth curves were determined for all fish cell lines to determine the doubling times. This was done because any changes in doubling time after radiation exposure are indicative of possible alterations in the viability of the cells.

Preliminary studies were completed, investigating the non-targeted effects of low ionizing radiation on fish cell lines, in order to determine if a bystander effect was present. From these results, lethal mutation experiments were conducted on the fish cell line showing the greatest reduction in survival, the rainbow trout gonad (RTG-2) cell line.

The six main objectives for this project are listed below. The details concerning these objectives will be explained in the methods section of this thesis:

(1) (a) Develop growth curves for all eight fish cell lines and the mammalian reporter cell line, HPV-G.

(b) Determine doubling times of all cell lines.

(2) Perform clonogenic assays on all fish cell lines to determine clonogenic ability. Determine plating efficiencies of all cell lines.

(3) Generate radiation survival curves for all cell lines that were found to have the ability to clone (from objective 2).

(4) Complete bystander experiments using both direct, donors and autologous recipients for the fish and mammalian clonogenic cell lines, using a clonogenic assay as the endpoint.

(5) Complete bystander experiments for non-clonogenic fish cell lines using these as the donor cells and the HPV-G cell line as the recipient cell line using a clonogenic assay as the endpoint.

(6) Use the fish cell line(s) that display the greatest reduction in survival, if any, to test for genomic instability using lethal mutation and cell kinetics experiments.

CHAPTER 2

BYSTANDER EFFECTS

2.1 MATERIALS AND METHODS

2.1 Materials and Methods:

2.1.1 Cell Lines

Eight different cell lines were investigated in this study. All cell lines studied were adherent in nature and included: CHSE-214, ZEB 2J, RT-Gill W1, RTG-2, EPC, PBLE, RTS-pBk+ and RTS-34st. All fish cell lines were a kind gift from Dr. Niels Bols at the University of Waterloo. Each of these cell lines will be described in detail in the following text.

CHSE-214: Chinook salmon embryo

This cell line is a teleost, salmonid cell line and has been used in the past as a model for an environmental study (Kilemade and Mothersill, 2003). This cell line has a natural cobblestone pattern of growth in culture, and has an epithelial-like morphology. It is derived from embryos of the Chinook salmon, *Oncorhynchus tshawytscha.*

EPC: Common carp (*Cyprinus carpio) Epithelioma papulosum cyprinid*

This cell line has an epithelial-like morphology and was isolated from a herpes virus-induced hyperblastic lesion on the common carp, *Cyprinus carpio* (O'Dowd *et al.*, 2006). It is often used for isolation and diagnosis of fish virus testing. Past studies have shown this cell line to be clonogenic, however, our laboratory did not find this to be the case.

ZEB 2J: Zebrafish (*Danio rerio*) mid-blastula-stage embryos

There are numerous zebrafish cell lines available due to the zebrafish itself being a well-established model for studies of vertebrate development (Fan *et al.*, 2004). This

cell line grows best at 26°C, but was grown in this study at 19°C and has an epithelial-like morphology (Collodi *et al.*, 1992).

PBLE: Peripheral blood leukocyte preparation of the American eel, *Anguilla rostrata.*

This relatively recent cell line was developed from the adherent cells of the peripheral blood leukocytes from the American eel, *A. rostrata.* At least three other cell lines have been derived from the genus Anguilla, but this is the first from this species (DeWitte-Orr, 2006). This cell line is fibroblastic in nature and can withstand temperatures from 5 to 36°C and can proliferate at temperatures from 19° to 30°C.

Rainbow trout (*Oncorhynchus mykiss):*

Of the eight fish cell lines, four are derived from rainbow trout (*Oncorhynchus mykiss*). Development of cell lines from rainbow trout has been accomplished since 1962 (Wolf and Quimby, 1962). Many cell lines deriving from rainbow trout have been successfully produced since this time (Ossum *et al.*, 2004). This is partially because these cells are easily able to undergo spontaneous immortalisation and develop into continuous cell lines (Ossum *et al.*, 2004), in comparison with human cell lines, which have proven difficult to develop from normal human tissues. This may be due to the high levels of telomerase activity in normal fish organs, including skin and muscle as opposed to humans, where the level of telomerase activity is mainly restricted to germ cells and stem cells (Ossum *et al.*, 2004). Telomerase activity may be linked to a high rate of spontaneous immortalisation, which would account for this occurring only in fish cell lines and not human cell lines.

RTG-2: Rainbow Trout Gonad

This cell line was first established by Wolf and Quimby (1962) and was originally derived from the primary culture of normal gonads of yearling fish. The morphology of RTG-2 is mostly fibroblast-like. This cell line proliferates at an optimum temperature of 20°C (Nehls and Segner, 2001). The cells were first obtained from the ECACC (European Collection of Animal Cell Cultures, UK)

RT-Gill W1: Rainbow trout gill

This cell line was developed originally from rainbow trout gills (Bols *et al.*, 1994). This cell line is polygonal or epithelial-like in nature and was found to grow best with at least 10% FBS.

RTS-34st: Rainbow trout spleen

This cell line was developed from an existing cell line, RTS34 that arose from long-term spleen hemopoietic cultures (Ganassin and Bols, 1999). Unlike the RTS34 cell line, which consists of a heterogeneous mixture of epithelial-like and fibroblast-like cells along with a layer of associated macrophage-like cells, the RTS-34st cell line is made up of only stromal components. This RTS-34st cell line allows for stromal cells to develop and proliferate on their own. Conditioned medium from this cell line allow for uptake of thymidine by leucocytes isolated from trout head kidney and peripheral blood, thus allowing this cell line to provide both the microenvironment and the soluble factors needed to promote rainbow trout macrophage growth *in vitro* (Ganassin and Bols, 1999). This cell line has been used as a feeder cell line for zebrafish stem cells (Fan *et al.*,

2004). The RTS34st cell line is fibroblastic in nature with areas of cobblestone, epithelial-like morphology and is found to grow best at 21°C.

RTS-pBk+: Rainbow trout spleen

This cell line is derived from the RTS-34st cell line but was transfected with a neo-expression cassette. This was done in order for this cell line to be able to survive drug selection using G418. As mentioned earlier, RTS-34st cell lines can be used as a feeder cell line for zebrafish stem cells. The lab using this cell line wanted to use a drug selection scheme using the drug, G418, in order to select for a successful zebrafish embryonic stem cells that have undergone homologous recombination. The goal was to allow the feeder cell line to be able to survive for this selection; hence the RTS-pBk+ cell line was developed. Essentially, cells have been 'knocked-in' as opposed to knocked out. This cell line is also fibroblastic in nature and was grown here at 19°C.

HPV-G: Human papillomavirus epithelial

A mammalian cell line, HPV-G was also used as a reporter system. This cell line was derived from human kerotinocytes and fibroblasts isolated from human foreskin and have a cobblestone, epithelial-like morphology. These cells were transformed by transfection with the human papillomavirus 16 (HPV16) DNA (Pirisi *et al.*, 1988).

2.1.2 Cell Culture

All cell culture was performed in a class two laminar flow cabinet. All fish cell lines used in these experiments were grown at 19°C in an incubator without CO_2. All cell lines were grown in Dulbecco's MEM F:12 (1:1) supplemented with 12% fetal

bovine serum, 200mM L-glutamine, 5ml of penicillin-streptomycin solution, 12.5ml of 1M hepes buffer solution and 1μg/ml of hydrocortisone (All products from Gibco Ltd., Ontario). Originally these fish cells were grown in Leibovitz's L-15 media but were adapted to grow in DMEM/F:12 because the mammalian reporter cell line used, HPV-G, grows optimally in DMEM/F:12, whereas the fish cell lines can grow in either medium without this significantly affecting the plating efficiencies of the cells. A simple clonogenic experiment was conducted to confirm that no obvious decrease in plating efficiency occurred in the clonogenic fish cell lines. This mammalian HPV-G cell line is known to produce the bystander effect from numerous past experiments (Reviewed by Mothersill and Seymour, 2004a) and served as a positive control. These cells were maintained in an incubator at 37°C in an atmosphere of 95% air/ 5% CO_2. Subculturing of all cell lines was performed using a 1:1 solution of 0.125% trypsin and 1mM EDTA (Both reagents from Gibco Ltd., Ontario). This was performed at 37°C for HPV-G cells and at room temperature for all fish cell lines. All cultures were used for experiments when they were 85-90% confluent. An aliquot of the cells was counted using a Coulter counter (model Z2) set at a precalibrated threshold that was appropriate for the cell lines used. Each count was repeated three times and the mean value was used.

2.1.3 Growth Curve

The growth curve experiments were conducted to determine the approximate time (in days) it takes for a particular cell population to double in number. Any changes in

this doubling time during radiation experiments are indicative of possible alterations in the viability of the cells. Thirty cell culture 25cm^2 growth area non-vented flasks (Sarstedt, ON, Canada) were set up for each fish cell line with 5ml of medium in each. Approximately 20 000 cells were plated in each of the thirty flasks for ZEB 2J, PBLE, RTS-34st, RTS pBk+ and CHSE-214 cell lines. The EPC cell line was plated with ~50 000, cells and RTG-2 and RT-Gill W1 were plated with ~30 000 cells as these cell lines were difficult to grow at lower densities. The number of cells per flask was calculated at appropriate time points by removing the attached cells with 2ml of 0.125% trypsin EDTA through pipetting techniques, the trypsin was then neutralized by adding 2mls of tissue culture medium to the cell suspension and the cells were counted. This cell number (cells/ml) is then multiplied by the total amount of cell solution (4mls) in order to get the total number of cells/flask. Every three days, three random flasks were taken from the incubator and cells were counted. This continued for 10 time points spread over approximately 45 days. Some cell lines grew slower than others, these cells were then counted every four to five days to ensure a good spread of points.

2.1.4 Clonogenic Assay Technique

Plated cell numbers ranged between 500-50 000 for all fish cell lines. Cells were diluted to give the appropriate cell numbers for cloning efficiency assay as described by Puck and Marcus (1956). Cells were plated into 5ml of culture medium in 25cm^2 non-vented flasks (Sarstedt, ON, Canada). Cultures were incubated for 12 days for the HPV-

G cell line and 3-8 weeks for the fish cell lines, depending on their individual growth rates. Mammalian and fish cell lines were grown in separate incubators at the different temperatures and conditions described earlier. Flasks were periodically checked for growth. When colonies were visible to the naked eye on control flasks, all were stained with Carbol Fuchsin (Ziehl-Nielsson, 1:15) and any colonies present were counted. A colony is defined as fifty or more cells in one grouping (Puck and Marcus, 1956). The percent plating efficiency (P.E.) of each cell line was then determined using the following calculation:

% P.E. = colony no. counted/ colony no. plated*100

Only four of the eight cell lines tested were found to be clonogenic and were used for bystander effect experiments where the signal and response could be measured in the same type of cell. The remaining four fish cell lines, which are not able to clone, were used in further bystander experiments using the mammalian HPV-G reporter system. Thus, for these only generation of the signal can be confirmed.

2.1.5 Irradiation

Cultures were irradiated at room temperature in the dark using a Cobalt 60 γ teletherapy unit at a flask-to-source distance of 80cm. Cells were placed back in their respective incubators immediately following irradiation to avoid large temperature fluctuations.

2.1.6 Survival Curves

Clonogenic cells were irradiated at doses of 0.5Gy, 1.0Gy, 2.5Gy, 5Gy, 10Gy and 15Gy, with a 0Gy sham irradiated. Three flasks per dose per cell line were irradiated using the same methods indicated earlier. Cells were placed back in their respective incubators until macroscopic colonies of 50-100 cells were seen, which were then stained with Carbol Fuchsin (Ziehl-Neelsen, 1:15). Colonies were counted and plating efficiencies were calculated and plotted against dose.

Bystander Effect Experiment:

2.1.7 Clonogenic Fish Cell Lines

It has been shown that HPV-G cells are able to produce and respond to the bystander signals (Lyng *et al.*, 2002; Mothersill and Seymour, 1997; Mothersill *et al.*, 2001a). In order to test if this was the case in this study a simple bystander experiment was conducted using HPV-G as the autologous recipient, donor and directly irradiated cell line. This HPV-G, mammalian cell line was used as a positive control for the experiments and was also used as recipients for the non-clonogenic cells. The bystander technique for the clonogenic fish cell lines, and the reporter mammalian cell line, HPV-G, are summarized below. The cell suspension obtained after trypsinization was diluted with medium and cells were plated with a total of 5ml of cell culture medium (donor flasks) with approximately 100 000 cells/ml for the HPV-G cell line and 300 000 cells/ml for the clonogenic fish cell lines. All recipient cell flasks contained 5ml of

media and were plated at cloning densities with 500 cells/flask for the HPV-Gs. The cell numbers plated varied for the recipient fish cell lines and are listed in Appendix I. A set of directly irradiated flasks was also seeded at appropriate densities as an additional control. Recipient, direct and donor flasks were seeded at the appropriate densities at least 6 hours before irradiations began. Cells were irradiated to 0.5, 1.0, 2.5 and 5Gy doses with a 0Gy sham irradiated control. Post-irradiation, the HPV-G cells were placed back in 37°C and the fish cell lines in the 19°C incubator until medium transfer. Medium transfer entailed the ICCM being filtered through a 0.22μm filter to ensure no cells would be transferred to the recipient flasks, and placed onto autologous recipient reporter fish cells as well as HPV-G mammalian reporter cells. This is done to investigate the separate processes of signal production and recipient response; using the fish cell lines as their own reporter and using the HPV-G mammalian cell line as another reporter system. The medium transfer took place one hour after irradiation for the mammalian cell line and two hours after the fish cell lines were irradiated. Past studies completed with fish cell lines that have tested positive for the bystander effect, have completed the transfer of ICCM to recipients as little as one hour post irradiation, and, mammalian studies have shown a bystander effect in as little as 0.5hr (Mothersill and Seymour, 1997). However, since fish cell lines have generally slower growth rates than the HPV-G cell line, to ensure any possible bystander signal that is created may be successfully transferred to the recipient cells, an increase in the number of cells plated and longer incubation time took place for the fish cell lines. This is also the reason why a greater number of cells were plated for the fish cell lines. The directly irradiated and recipient flasks were left to grow

in the incubator until macroscopic colonies could be seen. This time period was approximately 10-12 days for the HPV-G cell line and no less than 3 weeks for the clonogenic fish cell lines. The cultures were then stained with Carbol Fuchsin (Ziehl-Nielsson, 1:15).

As another control, flasks containing only media and no cells were irradiated and the media placed onto another set of recipient cells for all fish cell lines and the HPV-G cell line.

Non-cloning fish cell lines

The four remaining fish cell lines, which were non-clonogenic in our laboratory, were used in similar bystander experiments using the same doses as described above, however, the recipient reporter fish cell line could not be included because these cell lines do not clone. Instead the HPV-G cell line was used as a reporter recipient cell line in the same manner as described with the cloning fish cell lines, with the non-clonogenic fish cell lines being used as the donors. The non-cloning fish cell lines were plated at least 6 hours before treatment, irradiated, and the media was filter transferred onto HPV-G recipient cell lines after a 2-hour incubation period. As a positive control, HPV-G cells were also set up and directly irradiated at the same doses.

2.1.8 Statistical Analysis

Experiments conducted were performed with triplicate flasks and repeated three times unless otherwise stated. Significance was determined using the student's unpaired t-test. Level of significance was set to $p<0.05$.

CHAPTER 2

BYSTANDER EFFECTS

2.2 RESULTS

2.2 Results:

2.2.1 Growth Curve

Figure 7 shows the growth curves of the mammalian and fish clonogenic cell lines (a) and the non-clonogenic fish cell lines (b). Most grew in the same range except the ZEB 2J cell line that had a slower rate of growth. Next to HPV-G, EPC and CHSE-214 grew the fastest. Only EPC, and RT-Gill W1 began to decrease in cell number near the end of the growth curve experiment, whereas the other cell lines were continuing to increase in cell number. Table I shows the doubling times of all fish cell lines, which were calculated from the exponential portion of figure 7. Adjustments were made in determining the number of days in between counts following the first few counts as the growth rates for each cell line became apparent. After doubling times were calculated, the efficiency and ability of each cell line to produce clones was determined. For this, clonogenic assays were completed.

2.2.2 Clonogenic Assay

In order to investigate bystander effects for any of the cell lines it is desirable that each is able to produce clones when seeded at low density. It was found that only four of the eight original fish cell lines were able to produce clones with a repeatable plating efficiency.

Table II shows the results of the P.E. for all eight fish cell lines. The cell lines that were able to clone were CHSE-214, RTG-2, RT-Gill W1 and ZEB 2J, and the

mammalian cell line, HPV-G. Of these cell lines, CHSE-214, RTG-2 and HPV-G cloned the best, shown by a higher plating efficiency of these cell lines. ZEB 2J had the lowest plating efficiency and was the slowest growing of all the fish cell lines. ZEB 2J and RT-Gill W1 clonogenic assays also resulted in the smallest colonies of the five cell lines tested. The non-clonogenic fish cell lines were PBLE, RTS-pBk+, RTS-34st and EPC. Since no clonogenic assay was possible because these cell lines did not clone in this laboratory, the average amount of cells plated for the donor cell lines were divided by one to give the percent plating efficiency.

2.2.3 Survival Curve

RTG-2 is the most sensitive of the clonogenic fish cell lines at the lower doses (from 0-5Gy) as is shown by the result of the lowest percent survival fraction. After RTG-2, RT-Gill is the most sensitive fish cell line, followed by ZEB 2J and CHSE-214 (Figure 8). At the higher doses of 10-15Gy, CHSE-214 is the most radiosensitive (higher percent survival fraction) followed by RTG-2, and RT-Gill W1, which is the most sensitive (lowest percent survival fraction). RT-Gill W1 becomes more sensitive than the RTG-2 cell line at these higher doses, which was seen to be the most sensitive at lower doses. CHSE-214 seems to be the least sensitive or most resistant clonogenic fish cell line as there is a positive effect seen at 0.5Gy after direct irradiation. The HPV-G mammalian cell line is the most sensitive of all the cell lines tested at all doses. The ZEB 2J cell line was only accounted for up to 5Gy, as 10 and 15Gy did not yield any cells.

Table I.
Doubling Times of all cell lines. Cells were counted after trypsinization using a coulter counter (model Z2). Errors are ± S.E.M. of extrapolation from Figure 7 and cell counts were corrected for dilution factors.

Cell Line	Time (Days)
CHSE-214	2.20±0.30
ZEB 2J	6.75±2.75
RT-Gill W1	3.75±0.75
RTG-2	4.60±0.60
PBLE	3.35±0.15
RTS-34st	3.5±1.30
RTS-pBk+	4.13±0.78
EPC	2.16±0.60
HPV-G	1.70±0.20

Table II.
Plating efficiencies of all fish and mammalian cell lines. Errors are S.E.M. n = 6 for all clonogenic cell lines and n=3 for all non-clonogenic cell lines.

	Clonogenic Cell Lines					Non-clonogenic Cell Lines			
Cell Line	CHSE-214	RT-GillW1	RTG-2	ZEB 2J	HPV-G	PBLE	RTS-pBk+	RTS-34st	EPC
P.E. (%)	10.18±2.50	3.07±0.90	2.95±0.40	1.35±0.20	19.4±5.60	<0.00060 ±0.003	<0.00033 ±0.270	<0.00091 ±0.002	<0.00033 ±0.939

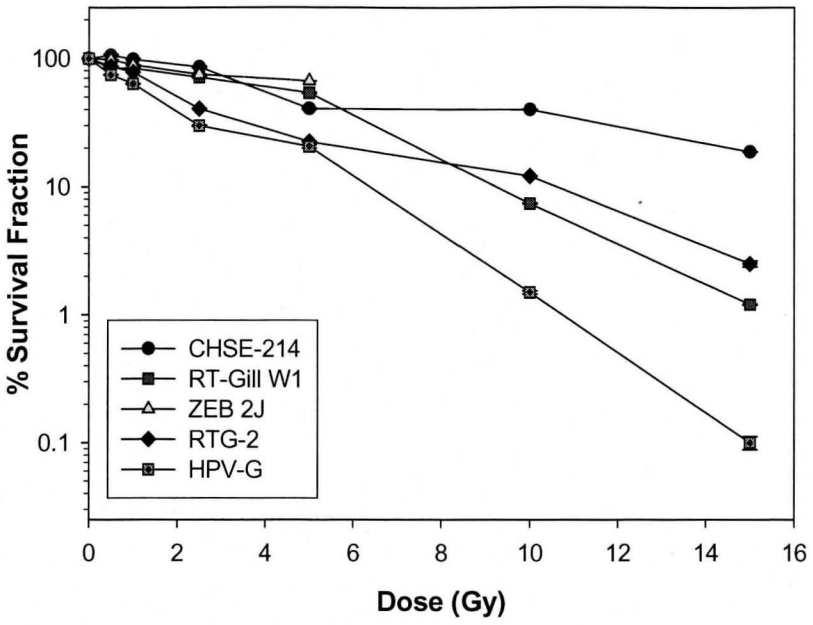


Figure 8. Survival curves of all clonogenic cell lines. Cells were directly irradiated and left undisturbed to form colonies. For doses ranging from 0-5Gy, n=9. For doses from 10-15Gy, n=3. Errors are ± S.E.M. Where error bars cannot be seen, they are contained within the symbol.

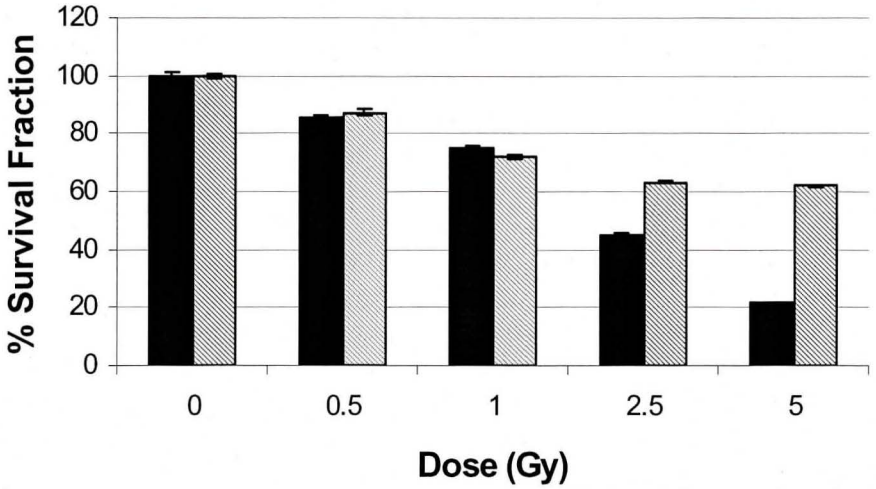


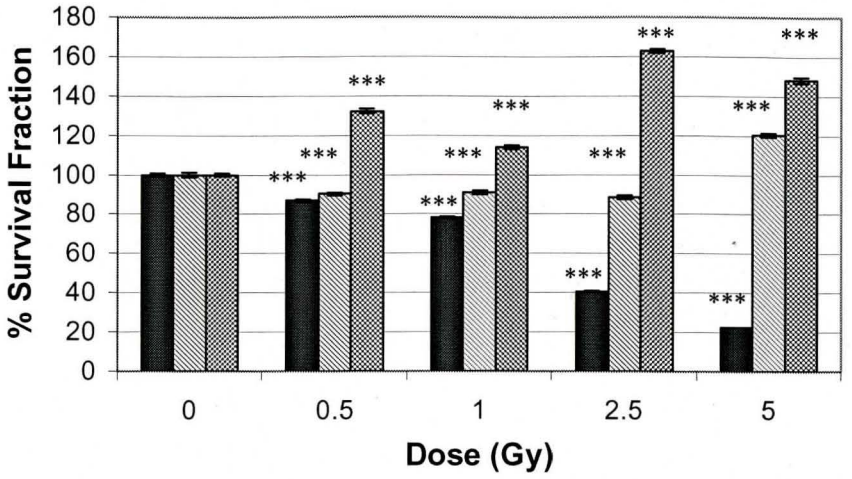
Figure 9. Bystander results for HPV-G mammalian cell line. Directly Irradiated cells (solid bars) and HPV-G cells receiving the ICCM (stripped bars) (n=6) are shown. Data represents the mean ± S.E.M. for N = 13. Significance of treatments is measured against their corresponding controls. All doses are $p<0.001$ as compared to their corresponding controls. Where error bars are not shown they are contained within the bar.

After repeating this experiment for this cell line, very few cells grew, possibly due to the media. This cell line was the only clonogenic fish cell line that appeared to grow less favourably in DMEM as compared to L-15 (personal observations). Additionally, this cell line has a very low plating efficiency initially, thus any factor that may decrease the plating efficiency even further would significantly reduce the number of no viable cells. One point of interest is all the results from 0-5Gy plotted were from experiments repeated at least 3 times, however, from 10-15Gy, these results are from one experiment only, thus, more repeats at these higher doses would eliminate any discrepancies.

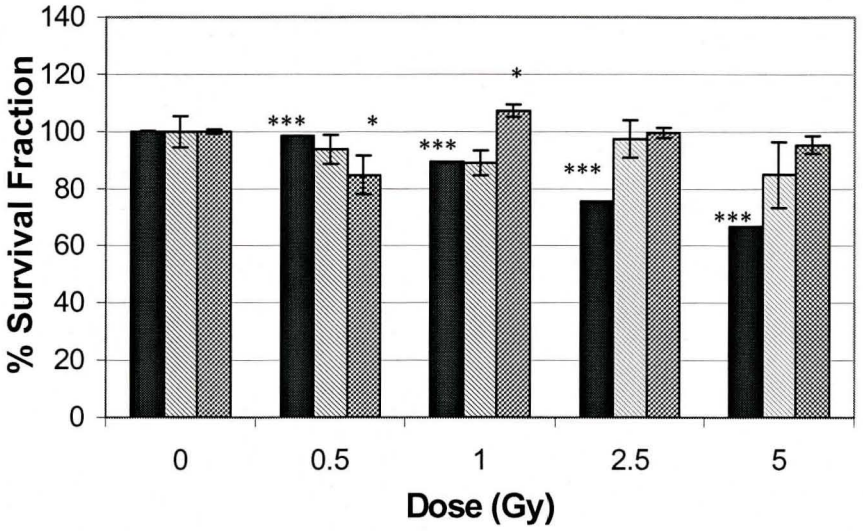
2.2.4 Clonogenic Cell Lines

The initial test to ensure that the bystander effect is occurring using only HPV-G cells was conducted and showed typical results as reported elsewhere (Figure 9) (Liu *et al.*, 2006; Lyng *et al.*, 2002; Mothersill and Seymour, 2002; Seymour and Mothersill, 2000). There was a negative correlation between dose and cell survival fraction for the directly irradiated HPV-G cells, with a result of 21.4±0.4866% survival at 5.0Gy. The recipient cells showed a decrease in survival as well, although less pronounced, with 62.0± 0.546% survival at 5.0Gy.

The bystander experiments conducted on the clonogenic fish cell lines are shown in figure 10. Of the ICCM autologous fish recipients, only RTG-2 showed a significant ($p<0.001$) decrease in cell survival at doses of 0.5Gy, 1.0Gy and 2.5Gy. CHSE-214 also showed a significant decrease in survival, but this was only seen at the 5Gy dose

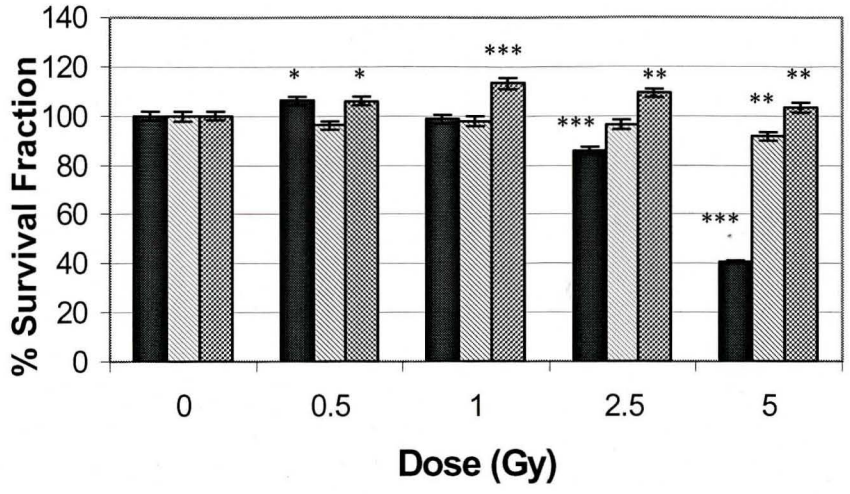


(a)

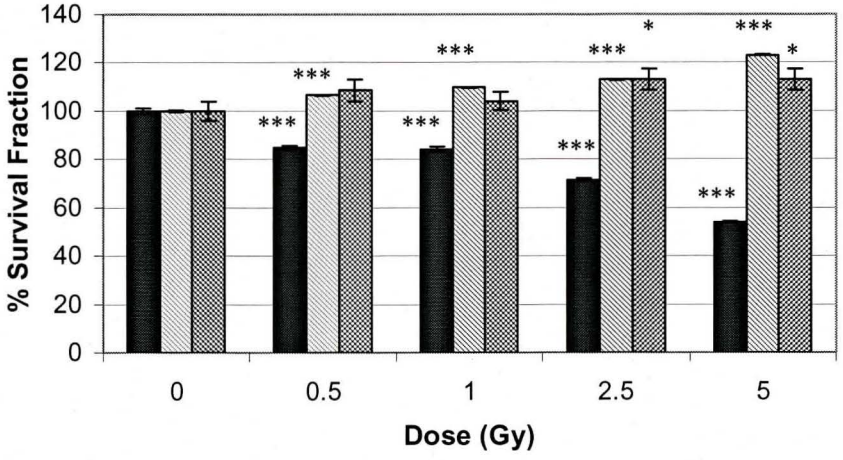


(b)

Figure 10. Bystander experiment results for all clonogenic fish cell lines. Each graph contains the HPV-G recipient reporter cell line results (dotted bars) as well as directly irradiated cells (solid bars) and ICCM autologous recipient fish cells (striped bars). Data represents the mean ± S.E.M. (a) RTG-2 cell line, n=9 (b) ZEB 2J cell line, n=6



(c)



(d)

Figure 10 Cont'd. (c) CHSE-214 cell line, n=9 (d) RT-Gill W1 cell line, n=9. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significance is measured against the corresponding controls for each dose. Where error bars are not shown they are contained within the bar.

($p<0.01$). RT-Gill W1 displayed a significant increase in cell survival at all doses ($p<0.001$). RTG-2 also showed an increase in percent cell survival for the ICCM harvested from 5.0Gy irradiated cells ($p<0.001$). ICCM of the ZEB 2J autologous recipients showed no significant effect across all doses tested.

Unlike the decrease in survival of the recipients found in past studies for HPV-G cells (Liu *et al.*, 2006; Lyng *et al.*, 2002; Mothersill and Seymour, 2002; Seymour and Mothersill, 2000), there was an increase in the percent survival fraction when HPV-G cells were used as reporters for some of the clonogenic fish cell lines. For RTG-2, this increase was seen at all doses ($p<0.001$). The P.E. of the recipient HPV-G cell line due to the ICCM from RT-Gill W1 only significantly increased at doses of 2.5Gy and 5.0Gy ($p<0.05$). The P.E. for HPV-G recipient cells for CHSE-214 was significantly greater than the 0Gy controls at all doses (0.5Gy $p<0.05$; 1.0Gy $p<0.001$; 2.5Gy, 5.0Gy $p<0.01$). The P.E. of the HPV-G recipients for the ZEB 2J cell line actually decreased at 0.5Gy ($p<0.05$) but increased significantly at 1.0Gy ($p<0.05$). For the remaining doses there was no significant difference from the HPV-G reporter cells to the corresponding ZEB 2J controls.

The dose responses for directly irradiated cells are as expected i.e. very resistant responses of decreasing survival with increasing dose. Only ZEB 2J and RT-Gill W1 cell lines had a percent survival fraction that remained greater than 50% for all doses. RTG-2 had the greatest effect with the final percent cell survival being 22.5±0.14% at 5.0Gy. CHSE-214 actually resulted in an increase in survival at the 0.5Gy dose

Table III. Plating efficiencies of 0Gy sham directly irradiated and recipients from clonogenic fish cell lines. There are no significant differences between the two treatments. Errors are shown as ± S.E.M for n=9.

	Direct	Recipient
CHSE-214	8.78±2.28	9.175±2.43
RTG-2	2.9±0.47	3.0±0.60
RT-Gill W1	2.53±0.73	2.7±1.15
ZEB 2J	1.25±0.04	1.9±1.00

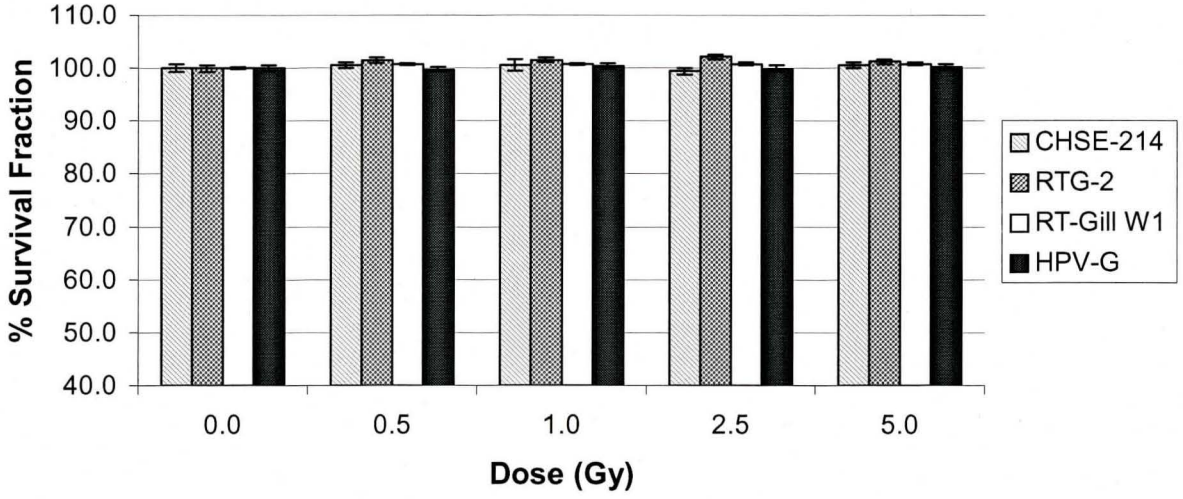


Figure 11. Clonogenic cell lines that received irradiated media containing no cells. Errors are ± S.E.M for n=3. Where error bars are not shown they are contained within the symbol. There are no significant differences between the treatments and the corresponding controls.

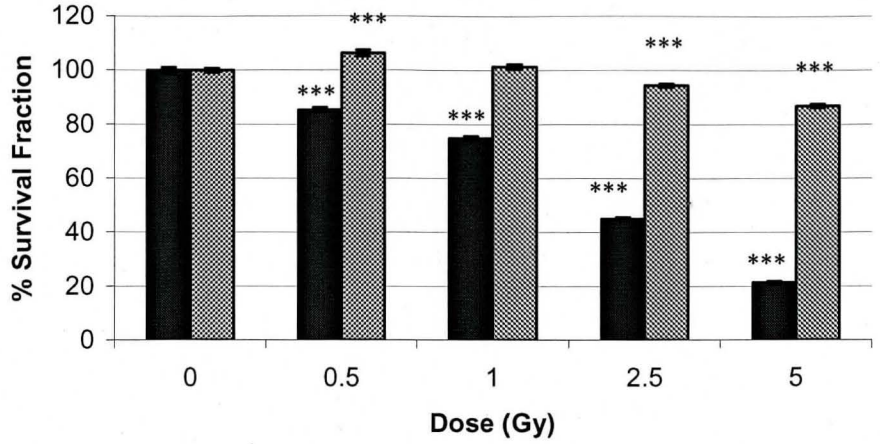
($p<0.05$); however, the higher doses followed the dose dependent decrease in survival with the final percent survival at 5Gy of 40.6±0.61%.

The media only experiments showed no effect of irradiated media added to any of the clonogenic cell lines (Figure 11). Since the non-clonogenic cell lines could not be tested in the only media experiments because of the end point used, the HPV-G mammalian cell line was used as the control. Media was irradiated and placed onto HPV-G recipient cells and left to form colonies. As with the clonogenic fish cell lines, no effect was seen.

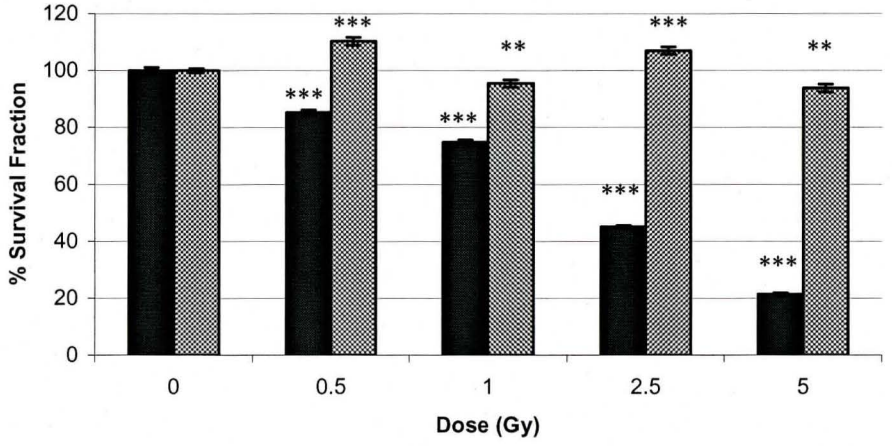
A direct control and a donor/recipient control were also included in all experiments to eliminate effects associated with media changes. Table III compares the 0Gy directly irradiated and autologous fish recipient P.E. No significant change is seen in the P.E. between these two groups. This shows that it is indeed the effects of radiation decreasing the survival of the recipients and not any media changes.

2.2.5 Non-clonogenic Fish Cell Lines

Figure 12 shows the results of the donor non-clonogenic fish cell lines using HPV-G cells as the recipient reporter and as the directly irradiated cells. The HPV-G recipient cells showed a significant increase in plating efficiency due to the ICCM from the non-clonogenic cell lines at most doses. The exception to this, however, was the RTS-pBk+ and RTS-34st cell lines that showed a slight decrease at most doses above 0.5Gy. This decrease was significant for both these cell lines at 5.0Gy (RTS-pBk+, p

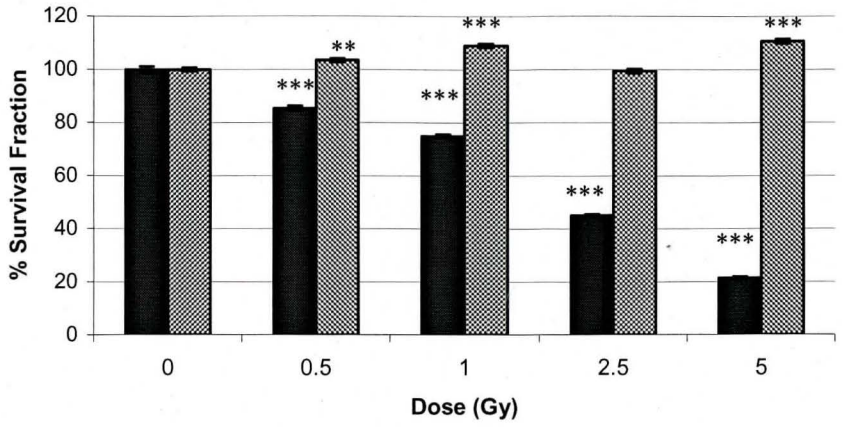


(a)

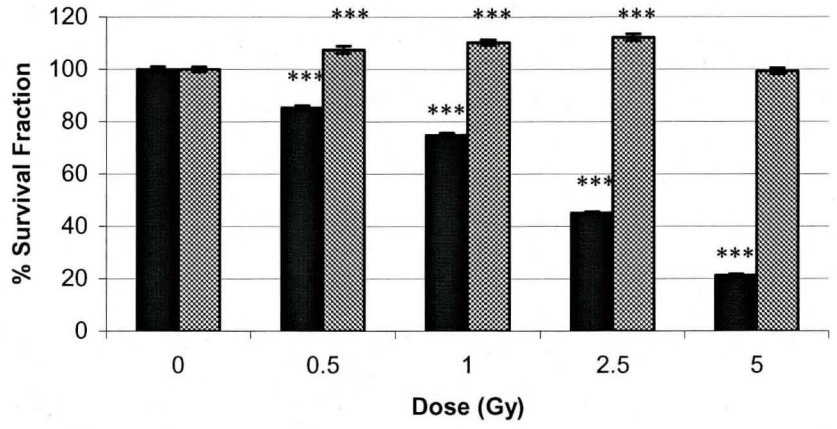


(b)

Figure 12. Bystander experiment results for all non-clonogenic fish cell lines. Each graph contains the HPV-G recipient reporter cell line with the corresponding non-clonogenic fish cell line as the donor (dotted bars) of the ICCM as well as directly irradiated HPV-G cells (solid bars). (a) RTS-pBk+, (b) RTS-34st.



(c)



(d)

Figure 12 Cont'd. (c) PBLE, (d) EPC. For all fish cell lines data represents the mean ± S.E.M. for n = 9. For the HPV-G cell line, N = 13. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Significance is measured against the corresponding controls for each dose. Where error bars are not shown they are contained within the bar.

<0.001; RTS-34st, $p<0.01$). There was also a decrease in survival seen for RTS-pBk+ at 2.5Gy ($p<0.001$) and for RTS-34st at 1.0Gy ($p<0.01$). ICCM from all of the cell lines induced an increase in P.E. of HPV-G reported at 0.5Gy ($p<0.001$) for all cell lines. RTS-34st displayed a significant increase in P.E. at 2.5Gy ($p<0.001$). EPC ICCM caused a significant increase in percent survival fraction of HPV-G recipient cells at all doses ($p<0.001$), with the exception of the ICCM harvested at 5.0Gy, which had no effect. PBLE ICCM caused a significant increase in survival at all doses, 1.0Gy and 5.0Gy ($p<0.001$), 0.5Gy ($p<0.01$), with the exception of 2.5Gy. As the directly irradiated HPV-G cells were under the same conditions as the bystander experiment that used only HPV-G cell lines (Figure 9), this data was pooled. Thus, the results for the directly irradiated HPV-G for this section are the same as in the HPV-G bystander experiment. There was a decrease in survival with increasing dose, that was significant at all doses ($p<0.001$), ending with a survival fraction of 21.4% ± 0.49 at a dose of 5Gy for N=13.

CHAPTER 2

BYSTANDER EFFECTS

2.3 DISCUSSION

2.3 Discussion:

This study demonstrates that fish cells and HPV-G reporter cells receiving media from irradiated fish cells have the potential to display the bystander effect and thus could be manifesting genomic instability (Morgan, 2003; Mothersill and Seymour, 2004a; Seymour and Mothersill, 1999). This study compared direct and non-targeted effects of ionizing radiation for eight fish cell lines on which there is little available data. Growth curve analysis and calculated doubling times indicate that of the eight fish cell lines, EPC, RTG-2, RT-Gill W1 and CHSE-214 display a standard growth curve pattern with a lag phase, log phase, stationary phase and death phase (Hutson *et al.*, 1988). Of these cell lines, EPC grew the fastest followed by CHSE-214, RT-Gill W1 and RTG-2. ZEB 2J was the slowest growing of all the cell lines. This may be due to the fact that these cells have an optimum growing temperature of 26°C (Collodi *et al.*, 1992) but were grown here at 19°C. This temperature was chosen, as it was in the optimal growth range for all the fish cell lines and was most economically sound. The remaining fish cell lines, RTS-34st, RTS-pBk+ and PBLE did not follow the standard growth curve pattern, however this may be because all cell lines were set up with only ten counts over a period of days, thus a greater sample size with more counts may reveal a clearer growth curve.

For the survival curves, HPV-G is shown as the most radiosensitive cell line for every dose tested. RTG-2 is the most radiosensitive fish cell line followed by, RT-Gill W1, CHSE-214, and ZEB 2J, at doses ranging from 0-5Gy. At the higher doses of 10 and 15Gy, however, RTG-2 becomes more radioresistant than RT-Gill W1. This may be an indication that there is a threshold dose between 5 and 10Gy for RTG-2 that may

make this cell line more radioresistant between these doses. Of particular interest is the CHSE-214 cell line survival fraction at lower doses. This cell line has had a direct survival curve completed in a past study, conducted by Dowling *et al.* (2005), using the same doses of 0-5Gy and under the same conditions such as incubation time, media used, serum concentration, and source of ionizing radiation. There was a temperature difference of only 1°C as Dowling *et al.* (2005) grew the CHSE-214 at 18°C, but in this study they were incubated at 19°C. However, in the study conducted by Dowling *et al.* (2005), it was found that CHSE-214 was at least 10% lower in survival fraction compared to the results found in this study. What is interesting to note is that the resulting P.E. found in our lab for the CHSE-214 cell line without any irradiation, was found to be 10.18±2.50%, whereas Dowling *et al.* (2005) found the P.E. to be 23.7±1.8%. As well, the doubling time for this cell line was found to be 50±0.3 hours in our lab, but was only 25.4±2.1 hours for Dowling *et al.* (2005). As this cell line is not from the same origin, a possible reason for these differences may be that the CHSE-214 cell line used in our lab may have mutated, thus resulting in more radioresistant factors. Another possibility is the number of passages that may affect the performance and stability of the cell line. As passage number was not recorded here, this may be an interesting future research project, to keep track of passage numbers and compare the same cell lines between few passage numbers or younger cells and large passage numbers or older cells and make note of any changes therein.

For the clonogenic autologous recipient fish cell lines, there is very little evidence of a toxic bystander effect occurring, with the most evident exception being RTG-2 at

most doses. This bystander effect for the RTG-2 is not as pronounced as past data completed using mammalian cell lines (Mothersill and Seymour, 1997), but still warrants further investigation of the possible mechanisms involved. Survival fractions for CHSE-214 and ZEB 2J autologous recipients showed little change from the controls. Interestingly, there was a strong increase in survival in the autologous recipient RT-Gill W1 cell line, suggesting a positive bystander effect. RT-Gill W1 caused a general increase in percent survival in the HPV-G recipients as well. It is interesting to note that a recent *in vivo* study using rainbow trout fish conducted by Mothersill *et al.* (2006), found bystander effects at doses as low as 0.5Gy. In the *in vivo* study it was found that the gill showed one of the most prominent responses of the bystander effect in comparison to the other organs examined using the same HPV-G reporter system. However, in the experiments described in this thesis, the RT-Gill W1 cell line actually showed an increase in survival in the mammalian reporter cell, and an increase in the autologous recipient cells for survival fraction. This may be due to numerous factors: the first being that a cell line is somewhat different than an explant derived directly from a fish, as these explant cells would have interacted with other systems and are more likely not to have been transformed or mutated compared to a cell line that has been passaged several times. The differences in results between these two studies are not likely to be due to a mutated HPV-G cell line as both of these studies were completed in the same lab, using the same cell line within a year of completing both experiments. This also shows that *ex vivo* systems may be more sensitive than cell lines, as doses as low 0.5Gy found bystander effects. A major reason for the differences seen in cell lines as

compared to explants may be that when a cell line is established, surviving and reproducing cells are selected for, where a toxic bystander response would not be beneficial.

For this study, the bystander experiment results suggest that some of the clonogenic fish cell lines are capable of producing the traditional decrease in survival, showing the bystander effect, but may not be able to express this themselves. In previous studies by Mothersill and Seymour (1997), bystander media transfer from one cell line to another showed that ICCM from human epithelial cells could kill fibroblastic human cells while ICCM from fibroblast cells had no effect on the epithelial cells. This suggests that some factor(s) is being secreted into the media in order to induce cell death and that this function is cell line dependent (Mothersill and Seymour, 1997). ICCM expressed in the mammalian reporter system that resulted in this traditional decrease in survival was only seen in the clonogenic fish cell line, ZEB 2J, at 0.5Gy ($p<0.05$). All other HPV-G recipient cells from the clonogenic ICCM resulted in either no response or an increase in survival. Only the RTG-2 clonogenic cell line was found to be the most capable of producing and expressing this bystander effect as seen in the autologous recipient cells.

Non-clonogenic fish cell line results also showed this increase in survival using the HPV-G cell line as the recipients of the ICCM. This was consistent for all non-clonogenic cell lines tested except for the RTS-pBk+ and RTS-34st cell lines at some doses. Even though these two cell lines are of the same tissue (rainbow trout spleen) they both gave different results, possibly due to the transfection of the neocassette

present in the RTS-pBk+ cell line. This suggests that even though all the ICCM from all fish are placed onto the common HPV-G cell line, each fish cell line seems to have a different reaction to the media transfer method that is dependent not only on dose but on the cell line in question.

As expected, the directly irradiated clonogenic cells showed a decrease in survival with increasing dose, except for the CHSE-214 cell line, which showed an increase in survival at 0.5Gy ($p<0.05$). This result is different from the results from Dowling *et al.* (2005), which showed a dose dependent decrease in survival at all doses measured (which were the same doses measured here). At all doses found here, the resulting survival fraction is greater than Dowling *et al.* (2005) findings by at least 10%. An explanation for this may be that the cell line itself came from different sources, thus, the CHSE-214 cell line that our group received may be different in age and/or radiosensitivity. All of the fish cell lines were more radioresistant compared to the HPV-G cell line, with RTG-2 being the closest to HPV-G in radiosensitivity.

Bystander experiment results using directly irradiated HPV-G media onto unirradiated HPV-G resulted in the typical decrease in cell percent survival as has been reported by numerous authors and confirmed in these experiments (Lyng *et al.*, 2002; Mothersill and Seymour, 1997). It was shown in these past studies that media from some mammalian cell lines that have been exposed to ionizing radiation could reduce the survival of unirradiated cells. The question arises when placing ICCM from fish cells onto mammalian cells if this is an appropriate approach in order to 'see' a bystander effect, since each are such physiologically, behaviourally and genetically different. As

both mammalian and fish cell lines were used as possible reporters in these experiments, any consistent effect seen, whether it be an increase or decrease in survival, would confirm the production of a signal induced by the fish cells. The use of the HPV-G reporter cell line was chosen because this cell line has been known to be capable of responding and producing the bystander effect (Mothersill *et al.*, 2001a). Thus, the comparison of the effect from fish cell ICCM on autologous cells or HPV-G cells allowed us to separate signal production from response.

All ICCM from the fish cell lines used for this study were able to induce some sort of effect in the recipient HPV-G cell line, but may not be able to express it themselves. The majority of recipient cells, both autologous and HPV-G, showed an increase in survival, suggesting a positive bystander response or perhaps a possible mutation resulted. This increase was mostly apparent in the HPV-G recipient cells. There may be certain receptors, which the mammalian cell line has that the fish cell lines do not, thus making the HPV-G cells able to express the bystander effect produced by the fish cell lines. Further study is needed to investigate these differences.

RTG-2 was found to be one of the most radiosensitive cell lines as well as the cell line that showed the most prominent cell death associated bystander effect. Thus, the RTG-2 cell line appears to be the most suitable candidate for a possible reporter system for the bystander effect in fish cell lines.

CHAPTER 3

LETHAL MUTATION EXPERIMENT

3.1 MATERIALS AND METHODS

3.1 Materials and Methods:

3.1.1 Cell Line

The cell line used for this study was the rainbow troud gonad (RTG-2) cell line. Details of this cell line are given in section 2.1.1

3.1.2 Cell Culture

The cell culture techniques are the same as described in section 2.1.2.

3.1.3 Irradiation

The fish cells were irradiated at room temperature using a Cobalt 60 γ teletherapy unit at a flask-to-source distance of 80cm as described in section 2.1.5. Doses of 0.5, 2.5 and 10Gy as well as a 0Gy sham control were used for both the lethal mutation and cell kinetics experiments. Flasks were returned to the incubator immediately following irradiation to minimize cell disturbance.

3.1.4 Lethal mutation/ delayed cell death (clonogenic assay) technique

A subconfluent flask of RTG-2 cells was trypsinised and the cell suspension was then counted using a Z2 coulter counter and appropriate numbers were plated in 5ml of culture medium in a 25cm^2 non-vented flask (Sarstedt) and incubated at 19°C in normoxic conditions at least six hours prior to irradiation to allow for cell attachment.

Six replicate flasks were set up, each containing approximately 3 000 cells per dose point. After cells had formed macroscopic colonies (approximately 4-5 weeks), three of the six flasks per dose point were stained with 15% carbol fuchsin (Ziehl-Neelson) and the number of macroscopic colonies were counted according to methods described in Puck and Marcus (1956) in order to determine the P.E. and initial survival fraction (ISF) per dose point. The remaining flasks were allowed to grow to subconfluence. These remaining flasks per dose point were then trypsinised, counted and replated with 3 000 cells into three new flasks without irradiation, resulting in nine flasks per dose point. Cells were then allowed to form colonies, stained with carbol fuchsin and the P.E. was determined for the progeny survival fraction (PSF).

3.1.5 Cell Kinetics Post Irradiation

Thirty flasks were plated with approximately 40 000 cells in each. The same dose points were used as in the lethal mutation methods. Ten sets of three flasks were set up accordingly in order to allow three flasks to be harvested per dose point at intervals of approximately four days, which was found to be the doubling time of this cell line in this laboratory (O'Neill-Mehlenbacher *et al.*, Submitted). Cell numbers were determined using a Z2 coulter counter. Cells were then replated at each time point with approximately 3 000 cells/ml per flask per dose point and allowed to form macroscopic colonies and then were stained with carbol fuchsin. Clonogenic survival was determined for the progeny of these cells.

3.1.6 Calculation of Delayed Cell Death Frequency

The toxic effects of radiation were measured by colony-forming assay of the P.E. of the cells during exposure. The percentage of colonies formed compared to controls was calculated for the progeny and any reduction in P.E. compared to the control progeny was taken as evidence of DCD (Seymour *et al.*, 1986). For the cell kinetics aspect, the DCD was examined in relation to time after irradiation.

3.1.7 Statistical Analysis

Triplicate cultures were set up for both the DCD/Lethal Mutation and Cell Kinetics experiments per dose point. Results are shown as the mean ± the standard error of the mean (S.E.M.) for n=3 replicate cultures for the initial survival fraction (ISF) and n=9 (each of the three replicates gave rise to three more replicates) for the progeny survival fraction (PSF). Cell counts from the cell kinetics are the mean ± S.E.M. for n=3 replicate cultures for the uncorrected data. Progeny cell P.E. data, or the corrected growth curves, are the mean ± S.E.M. for n=9 replicate cultures. For the cell kinetics comparison, the difference in slopes was evaluated using regression lines with 95% confidence limits.

CHAPTER 3

LETHAL MUTATION EXPERIMENT

3.2 RESULTS

3.2. Results:

3.2.1 Lethal Mutation

Figure 13 shows the initial and progeny survival fraction post exposure. For the ISF, there is a dose dependent decrease post irradiation at all doses ($p<0.001$), except at 0.5Gy dose, where there is a significant increase in survival compared to corresponding controls ($p<0.001$). For the progeny survival fraction, there is a decrease in survival that is dose dependent at the lower doses (0.5-1Gy) but then saturates at the higher doses (2.5-10Gy) ($p<0.001$).

3.2.2 Cell Kinetics

Figure 14a shows the growth curves of both corrected and uncorrected values over forty days. Table IV shows the slopes ± S.E.M. of both corrected and uncorrected values for the exponential portion of the growth curve (shown as days 4-24). The purpose of the corrected growth curves is to make the exponential phase of each growth curve parallel, then any change that is seen between the treatments and controls is due to lethal mutations or delayed cell death and not just slower growing cells, which are alive. Slopes ranging from 0-2.5Gy were grouped as there was little difference seen between these slopes. There was a significant difference found between doses ranging from 0-2.5Gy and the 10Gy slopes of the uncorrected data, as seen by the regression lines

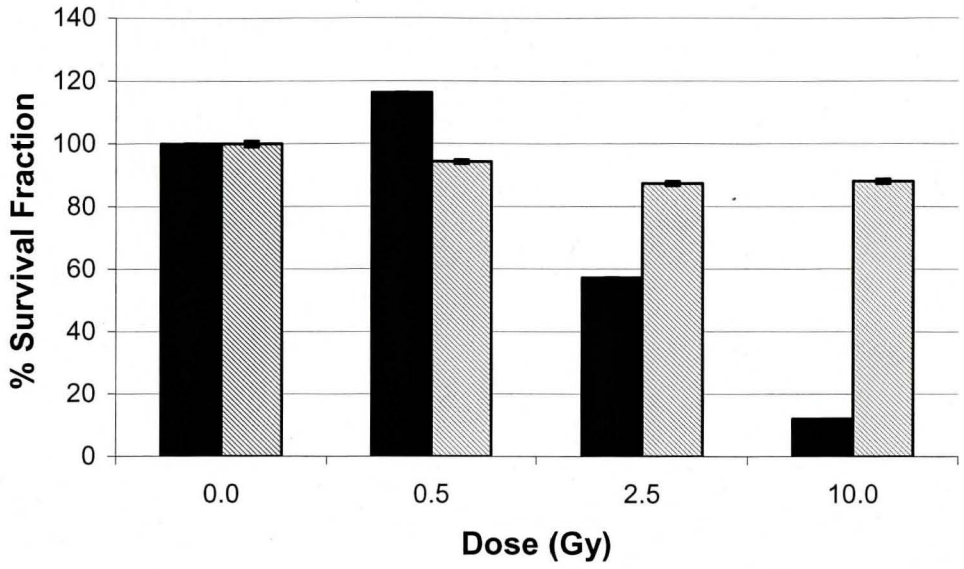
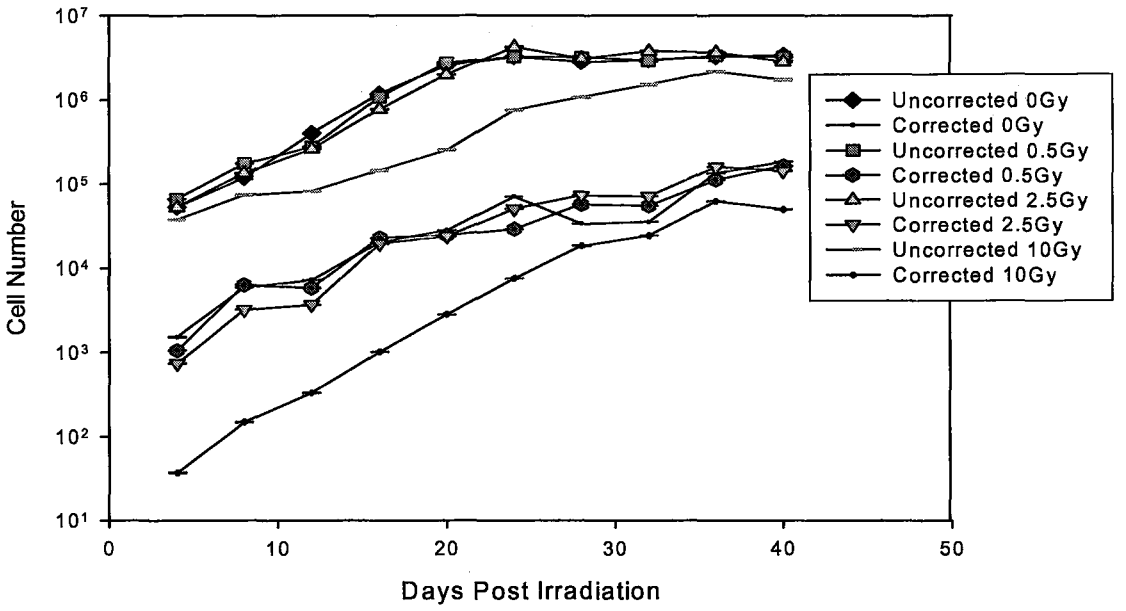


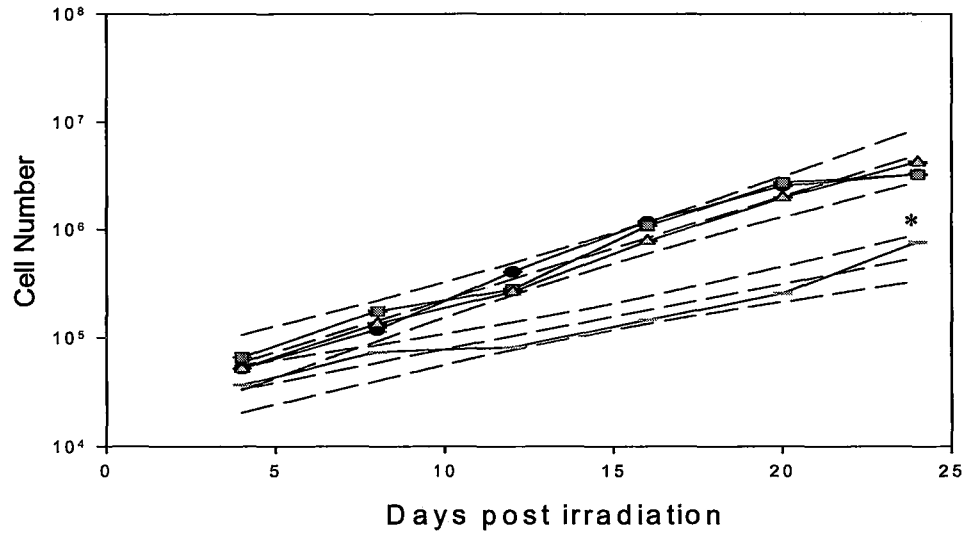
Figure 13. Initial Survival Fraction (ISF) and Progeny Survival Fraction (PSF) of RTG-2 cells. The solid bars are ISF, n=3, the stripped bars are PSF, n=9. ISF were irradiated directly and left to form colonies, replated to produce progeny, which were allowed to form colonies. P.E. and survival fraction were then determined. Errors are ± S.E.M. Where error bars are not shown they are contained within the bar. All doses are $p<0.001$ as compared to their corresponding controls.

Table IV. Slopes of uncorrected and corrected values derived from the exponential phase of figure 14. Slopes from doses of 0-2.5Gy were grouped, as these were not significantly different from each other ($p>0.05$). *$p<0.05$ for 10Gy compared to groups for corresponding uncorrected and corrected values.

Dose (Gy)	Uncorrected	Corrected
0	0.2199	0.1772
0.5	0.2068	0.1566
2.5	0.2216	0.2054
10	0.1383	0.2611
Average		
0-2.5Gy	0.2161±0.0047	0.1797±0.014
10 Gy	0.1383*	0.2611*

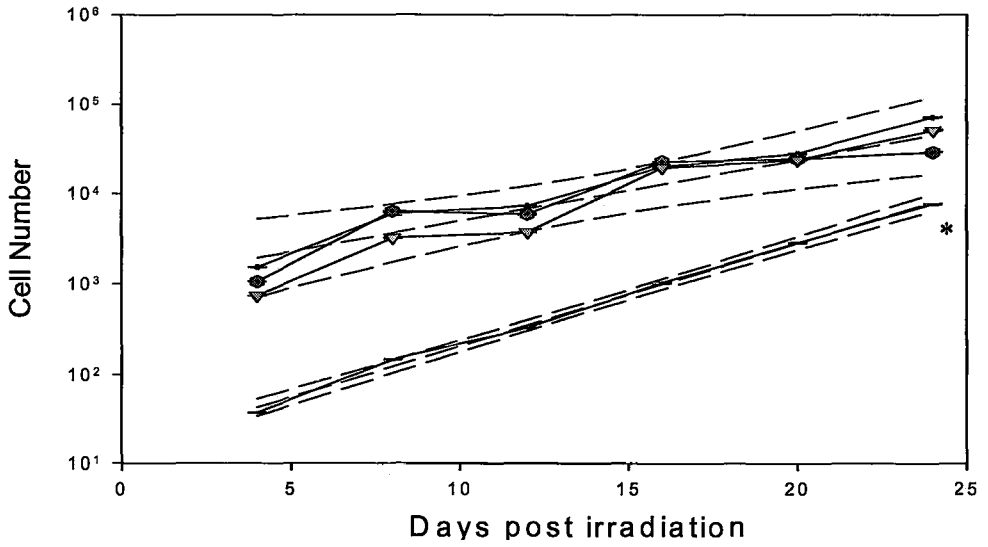


(a)



(b)

Figure 14a. Uncorrected (directly irradiated) and Corrected (progeny of directly irradiated) growth curves over a period of 40 days. Errors are ± S.E.M. Where error bars are not shown they are contained within the symbol. Exponential portion of all plots were taken as days 4-24. (b). Mean regression lines with 95% confidence limits for the growth curves of uncorrected data for the exponential phase. Slopes from 0-2.5Gy were significantly from the 10Gy slope at the higher doses (from day 12-day 24), $*p<0.05$. Symbols are the same as shown in (a).



(c)

Figure 14 Cont'd. (c) Growth curves of the Corrected data for the exponential phase. The average doses of 0-2.5Gy are significantly different from the 10Gy slope, $*p<0.05$. Errors are ±S.E.M. Where error bars are not shown they are contained within the symbol. Symbols are the same as shown in (a).

($p<0.05$) starting from day 12-24 (Figure 14b). When the corrected data were compared, significant differences between these two groups was still present ($p<0.05$). When comparing the uncorrected growth curves to the corresponding corrected growth curves, there were no significant differences found between the grouped data from 0-2.5Gy. There is a difference between the 10Gy uncorrected growth curve and the 10Gy corrected growth curve that can be seen simply by comparing the difference in slopes shown in table IV, however, this particular slope cannot be tested for significance as there is only one set of data.

Since the DCD experiment only shows the results of the final clonogenic progeny survival, it would be interesting to see what difference in survival is seen throughout the growth period of the progeny. Figure 15 shows the results from plotting the survival fraction for each dose over the 40 days of growth for the progeny. There is a distinct pattern seen for each dose, in that, there are two waves that form a decrease in survival, followed by a steep increase, that is repeated twice over the course of the forty days that these cells have been re-plated. The final survival fraction result for the progeny at forty days should resemble the progeny survival fraction of figure 13. This is due to the cell kinetics being the progression of the growth of these cells over the course of the forty days thus, the results of the cells that were stained from the last day these cells were plated should be the same as the PSF. Figure 16 shows that these data points from the last day are very similar.

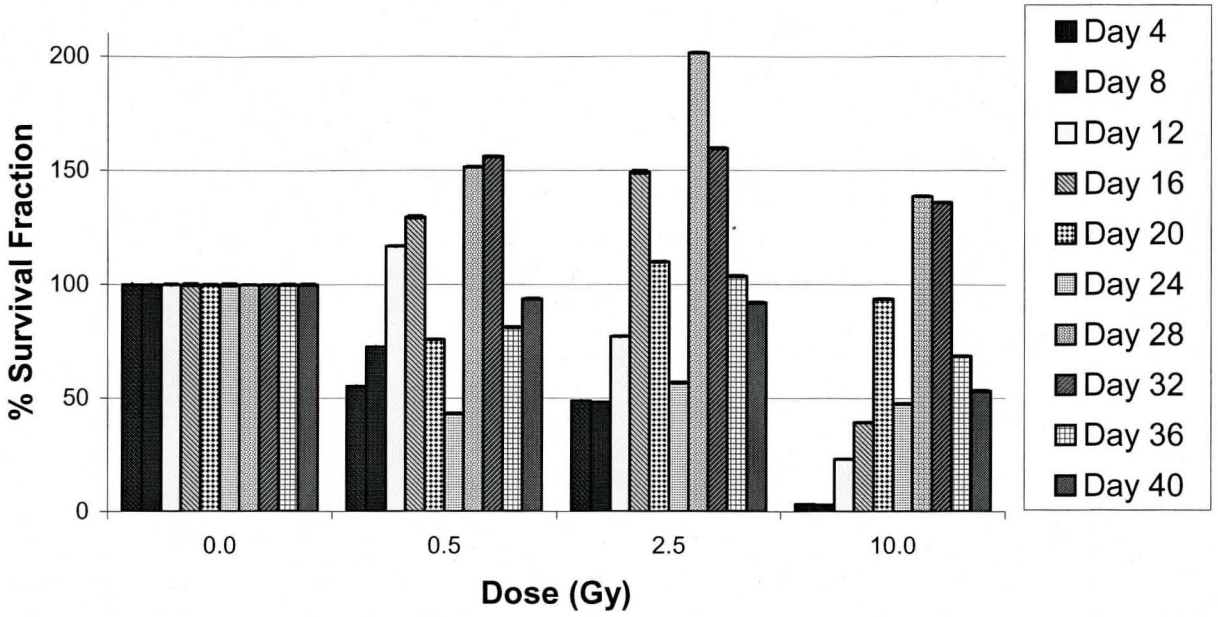


Figure 15. Cell kinetics of the progeny of irradiated cells during the growth of cells plated over forty days. Errors are ± S.E.M for n=9. Where error bars are not shown, they are contained within the symbol. All doses show $p<0.001$ compared to the corresponding controls for all days counted.

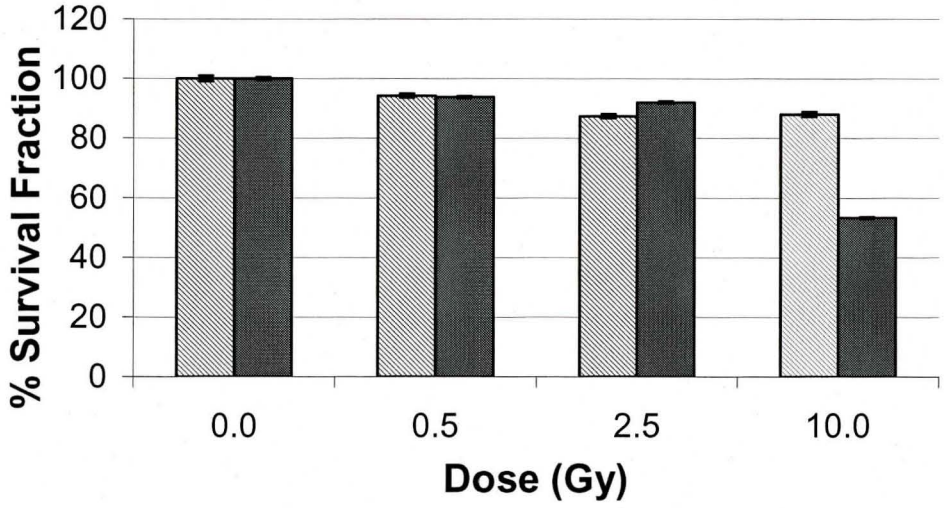


Figure 16. Comparison of PSF for delayed cell death experiment with last progeny count in cell kinetics. The PSF are the striped bars and the last progeny cell count for the cell kinetics experiment (at day forty) are the solid bars. All doses are $p<0.001$ compared to corresponding controls.

CHAPTER 3

LETHAL MUTATION EXPERIMENT

3.3 DISCUSSION

3.3 Discussion:

Lethal mutations are known to occur in the progeny of human cells that have been exposed to ionizing radiation (Seymour and Mothersill, 1986). Little lethal mutation work has been done by this group on fish cell lines with ionizing radiation, as most work done thus far on fish cell lines has been on the toxicity of chemicals (Dowling *et al.*, 2005; Kilemade and Mothersill, 2003). The results from the current study show that DCD or lethal mutations occurred in the progeny of a fish cell line that survived ionizing radiation exposure.

This study shows that genomic instability effects were induced in the RTG-2 cells surviving radiation insult and that the progeny of these cells express the DCD phenotype. In the delayed cell death experiment (Figure 13), showing Initial Survival Fraction (ISF) and Progeny Survival Fraction (PSF), if no DCD occurred, it would be expected that all PSF results would be equal to the corresponding PSF control. However, at all doses (0.5-10Gy), there is a decrease in cell survival ($p<0.001$), suggesting a lethal mutation or DCD event has taken place. This decrease in survival does not appear to be dose-dependent and the level of DCD expression was relatively constant with respect to dose. Similar responses were seen in past studies following irradiation of mammalian cells (Seymour *et al.*, 1986; Stamato *et al.*, 1987; Alper *et al.*, 1988; Mothersill and Seymour, 1993; O'Reilly *et al.*, 1994b). If the expression of these DCD effects is constant with respect to dose, it may indicative of the possible mechanism(s) involved (Kilemade and Mothersill, 2003). For instance, there may be a defined lethal mutator gene that is activated in certain parental cells, causing their progeny to have a decreased probability

of successful division (O'Reilly *et al.*, 2004). This may also indicate that only cells with certain levels of damage can initially survive the irradiation, similar to a threshold level, and that the DCD fraction consists of those cells in the population which suffered this degree of damage (Kilemade and Mothersill, 2003).

An interesting result is seen in the ISF at the 0.5Gy dose where there is an increase in survival ($p<0.001$) compared to the corresponding control. This may be a type of hormetic response in which the cells are overcompensating the induction of repair mechanisms to ensure a full recovery of homeostasis (Calabrese and Baldwin, 1999). These repair mechanisms may not be as efficient at the higher doses of radiation, as more damage is likely to be induced.

The slopes of the lines for the uncorrected growth curves and corrected growth curves were compared in figure 14. Significant differences were found among the slopes tested in the uncorrected growth curves for doses ranging from 0-2.5Gy and 10Gy. This suggests that the directly irradiated cells were growing at the same rate independent of dose, except at the 10Gy, which showed a significant decrease in growth rate compared to the controls beginning around day 12. There was significance found when comparing the corrected slopes. The 10Gy slope was significantly different from all other growth curve slopes ($p<0.05$). Interestingly, this significant difference is due to an increase in slope for the 10Gy as opposed to a decrease, suggesting an improvement in cell growth in the progeny. The lethally mutated cells have died and the cells that have remained show an increase in survival that actually surpasses the controls' growth rate. This may in fact coincide with the results found for the survival curves of the clonogenic cell lines

(Figure 8), where the RTG-2 cell line became more radioresistant between doses of 5Gy-10Gy compared to the RT-Gill W1 cell line that became more radiosensitive. It may be that at this very high dose, cells need to overcompensate for the high level of lethal mutations and thus increase the growth rate of the remaining healthy cells. It is evident that the cell type and cell line used as well as doses employed when testing for any direct, non-targeted or delayed cell death effect need to be taken into consideration.

When comparing uncorrected growth curves to their corresponding corrected growth curves, significance was not found for all doses ranging from 0-2.5Gy (p>0.07). At all these doses tested (0-2.5Gy), since there is no difference compared to the controls, this suggests that no lethal mutation is occurring. This may be because the growth curves may not reflect the survival of the cells accurately enough to see any effect of lethal mutations that may be occurring for this cell line. It may be that since there is large fluctuations in cell survival over the course of the growth period (seen in figure 15), the slope of the lines are not able to display this large change in survival accurately, but at the 10Gy dose, the reaction of the cells to the radiation is large enough to show an effect that can be seen in the growth curves.

When considering the lethal mutation effects that are prominent in the cell kinetics study, in which the survival of the progeny is seen over the forty days of growth, it is clear that there are fluctuations occurring across all doses that do not show a dose dependent relationship (Figure 15). During the earlier stages of progeny growth, it is evident that there are lethal mutations occurring because of the significant decrease in survival at all three irradiation doses. Further in the growth of the cells, there is an

increase in survival, resulting in an improvement in survival fraction compared to the control. This may be due to the cells overcompensating for the delayed effects of the radiation that took place during the earlier stages of progeny growth, thus repair mechanisms may have been activated in order to compensate for this decrease in survival. Past studies have shown that fish cells have the ability to induce repair mechanisms after such damage as that resulting from UV radiation (O'Reilly and Mothersill, 1997). In a study conducted by Ahmed *et al.* (1993), repair mechanisms in fish after UV radiation exposure were investigated. It was found that a fish fibroblastic cell line had an efficient photoreactivation system that was activated after certain wavelengths of UV B light and that this reversed cytotoxicity and dimer formation. As the study performed by Ahmed *et al.* (1993) showed the induction of repair mechanisms in fish after radiation, this also may be occurring at certain times during the growth of the fish cells over the forty days, however, further investigation into these results is necessary. As mentioned earlier, past studies have suggested that the induction of DCD occurs at a constant rate independent of dose (Kilemade and Mothersill, 2003). This suggests that a particular cell response is involved and that the response induced by radiation causes cell death in the progeny at a constant rate. As this past study conducted by Kilemade and Mothersill (2003) only looked at the final colony survival using a clonogenic assay, this statement appears to be true. However, by following the survival fraction of the cells over the course of the forty days of growth, it is apparent that DCD does not occur at a constant rate during the growth of the cells. The survival fraction of the progeny cells varies after each doubling time (approximately every four days for

RTG-2), most likely due to the cells coping with the non-targeted effects of the radiation or to due to the normal progression of the cell cycle. Thus, the proposed constant rate of DCD that is independent of dose is true only when examining the final colony survival. However, when investigating the survival of the cells over the entire growth period, different mechanisms may be involved that are induced before the appearance of the effects of DCD that are independent of dose.

Figure 16 compares the results of the last count on day forty for the cell kinetics experiment with the PSF in the DCD experiment, which was also allowed to grow for forty days. The difference between these two methods is the PSF (Figure 13) was left undisturbed for the entire forty days of growth, whereas the cell kinetics measured the progression of growth of the progeny cells over the course of the forty days. As only a select number of flasks were taken up for every count in the cell kinetics experiment, the cells stained on day forty would be under the same conditions as the cells resulting in the PSF from the DCD experiment. The results comparing these two methods reveal that DCD occurs at all radiation doses tested in both experiments, as the treatments are significantly less than the corresponding controls ($p<0.001$). This also adds evidence that DCD does occur in this cell line at the doses tested as the same result was achieved in two experiments with the same endpoint. In order to see the effects of DCD, there may be a need to display the survival fractions for the corrected growth curves for every study as DCD effect may not be detectable by only analyzing the slope of the line.

CHAPTER 4

BYSTANDER AND LETHAL MUTATION EXPERIMENTS

GENERAL CONCLUSIONS

4.1 General Conclusions:

This study was the first to investigate the cloning abilities of several different fish cell lines as well as producing preliminary bystander experimental results. This advances studies in radiobiology of non-targeted effects of radiation in fish. Studies have been conducted in the past that demonstrated the differences between chemical toxicity in *in vitro* and *in vivo* studies on rainbow trout (Kilemade *et al.*, 2002). It was found that *in vivo* exposures to an aromatic amine resulted in increased sensitivity in cellular proliferation, as much as two-fold, as compared to the *in vitro* effects (Kilemade *et al.*, 2002). Similarly, the recent *in vivo* study conducted by Mothersill *et al.* (2006), showed bystander effects in the rainbow trout gill tissue at doses of 0.5Gy. However, in the current study, the RT-Gill W1 cell line actually showed an increase in survival in the autologous recipient cells and the mammalian reporter cells. This may indicate that *ex vivo* systems are more radiosensitive and may be more suited to relate to *in vivo* studies as compared to fish cell lines or that there is a need to find a radiosensitive fish cell line that would be just as reliable as an *ex vivo* system and serve as a suitable alternative to whole animal studies. However, cell lines can also vary in radiosensitivity even amongst the same cell line from different origins. This is seen in the different results comparing the CHSE-214 results from direct ionization in the study conducted by Dowling *et al.* (2005) to the results found in the current study. The current study showed 10% more radioresistance in the CHSE-214 cell line compared to the results found by Dowling *et al.* (2005) in the same cell line. Evidence exists from previous studies to suggest that signal(s) created by an irradiated cell and response(s) to that signal by a recipient cell

may be separate processes and that these processes differ depending on the system used such as *in vitro*, *in vivo* or *ex vivo* or the species being investigated (Mothersill *et al.*, 2001a). Thus, the system that is used as an indicator for the non-targeted effects of radiation must be suitable to relay any signal(s) that is possibly induced in radiation studies. The current study suggests that the cell line, RTG-2, may fit these parameters.

It has been hypothesized that there is a link between bystander effects and delayed cell death as both these non-targeted effects of radiation involve the expression of genomic instability (Dowling *et al.*, 2005; Lyng *et al.*, 2002; Mothersill *et al.*, 1998; Seymour *et al.*, 1986). It is interesting to note that at the 5Gy dose in the autologous recipients for the bystander study of RTG-2, there is an increase in cell survival as opposed to the lower doses of 0.5-2.5Gy. This may suggest that repair mechanisms are being induced. This may be related to the cell kinetics experiment, in which there is an increase in the slope of the 10Gy corrected growth curve or progeny plating efficiency. Perhaps at doses between 5-10Gy, there is a threshold region where the cells have been highly damaged, thus the cells need to over compensate for this by increasing cell growth rate. This increase in survival is seen in both the cell kinetics progeny survival and in the bystander autologous fish recipient cells. This is also seen in the survival curve in which the RTG-2 cell line becomes more radioresistant than the RT-Gill W1 cell line between the same threshold doses. Past studies have shown that different repair mechanisms are elicited depending on the dose hitting the cells (Yin *et al.*, 2003). Yin *et al.* (2003) performed in vivo studies in mice on low doses of irradiation. At low doses (0.1Gy), brain tissue was found to induce repair mechanisms including changes in gene

expression involved in stress response, cell cycle control and DNA synthesis. These changes were significantly different than those seen at a higher dose of 2Gy (Yin *et al.* 2003). This leads to the possibility that different mechanisms may be activated depending on the dose used, thus making extrapolations from low to high doses (as is suggested in the LNT model) inappropriate. The findings that both bystander effects and lethal mutations are seen at the same doses in the same cell line support the hypothesis that bystander effects and lethal mutations are linked. As well, the findings that the RTG-2 cell line appears to increase in cell growth rate at higher doses in the progeny of irradiated cells, suggests that repair mechanisms may be employed earlier at these higher doses to overcompensate for the large amount of damage that would occur due to these high levels of radiation. In addition, the RTG-2 cell line was the most radiosensitive at lower doses and showed the most prominent bystander effect, suggesting a link between radiosensitivity of the cell line in question and the induction of a bystander signal production and response.

The role of bystander-related events in the induction of genomic instability needs to be examined in relation to risk assessments. Non-targeted irradiated cells that result in genomic instability allow for important implications for studies of mechanisms and risk assessments. This report further adds to the numerous past studies that make extrapolation from high to low doses for risk assessments irrelevant. In addition, a potential reporter system for determining the presence of bystander effects and delayed cell death may be present in the RTG-2 cell line using the donor and recipient method.

There is much research yet to be completed in this field, however, this study shows that the reporter recipient cell line used is as important as the cell line producing the secreted bystander factor through ionizing radiation as well as the direct radiosensitivity of the cell line. This also emphasizes the importance of signal production and cellular response as both are involved in the final outcome. Although the BIER VII report stated that it is too early to assess the relevance to risk from low dose exposure, this study adds to the numerous amounts of data that may aid in finding a method that may prove to be efficient in determining risk at low doses. This study is one of the first to show the non-targeted effects of radiation across several fish cell lines and species.

4.2 Future Directions:

In the future, it would be interesting to see if these bystander effects and corresponding lethal mutation results would occur *in vivo* and/or in primary fish cell lines, as results *in vivo* would be more applicable to actual environmental situations. It would also be interesting to see if a synergistic effect occurs if more than one stress factor is applied to these systems. Examples of multiple stress factors could be radiation and toxic chemicals. The methodology to carry out this experiment cannot be performed using the same clonogenic assay end point used in the current study, as primary cell lines do not clone. However, using a different end point may solve this dilemma.

Different end points such as ROS, or up regulation of Bcl2 or c-myc, may also aid in further determining the mechanics that create the bystander effect as well as all non-targeted effects of radiation. Studies designed to test for the presence and/or change

in ROS during these processes may also solidify the hypothesis that bystander effects and lethal mutations are linked.

As this study only recorded effects of radiation at doses greater than or equal to 0.5Gy, it would be interesting to apply lower does in the cGy region and record the changes that may occur and if these effects differ from the higher doses used in this study. In addition to the lower doses, however, another study could investigate the proposed threshold region (5-10Gy) to find if there is an increase in induction of repair mechanisms that could explain this increase in progeny survival seen in the RTG-2 cell line at these higher doses in the lethal mutation experiment. As well, future studies should use the RTG-2 cell line as the donor and/or recipient for other fish cell lines and observe potential changes, as this would be beneficial in determining if this cell line is truly a suitable reporter system. As well as testing this cell line, using the same methods for other non-targeted effects such as adaptive responses, hyper-radiosensitivity and hormesis would also prove beneficial.

For the bystander effect experiments, more tests should be conducted using different donor and recipient fish cell lines to observe if this effect can be seen across different species of the same family or order.

It would be interesting to investigate the repair mechanisms in fish that may be induced after radiation insult. In fish, it has been found that the main factors involved in the cellular immune system are phagocytes (macrophages, monocytes, neutrophils) as these are the first line of defence (Betoulle *et al.*, 2000). These phagocytes have been known to secrete a variety of active molecules and ROS. In a study conducted by Olwell

et al. (2005), it was found that phagocytosis levels occurred at a lower rate after a dose of 0.5Gy using γ-radiation on primary cell rainbow trout hemopoietic tissue cultures. It was hypothesized that cells from the immune system of the fish were reacting to radiation in a similar manner as when commencing the immune response of phagocytosis (Olwell *et al.*, 2005). This suggests that if repair mechanisms (ex. phagocytes) were damaged, ROS would increase, resulting in lethal mutations. Thus, at low levels (such as 0.5Gy), repair mechanisms may still be functional, but are occurring at a lower rate due to some damage that occurs at this dose, and thus, such as the case may be in this study, are able to activate the proper immune systems. Evidence of this is seen in past studies that have found that the production of ROS is correlated with lethal mutations (Mothersill *et al.*, 1998). Any modification of repair mechanisms is critical to the cell, which leads to the conclusion that lethal mutations arise as a result of incomplete or defective repair (O'Reilly *et al.*, 1994a). Studies investigating these hypotheses would be beneficial in the pursuit of further understanding the mechanisms behind this phenomenon.

As most results showed increased in survival for the bystander experiments, such as for the cell line RT-Gill W1, it would be interesting to see if there is a connection between radiosensitivity of fish cell lines and their ability to show a cell death associated bystander effect or an increased survival associated bystander effect.

As well, all fish cell lines tested here were kept at a constant temperature of 19°C, as this was the temperature that most cell lines grew best at. It would be interesting to see if all cell lines grown in their optimal temperature has an effect on the production and response to irradiation in bystander effect experiments.

References:

Ahmed, F.E., Setlow, R.B., Grist, E. and Setlow, N. 1993. DNA damage photorepair, and survival in fish and human cells exposed to UV radiation. *Environmental Molecular Mutagenesis*. **22**: 18-25.

Alper, T. 1979. Cellular Radiobiology. Cambridge University Press.

Alper, T., Mothersill, C and Seymour, C.B. 1988. Lethal mutations attributable to misrepair of Q-lesions. *International Journal of Radiation Biology*. **54**: 525-530.

Altland, P.D., Highman, B. and Wood, B. 1951. Some effects of X-irradiation on turtles. *Journal of Experimental Zoology*. **118**: 1-19.

Amundson, S.A. Bittner, M., Meltzer, P., Trent, J., Fornace, A.J., Jr. 2001. Biological indicators for the identification of ionizing radiation exposure in humans. *Expert Review of Molecular Diagnostics*. **1**: 211-9.

Avila, R., Beresford, N.A., Aguero, A., Broed, R., Brown, J., Iospje, M., Robles, B., Suanez, A. 2004. A study of the uncertainty in estimation of the exposure of non-human biota to ionising radiation. *Journal of Radiation Protection*. **24**: A105-122.

Azzam, E.I., de Toledo, S.M, Gooding, T. and Little, J.B. 1998. Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. *Radiation Research*. **150**: 497-504.

Azzam, E.I., de Toledo, S.M, Waker, A.J. and Little, J.B. 2000. High and low fluences of alpha-particles induce a G1 checkpoint in human diploid fibroblasts. *Cancer Research*. **60**: 2623-2631.

Azzam, E.I., de Toledo, S.M. and Little, J.B. 2001. Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from α-particle irradiated to nonirradiated cells. *Proceedings of the National Academy of Science USA*. **98**: 473-478.

Azzam, E.I., de Toledo, S.M., Spitz, D.R. and Little, J.B. 2002. Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from α-particle-irradiated normal human fibroblast cultures. *Cancer Research*. **62**: 5436-5442.

Beam, C.A., Mortimer, R.K., Wolfe, R.G. and Tobias, C.A. 1954. The relation of radioresistance to budding in Saccharomyces cerevisiae. *Archives of Biochemistry*

and Biophysics. **49**: 110-122.

Belyakov, O.V., Malcolmson, A.M., Folkard, M., Prise, K.M. and Michael, B.D. 2001. Direct evidence for a bystander effect of ionizing radiation in primary human fibroblasts. *British Journal of Cancer*. **84**: 674-679.

Betoulle, S., Duchiron, C. and Deschaux, P. 2000. Lindane increases in vitro respiratory burst activity and intracellular calcium levels in rainbow trout (*Oncorhynchus mykiss*) head kidney phagocytes. *Aquatic Toxicology*. **48**: 211-221.

BEIR VII Report. 2006. Committee on the Biological Effects of Ionizing Radiations. Health effects of exposure to low levels of ionizing radiation. National Academy Press, USA: Washington, D.C.

Bols, N.C., Barlian, A., Chirino-Trejo, M., Caldwell, S.J., Goegan, P. and Lee, L.E.J. 1994. Development of a cell line from primary cultures of rainbow trout, *Oncorhynchus mykiss* (Walbaum), gills. *Journal of Fish Diseases*. **17**: 601-611.

Bosi, A. and Olivieri, G. 1989. Variability of the adaptive response to ionizing radiations in humans. *Mutation Research*. **211**: 13-17.

Branch, P., Aquilina, G., Bignimi, M. and Karran, P. 1993. Defective mismatch binding and a mutator phenotype in cells tolerant to DNA damage. *Nature*. **362**: 652-654.

Brooks, A.L. 2005. Paradigm shifts in radiation biology: Their impact on intervention for radiation-induced disease. *Radiation Research*. **164**: 454-461.

Cai, L. and Liu, S.Z. 1990. Induction of cytogenetic adaptive response of somatic and germ cells *in vivo* and *in vitro* by low dose X-irradiation. *International Journal of Radiation Biology*. **58**: 187-194.

Cai, L. 1999. Research of the adaptive response induced by low-dose radiation: where have we been and where should we go? *Human and Experimental Toxicology*. **18**: 419-425.

Calabrese, E.J. and Howe, K.J. 1976. Stimulation of growth of peppermint (*Mentha piperita*) by phosfon, a growth retardant. *Physiologia Plantarum*. **37**: 163-165.

Calabrese, E.J., McCarthy, M.E. and Kenyon, E. 1987. The occurrence of chemically induced hormesis. *Health Physics*. **52**: 531-541.

Calabrese, E.J. and Baldwin, L.A. 1999. Re-evaluation of the fundamental dose-response Relationship: A new database suggests that the U-shaped, rather than the sigmoidal, curve predominates. *Bioscience*. **49**: 725-732.

Chiba, S., Saito, A., Ogawa, S., Takeuchi, K., Kumano, K., Seo, S., Suzuki, T., Tanaka, Y., Saito, T., Izutsu, K., Yuji, K., Masuda, S., Futami, S., Nishida, M., Suzuki, G., Gale, R.P., Fukayama, M., Maekawa, K. and Hirai, H. 2002. Case Report: Transplantation for accidental acute high-dose total body neutron- and γ-radiation exposure. *Bone Marrow Transplantation.* **29**: 935-939.

Chilmonczyk, S. and Oui, E. 1988. The effects of gamma irradiation on the lymphoid organs of rainbow trout and subsequent susceptibility to fish pathogens. *Veterinary Immunology and Immunopathology.* **18**: 173-180.

Clutton, S.M., Townsend, K.M.S, Walker, C., Ansell, J.D. and Wright, E.G. 1996. Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. *Carcinogenesis.* **17**:1633-1639.

Collodi, P., Kamei, Y., Ernst, T., Miranda, C., Buhler, D.R. and Barnes, D.W. 1992. Culture of cells from zebrafish (*Brachydanio rerio*) embryo and adult tissues. *Cell Biology and Toxicology.* **8**: 43-61.

Cramers, P., Atanasova, P., Vrolijk, H., Darroudi, F., Van Zeeland, A.A., Huiskamp, P., Mullenders, L.H. and Kleinjans, J.C. 2005. Pre-exposure to low doses: modulation of X-ray-induced dna damage and repair? *Radiation Research.* **164**: 383-390.

Curtis, S.B., Hazelton, W.D., Luebeck, E.G. and Moolgavkar, S.H. 2004. From mechanisms to risk estimation – bridging the chasm. *Advances in Space Research.* **34**: 1404-1409.

Deshpande, A., Goodwin, E.H., Bailey, S.M., Marrone, B.L. and Lehnert, B.E. 1996. α-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. *Radiation Research.* **145**: 260-267.

DeWitte-Orr, S.J. 2006. A study of innate antiviral mechanisms using fish cell lines. Doctor of Philosophy Thesis in Biology. University of Waterloo, Ontario.

Dowling, K., Seymour, C. and Mothersill, C. 2005. Delayed cell death and bystander effects in the progeny of Chinook Salmon Embryo cells exposed to radiation and a range of aquatic pollutants. *International Journal of Radiation Biology.* **81**: 89-95.

Durante, M., George, K. and Yang, T.C. 1996. Biological dosimetry by interphase chromosome painting. *Radiation Research.* **145**: 53-60.

Durante, M. 2005. Biomarkers of Space Radiation Risk. *Radiation Research.* **164**: 467-473.

Elkind, M.M. and Whitemore, G.F. 1967. The Radiobiology of Cultured Mammalian Cells, New York: Gordan and Breach.

Elkind, M.M. and Sutton H. 1959. X-ray damage and recovery in mammalian cells in culture. *Nature* **184**: 1293-1295.

Eriksson, G. 1963. Induction of waxy mutants in maize by acute and chronic gamma-irradiation. *Hereditas*. **50**: 161-178.

Fan, L., Crodian, J., Liu, X., Aleström, A., Aleström, P. and Collodi P. 2004. Zebrafish embryo cells remain pluripotent and germ-line competent for multiple passages in culture. *Zebrafish*. **1**: 21-26.

Fox, M. 1985. Assay of colony-forming ability in an established cell line. Cell Clones. Eds: C.S. Potten, J.H. Hendry. Edinburgh: Churchill, Livingstone.

Gajendiran, N., Tanaka, K., Kumaravel, T.S. and Kamada, N. 2001. Neutron-induced adaptive response studied in G0 human lymphocytes using the comet assay. *Journal of Radiation Research (Tokyo)* **42**: 91-101.

Ganassin, R.C. and Bols, N.C.1999. A stromal cell line from rainbow trout spleen, RTS34st, that supports the growth of rainbow trout macrophages and produces conditioned medium with mitogenic effects on leukocytes. *In Vitro Cellular and Developmental Biology - Animal*. **35**: 80-86.

Goh, K and Sumner, H. 1968. Breaks in normal human chromosomes: are they induced by a transferable substance in the plasma of persons exposed to total body irradiation? *Radiation Research*. **35**: 171-181.

Grote, S.J., Joshi, P.G. and Revell, S.H. 1981. Observations of radiation induced chromosome fragment loss in live mammalian cells in culture, and its effect on their colony-forming ability. *International Journal of Radiation Biology*. **39**: 395-408.

Hain, J., Jaussi, R., Burkart, W. 1992. Lack of adaptive response to low doses of ionizing radiation in human lymphocytes from five different donors. *Mutation Research*. **283**: 137-144.

Hall, E. J. 2000. Radiation and Life. Retrieved December 5th, 2006, from http://www.uic.com.au/ral.htm.

Hart, R.W. and Frome, L.T. 1996. Toxicological defense mechanisms and how they may affect the nature of the dose-response relationship. *BELLE Newsletter*. **5**: 1-16.

Hinton, T.G., Bedford, J.S., Congdon, J.C. and Whicker, F.W. 2004. Effects of radiation on the environment: A need to question old paradigms and enhance collaboration among radiation biologists and radiation ecologists. Commentary. *Radiation Research*. **162**: 332-338.

Hollowell, J.G. and Littlefield, L.G. 1968. Chromosome damage induced by plasma of X-rayed patient: an indirect effect of radiation. *Proceedings of the Society for Experimental Biology and Medicine*. **129**: 240-244.

Hutson, T.B., Mitchell, M.L., Keller, J.T., Long, D.J. and Chang, M.J.W. 1988. A technique for monitoring mammalian cell growth and inhibition *in situ* via fourier transform infrared spectroscopy. *Analytical Biochemistry*. **174**: 415-422.

IAEA (International Atomic Energy Agency). 2002. Ethical Considerations in Protecting the Environment from the Effects of Ionizing Radiation, IAEA-TECDOC-1270, IAEA, Vienna, Austria.

ICRP (International Commission on Radiological Protection). 1966. The Evaluation of Risks from Radiation. ICRP Publication 8. Oxford, England: Pergammon Press.

ICRP (International Commission on Radiological Protection). 1977. Recommendations of the International Commission on Radiological Protection. Annals of the ICRP 1(3). ICRP Publication 26. Oxford, England: Pergammon Press.

ICRP. Recommendations of the International Commission on Radiological Protection.1991. Annals of the ICRO 21(1-3): 1-201. ICRP Publication 60. Oxford, Enlgand: Pergammon Press,

ICRP. Recommendations of the International Commission on Radiological Protection. 2003. A framework for assessing the impact of ionising radiation on non-human species. Publication 91. Pergammon Press, Oxford and New York.

Iyer, R. and Lehnert, B.E. 2000. Factors underlying the cell growth-related bystander responses to alpha particles. *Cancer Research*. **60**: 1290-1298.

Joiner, M.C. 1994. Induced radioresistance: an overview and historical perspective. *International Journal of Radiation Biology*. **65**: 79-84.

Joiner, M.C., Marples, B., Lambin, P., Short, S.C. and Turesson, I. 2001. Low-dose hypersensitivity: current status and possible mechanisms. *International Journal of Radiation Oncology, Biology, Physics*. **49**: 379-389.

Kadhim, M., MacDonald, D.A., Goodhead, S.A., Lorimore, S.A., Marsden, S.J. and

Wright, E.G. 1992. Transmission of chromosomal instability after plutonium alpha-particle irradiation. *Nature.* **355**: 738-740.

Kadhim, M., Lorimore, S.A., Townsend, K.M., Goodhead, D.T., Buckle, V.J. and Wright, E.G. 1995. Radiation-induced genomic instability: delayed cyotgenetic aberrations and apoptosis in primary human bone marrow cells. *International Journal of Radiation Biology.* **67**: 287-293.

Karran, P and Hampson, R. 1996. Genomic instability and tolerance to alkylating agents. *Cancer Surveys.* **28**: 69-85.

Karrow, N.A., Boermans, H.J., Dixon, D.G., Hontella, A., Solomon, K.R., Whyte, J.J. and Bols, N.C. 1999. Characterizing the immunotoxicity of creosote to rainbow trout (*Oncorhynchus mykiss*): a microcosm study. *Aquatic Toxicology.* **45**: 223-239.

Kilemade, M., Lyons-Alcantra, M., Rose, T., Fitzgerald, R. and Mothersill, C. 2002. Rainbow trout primary epidermal cell proliferation as an indicator of aquatic toxicity: an in vitro/in vivo exposure comparison. *Aquatic Toxicology.* **60**: 43-59.

Kilemade, M. and Mothersill, C. 2003. Expression of delayed cell death (DCD) in the progeny of fish cells surviving 2,4-dichloroaniline (2,4-DCA) exposure. *Aquatic Toxicology.* **63**: 207-219.

Knowles, J.F. 1992. The effect of chronic radiation on the humoral immune response of rainbow trout (Oncorhynchus mykiss Walbaum). *International Journal of Radiation Biology.* **62**: 239-248.

Limoli, C.L., Kaplan, M.I., Corcoran, J., Meyers, M., Boothman, D.A. and Morgan, W.F. 1997. Chromosomal instability and its relationship to other end points of genomic instability. *Cancer Research.* **57**: 4048-4056.

Little, J.B. and Morgan, W.F. 2003. Guest editors. Special Issue. *Oncogene.***13**: 22-6977.

Little, J.B., Nagasawa, H., Li, G.C. and Chen, D.J. 2003. Involvement of the nonhomologous end joining DNA repair pathway in the bystander effect for chromosomal aberrations. *Radiation Research.* **159**: 262-267.

Liu, Z., Mothersill, C.E., McNeill, F.E., Lyng, F., Byun, S.H., Seymour, C.B. and Prestwich, W.V. 2006. A dose threshold for a medium transfer bystander effect for a human skin cell line. *Radiation Research.* **166**: 19-23.

Loeb, L.A. 1991. Mutator phenotype may be required for multistage carcinogenesis. *Cancer Research.* **51**: 3075-3079.

Loosli, F., Köster, R.W., Carl, M., Kühnlein, R., Henrich, T., Mücke, M., Krone, A. and Wittbrodt, J. 2000. A genetic screen for mutations affecting embryonic development in medaka fish (*Oryzias latipes*). *Mechanisms of Development*. **97**: 133-139.

Lorimore, S.A., Kadhim, M.A., Pocock, D.A., Papworth, D., Stevens, D.L., Goodhead, D.T. and Wright, E.G.. 1998. Chromosomal instability in the descendants of unirradiated surviving cells after α-particle irradiation. *Proceedings of the National Academy of Sciences USA*. **95**: 5730-5733.

Lorimore, S.A. and Wright, E.G. 2003. Radiation-induced genomic instability and bystander effects: related inflammatory-type responses to radiation-induced stress and injury? A review. *International Journal of Radiation Biology*. **79**: 15-25.

Lorimore, S.A., Coates, P.J. and Wright, E.G. 2003. Radiation-induced genomic instability and bystander effects: inter-related nontargeted effects of exposure to ionizing radiation. *Oncogene*. **22**: 7058-7069.

Luckey, T.D. Hormesis with Ionizing Radiation. CRC Press, Boca Raton, Florida. 1980.

Luckey, T.D. 1982. Physiological benefits from low levels of ionizing radiation. *Health Physics*. **43**: 771-789.

Luckey, T.D. Radiation Hormesis. CRC Press, Boca Raton, Florida. 1991.

Lyng, F.M., Seymour, C.B. and Mothersill, C. 2002. Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: A possible mechanism for bystander-induced genomic instability? *Radiation Research*. **157**: 365-370.

Lyng, F.M., Lyons-Alcantara, M., Olwell, P., Shuilleabhain, S.N., Seymour, C., Cottell, D.C. and Mothersill, C. 2004. Ionizing radiation induces a stress response on primary cultures of rainbow trout skin. *Radiation Research*. **162**: 226-232.

Maguire, P., Mothersill, C., Seymour, C. and Lyng, F.M. 2005. Medium from irradiated cells induces dose-dependent mitochondrial changes and BCL2 responses in unirradiated human keratinocytes. *Radiation Research*. **163**: 384-390.

Marder, B.A. and Morgan, W.F. 1993. Delayed chromosomal instability induced by DNA damage. *Molecular and Cell Biology*. **13**: 6667-6677.

Marples, B. and Joiner, M.C. 1995. The elimination of low-dose hypersensitivity in Chinese hamster V79-379A cells by pre-treatment with X-rays or hydrogen peroxide. *Radiation Research*. **141**: 160-169.

Marples, B., Wouters, J.G. and Joiner, M.C. 2003. An association between the radiation-induced arrest of G2 phase cells and low-dose hyper-radiosensitivity: A plausible underlying mechanism? *Radiation Research.* **160**: 38-45.

Marples, B. 2004. Is low-dose hyper-radiosensitivity a measure of G2-phase cell radiosensitivity? *Cancer and Metastasis Reviews.* **23**: 197-207.

Matsumoto, H., Hayashi, S., Hatashita,M., Ohnishi, K., Shioura, H., Ohtsubo, T., Kitai, R., Ohnishi, T. and Kano, E. 2001. Induction of radioresistance by a nitric oxide-mediated bystander effect. *Radiation Research.* **155**: 387-396.

McCarty, L.S., Power, M. and Munkittrick, K.R. 2002. Bioindicators versus biomarkers in ecological risk assessment. *Human and Ecological Risk Assessment.* **8**: 159-164.

Morgan, W.F. 2003. Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. A review. *Radiation Research.* **159**: 567-580.

Morgan, W.F. and Sowa, M.B. 2005. Effects of ionizing radiation in nonirradiated cells. *Proceedings of the National Academy of Sciences, USA.* **102**: 14127-14128.

Mothersill, C. and Seymour, C. 1993. Recovery of the radiation survival curve shoulder in repair deficient cell populations. *Mutation Research.* **285**: 259-266.

Mothersill, C and Seymour, C. 1997. Medium from irradiated human epithelial cells but not human fibroblasts reduced the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology.* **71**: 421-427.

Mothersill, C., Crean, M., Lyons, M., McSweeney, J., Mooney, R., O'Reilly, J. and Seymour, C.B. 1998. Expression of delayed toxicity and lethal mutations in the progeny of human cells surviving exposure to radiation and other environmental mutagens. *International Journal of Radiation Biology.* **74**: 673-680.

Mothersill, C. and Seymour, C.B. 2001. Radiation-induced bystander effects: Past history and future directions. *Radiation Research.* **155**: 759-767.

Mothersill. C., Rea, D., Wright, E.G., Lorimore, S.A., Murphy, D., Seymour, C.B. and O'Malley, K. 2001a. Individual variation in the production of a 'bystander signal' following radiation of primary cultures of normal human urothelium. *Carcinogenesis.* **22**: 1465-1471.

Mothersill, C., Lyng, F., Mulford, A., Seymour, C., Cottell, D., Lyons, M. and Ausin, B. 2001b. Effect of low doses of ionizing radiation on cells cultured from the hemotopoietic tissue of the Dublin Bay prawn, *Nephrops norvegicus*. *Radiation Research*. **156**: 241-250.

Mothersill, C. and Seymour, C.B. 2002. Bystander and delayed effects after fractionated radiation exposure. *Radiation Research*. **158**: 626-633.

Mothersill, C., O'Malley, K., Seymour, C.B. 2002. Characterization of a bystander effect induced in human tissue explant cultures by low LET radiation. *Radiation Protection Dosimetry*. **99**: 163-167.

Mothersill, C and Seymour, C. 2004a. Radiation-induced bystander effects and adaptive responses – The Ying and Yang of low dose radiobiology? Review. *Mutation Research*. **568**: 121-128.

Mothersill, C and Seymour, C. 2004b. Radiation-induced bystander effects – implications for cancer. *Nature Reviews, Cancer*. **4**: 158-164.

Mothersill, C., Bucking, C., Smith, R.W., Agnihotri, N., O'Neill, A., Kilemade, M. and Seymour, C.B. 2006. Communication of radiation-induced stress or bystander signals between fish *in vivo*. *Environmental Science and Technology*. **40**: 6859-6864.

Mulgrew, R.S. 1988. Investigation of methodologies for measuring survival of irradiated skin cells in culture. MSc Thesis, University of Dublin.

Murphy, J.B., Nakahara, W. and Sturm, E. 1921. Studies on lymphoid activity. *Journal of Experimental Medicine*. **33**: 423-428.

Nagasawa, H. and Little, J.B. 1992. Induction of sister chromatid exchanges by extremely low doses of α-particles. *Cancer Research*. **52**: 6394-6396.

Nagasawa, H. and Little, J.B. 2002. Bystander effect for chromosomal aberrations induced in wild-type and repair deficient CHO cells by low fluences of α-particles. *Mutation Research*. **508**: 121-129.

Nais, A.H.W., Gilbert, C.W., Lajtha, L.G. and Lange, C.S. 1965. Clone size analysis in the study of cell growth following single or during continuous irradiation. *International Journal of Radiation Biology*. **9**: 275-290.

Narayanan, P.K., Goodwin, E.H. and Lehnert, B.E. 1997. Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Research*. **57**: 3963-3971.

NAS/NRC (National Academy of Sciences/National Research Council). 1959. Committee on Pathological Effects of Atomic Radiation. A Commentary on the Report of the United Nations Scientific Committee on the Effects of Atomic Radiation. NAS/NRC Publication 647. Washington, D.C: National Academy Press.

NAS/NRC (National Academy of Sciences/National Research Council). 1972. Committee on Pathological Effects of Atomic Radiation. A Commentary on the Report of the United Nations Scientific Committee on the Effects on Populations of Exposure to Low Levels of Ionizing Radiation. Washington, D.C: National Academy Press.

Nehls, S. and Segner, H. 2001. Detection of DNA damage in two cell lines from rainbow trout gonad, RTG-2 and RTL-W1, using the comet assay. *Environmental Toxicology*. **16**: 321-329.

O'Dowd, C., Mothersill, C.E., Cairns, M.T., Austin, B., McClean, B., Lyng, F.M., Murphy, J.E.J. 2006. The release of bystander factor(s) from tissue explant cultures of rainbow trout (*Onchorhynchus mykiss*) after exposure to γ radiation. *Radiation Research*. **166**: 611-617.

Olivieri, G., Bodycote, J. and Wolff, S. 1984. Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. *Science*. **223**: 594-597.

Olwell, P.M., Cottell, D.C., Shúilleabháin, S.N., Maderna, P., Seymour, C., Mothersill, C. and Lyng, F.M. 2005. Cytoskeletal reorganization and altered phagocytotic ability in primary cultures of rainbow trout hemopoietic tissue exposed to low-level ionizing radiation. *Radiation Research*. **164**: 45-52.

O'Neill-Mehlenbacher, A., Kilemade, M., Elliott, A., Mothersill, C. and Seymour, C.B. Comparison of direct and bystander effects induced by ionizing radiation in eight fish cell lines. Submitted Nov. 2006.*International Journal of Radiation Biology*.

O'Reilly, J.P., Mothersill, C. and Seymour, C.B. 1994a. Postirradiation expression of lethal mutations in an immortlaized human keratinocyte cell line. *International Journal of Radiation Biology*. **66**: 77-83.

O'Reilly, S., Mothersill, C. and Seymour, C.B. 1994b. Lethal mutations occur at a constant rate in human keratinocytes following carcinogen treatment. *International Journal of Radiation Biology*. **66**: 134-142.

O'Reilly, J.P. and Mothersill, C. 1997. Comparative effects of UV A and UV B on clonogenic survival and delayed cell death in skin cell lines from human and fish.

International Journal of Radiation Biology. **72**: 111-119.

Ossum, C.G., Hoffmann, E.K., Vijayan, M.M., Holts, S.E. and Bols, N.C. 2004. Characterization of a novel fibroblast-like cell line from rainbow trout and responses to sublethal anoxia. *Journal of Fish Biology*. **64**: 1103-1116.

Parsons, W.B., Watkins, C.H., Pease, G.L. and Childs, D.S. 1954. Changes in sternal bone marrow following roentgen-ray therapy to the spleen in chronic granulocytic leukemia. *Cancer*. **7**: 179-189.

Parsons, P.A. 2003. Energy, stress and the invalid linear no-threshold premise: A generalization illustrated by ionizing radiation. *Biogerontology*. **4**: 227-231.

Pirisi L, Yasumoto T, Feller S, Doniger J, DiPaolo J. 1988. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *Journal of Virology*. **61**:1061-1066.

Poncy, J.L., Walter, C., Fritsch, P., Masse, R. and La Fuma, J. 1980. Delayed SCE frequency in rat bone-marrow cells after radon inhalation. Eds. Sanders, C.L., Cross, F.T., Dagle, G.E. and Mahaffrey, J.A. CONF-791002, Technical Information Center, IS Department of Energy.

Ponnaiya, B., Jenkins-Baker, G., Brenner, D.J., Hall, E.J., Randers-Pehrson, G. and Geard, C.R. 2004a. Biological responses in known bystander cells relative to known microbeam-irradiated cells. *Radiation Research*. **162**: 426-432.

Ponnaiya, B., Jenkins-Baker, G., Bigelow, A., Marino, S. and Geard, C.R. 2004b. Detection of chromosomal instability in α-irradiated and bystander human fibroblasts. *Mutation Research*. **568**: 41-48.

Prise, K.M., Belyakov, O.V., Folkard, M., Ozols, A., Shettino, G., Vojnovic, B. and Michael, B.D. 2002. Investigating the cellular effects of isolated radiation tracks using microbeam techniques. *Advanced Space Research*. **30**: 871-876.

Prise, K.M., Folkard, M. and Michael, B.D. 2003. A review of the bystander effect and its implications for low-dose exposure. *Radiation Protection Dosimetry*. **104**: 347-355.

Prise, K.M., Schettino, G., Folkard, M. and Held, K.D. 2005. New insights on cell death from radiation exposure. *The Lancet Oncology*. **6**: 520-528.

Puck, T.T. and Marcus, P.I. 1956. Action of X-rays on mammalian cells. *Journal of Experimental Medicine*. **103**: 653-666.

Rackham, B.D. and Woodhead, D.S. 1984. Effects of chronic gamma-irradiation on the gonads of adult *Ameca splendens* (Osteichthyes: Teleostei). *International Journal of Radiation Biology*. **45**: 645-656.

Reznikoff, C.A., Belair, C., Savelieva, E., Zhai, Y., Pfeifer, K., Yeager, T., Thompson, K.J., DeVries, S., Bindley, C. and Newton, M.A. 1994. Long-term genome stability and minimal genotypic and phenotypic alterations in HPV16 E7- but not E6-, immortalised human uroepithelial cells. *Genes and Development*. **8**: 2227-2240.

Sabatier, L., Dutrillaux, B. and Martin, M.B. 1992. Chromosomal instability. *Nature*. **357**: 548.

Sasaki, M.S. 1995. On the reaction kinetics of the radioadaptive response in cultured mouse cells. *International Journal of Radiation Biology*. **68**: 281-291.

Sawant, S.G., Randers-Pehrson, G., Geard, C.R., Brenner, D.J. and Hall, E.J. 2001. The bystander effect in radiation oncogenesis: I. Transformation in C3H 10T1/2 cells *in vitro* can be initiated in the unirradiated neighbors of irradiated cells. *Radiation Research*. **155**: 397-401.

Schettino, G., Folkard, M., Prise, K.M., Vojnovic, B., Held, K.D. and Micheal, B.D. 2003. Low-dose studies of bystander cell killing with targeted soft X-rays. *Radiation Research*. **160**: 505-511.

Schettino, G., Folkard, M., Micheal, B.D. and Prise, K.M. 2005. Low-dose binary behaviour of bystander cell killing after microbeam irradiation of a single cell with focused Ck X Rays. *Radiation Research*. **163**: 332-336.

Seymour, C.B., Mothersill, C. and Alper, T. 1986. High yields of lethal mutations in somatic mammalian cells that survive ionising radiation. *International Journal of Radiation Biology*. **50**: 167-179.

Seymour, C.B and Mothersill, C. 1989. Lethal mutations, the survival curve shoulder and split-dose recovery. *International Journal of Radiation Biology*. **56**: 999-1010.

Seymour, C.B. and Mothersill, C. 1992. All colonies of CHO cells surviving gamma radiation contain mutated cells. *Mutation Research*. **267**: 19-30.

Seymour, C. and Mothersill, C. 1999. Cell communication and the "bystander effect". *Radiation Research*. **151**: 505-506.

Seymour, C.B. and Mothersill, C.E. 2000. Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve.

RadiationResearch. **153**: 508-511.

Shadley, J.D. and Wiencke, J.K. 1989. Induction of the adaptive response by X-rays is dependent on radiation sensitivity. *International Journal of Radiation Biology*. **56**: 107-118.

Shao, C., Furusawa, Y., Aoki, M., Matsumoto, H. and Ando, K. 2002. Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells. *International Journal of Radiation Biology*. **78**: 837-844.

Shao, C., Furusawa, Y., Kobayashi, Y., Funayama, T. and Wada, S. 2003. Bystander effect induced by counted high-LET particles in confluenct human fibroblasts: A mechanistic study. *Federation of American Societies for Experimental Biology*. **17**: 1422-1427.

Shima, A. and Shimada, A. 1991. Development of a possible nonmammalian test system for radiation-induced germ-cell mutagenesis using a fish, the Japanese medaka (*Oryzias latipes*). *Proceedings of the National Academy of Science USA*. **88**: 2545-2549.

Short, S.C., Woodcock, M., Marples, B. and Joiner, M.C. 2003. The effects of cell cycle phase on low dose hyper-radiosensitivity. *International Journal of Radiation Biology*. **79**: 99-105.

Sonveaux, P., Dessy, C., Brouet, A., Jordan, B.F., Gregoire, V., Gallez, B., Balligand, J.L. and Ferron, O. 2002. Modulation of the tumor vasculature functionality by ionizing radiation accounts for tumor radiosensitization and promotes gene delivery. *FASEB Journal*. **16**: 1979-1981.

Southam, C.M. and Erhlich, J. 1943. Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture. *Phycopathology*. **33**: 517-724.

Stamato, T., Weinstein, R., Peters, B., Hu, J., Giacca, A. 1987. Delayed mutation in Chinese hamster cells. *Somatic Cell Molecular Genetics*. **13**: 57-66.

Stebbing, ARD. 1981. The kinetics of growth control in a colonial hydroid. *Journal of the Marine Biology Association of the United Kingdom*. **61**: 35-63.

Stern, H.M and Zon, L.I. 2003. Cancer genetics and drug discovery in the zebrafish. *Nature Reviews, Cancer*. **3**: 1-7.

Tlsty, T.D., Margolin, B.H. and Lum, K. 1989. Differences in the rates of gene amplification in nontumourigenic and tumourigenic cell lines as measured by Luria-Delbruck fluctuation analysis. *Proceedings of the National Academy of*

Sciences, USA. **86**: 9441-9445.

Ulsh, B., Hinton, T.G., Congdon, J.D., Dugan, L.C., Whicker, F.W. and Bedford, J.S. 2003. Environmental biodosimetry: a biologically relevant tool for ecological risk assessment and biomonitoring. *Journal of Environmental Radioactivity.* **66**: 121-139.

Ulsh, B.A., Miller, S.M., Mallory, F.F., Mitchel, R.E., Morrison, D.P. and Boreham, D.R. 2004. Cytogenetic dose-response and adaptive response in cells of ungulate species exposed to ionizing radiation. *Journal of Environmental Radioactivity.* **74**: 73-81.

UNSCEAR (United Nations Scientific Committee on the Effects of Atomic Radiation). 1958. Report of the United Nations Scientific Committee on the Effects of Atomic Radiation. General Assembly Official Records: Thirteenth Session Supplement No. 17 (A/3838). New York, NY: United Nations.

Vijayalaxmi, Leal, B.Z., Deahl, T.S. and Meltz, M.L. 1995. Variability in adaptive response to low dose radiation in human blood lymphocytes: consistent results from chromosome aberrations and micronuclei. *Mutation Research.* **348**: 45-50.

Watson, G.E., Lorimore, S.A., MacDonald, D.A. and Wright, E.G. 2000. Chromosomal instability in unirradiated cells induced in vivo by a bystander effect of ionizing radiation. *Cancer Research.* **60**: 5608-5611.

Wolf, K. and Quimby, M. 1962. Established eurythermic line of fish cells *in vitro.* *Science.* **135**: 1065-1066.

Wolff, S. 1998. The adaptive response in radiobiology: evolving insights and implications. *Environmental Health Perspectives.* **106S**: 277-283.

Woodhead, D.S. 1970. The assessment of the radiation dose to developing fish embryos due to the accumulation of radioactivity by the egg. *Radiation Research.* **43**: 582-597.

Yin, E., Nelson, D.O., Coleman, M.A, Peterson, L.E and Wyrobek, A.J. 2003. Gene expression changes in mouse brain after exposure to low-dose ionizing radiation. *International Journal of Radiation Biology.* **79**: 759-775.

Yonezawa, M., Misonoh, J. and Hosokawa, Y. 1996. Two types of X-ray induced radioresistance in mice: presence of 4 dose ranges with distinct biological effects. *Mutation Research.* **358**: 237-243.

Zhang, L. 1995. Cytogenetic adaptive response induced by pre-exopsure in human

lymphocytes and marrow cells of mice. *Mutation Research*. **334**: 33-37.

Zhou, H., Randers-Pehrson, C.A., Waldren, D., Vannais, E., Hall, E.J. and Hei., T. 2000. Induction of a bystander mutagenic effect of α-particles in mammalian cells. *Proceedings of the National Academy of Sciences, USA*. **97**: 2099-2104.

Zhou, H., Randers-Pehrson, G., Suzuki, M., Waldren, C.A. and Hei, T.K. 2002. Genotoxic damage in non-irradiated cells: Contribution from the bystander effect. *Radiation Protection Dosimetry*. **99**: 227-232.

Appendix I: Cell numbers plated for all directly irradiated clonogenic cell lines

Dose (Gy)	RTG-2	RT-GillW1	CHSE-214	ZEB 2J	HPV-G
0 and all Recipient Cells for each corresponding cell line	3,000	2,000	600	20,000	500
0.5	3,000	2,000	800	20,000	500
1.0	4,000	2,000	1,000	30,000	500
2.5	5,000	3,000	1,500	40,000	700
5.0	10,000	5,000	5,000	50,000	1,000

Appendix II. Raw data for growth curves for clonogenic cell lines (Figure 7a)

CHSE-214

Cell Number	S.E.M.	Time (Days)
44032	24073.684	1
378686	97497.296	4
415968.668	20130.388	7
1971486	287228.19	11
3847810.668	999964.01	13
5188716	1351384.9	15
7993972.668	1122886	18
10734824	1120057.1	21
12630372	1238580	25
15906766.67	243634.11	29

RT-Gill W1

Cell Number	S.E.M.	Time (Days)
22206.66667	2404.628	1
56752.88932	5033.476	5
84972	16372.824	9
139528.444	36717.232	12
276143.112	28807.568	16
590543.1108	71640.288	21
750949.332	294754.9	26
2756586.666	296777.43	31
4237276	410234.13	36
4583918.668	188934.22	41

ZEB 2J S.E.M.

Cell Number	S.E.M.	Time (Days)
24020	2856.108	1
30439.112	5282.092	4
32342.668	2299.684	8
69788.444	16347.128	12
84995.78	14519.768	17
101841.332	15053.62	22
93719.556	10875.472	27
108478.216	9586.08	32
127807.112	9025.908	37
252186	32381.248	42

HPV-G

Cell Number	S.E.M.	Time (Days)
16016	351.6	3
30520	2330	4
51220	1114	5
134380	9283.2	6
131676	9313.6	7
535544	12825.2	8
910912	33270.8	9
1329732	52366	10
2040976	61793.6	11
2557688	19178	12
3248252	19966.8	13
4732000	78232.4	14
5368000	158373.2	15
8212000	248946.8	16
10348000	126214.4	17
10188000	166736	18
11132000	241353.2	19
11264000	42240	20

RTG-2

Cell Number	S.E.M.	Time (Days)
72797.776	31240.392	1
94325.332	26211.768	4
174908.444	23545.116	7
451473.332	180390.99	11
747056.444	249125.69	14
1302227.556	462008.96	18

2637628	424114.16	21
3270777.332	120504	25
6401458.668	402991.04	28
8954185.336	2416324.3	34
16825770.67	5512791.9	38

10824000	95786.8	21

Appendix III. Raw data for growth curves for non-clonogenic cell lines (Figure 7b)

PBLE

Cell Number	S.E.M.	Time (Days)
20038	799.032	1
149490	934.796	4
148123.334	4132.804	7
280746.666	88768.3	11
206646	51600.412	13
358809.334	76035.664	15
539420	129544.792	18
1094784	49208.976	21
1691475.334	188212.496	25
1953434	234865.516	28

EPC

Cell Number	S.E.M.	Time (Days)
24567.112	2489.376	1
25100.888	5383.64	4
100794.224	15327.176	8
370992.888	117787.68	12
855070.668	247027.308	16
2000309.332	428792.332	19
8521699.112	2164541.788	23
21844400	3900405.696	26
30660863.11	2486397.392	29
23114805.34	2755749.436	32

RTS-34st

Cell Number	S.E.M.	Time (Days)
58580	4259.612	1
60421.334	10689.568	5
305164.666	130792.6	7
131558.666	9909.864	9
392399.334	40802.42	12
218834	25926.776	15
274550	37050.98	19
512162	166697.596	22
612472	17243.288	26
594020	105493.032	29
3590261.332	109862.836	34
5253893.332	655318.612	37

RTS-pBk+

Cell Number	S.E.M.	Time (Days)
68864	11573.924	1
72439.334	9307.884	5
247402	81921.148	7
330282.666	33020.944	9
399076	58064.78	12
603782	83244.852	15
1462728	421789.196	19
3039820	1148579	22
4279158	1019827.584	26

Appendix IV. Raw data for Survival curves of all clonogenic cell lines. (Figure 8).

CHSE-214

Dose (Gy)	Mean	% S.F.	S.E.M.
0	42.2	100	1.8486292
0.5	60.8	106.2	1.9249168
1	81.3	98.7	1.6921684
2.5	119.3	85.9	1.4654355
5	356.8	40.6	0.6082195
10	442	40	0.2407756
15	395.2	18.7	0.1154252

RT-Gill W1

Dose (Gy)	Mean	% S.F.	S.E.M.
0	141.8	100	1.0655149
0.5	176.9	84.7	0.9289436
1	250.4	84.2	0.9352462
2.5	272.5	71.5	0.7043334
5	327.1	53.9	0.496565
10	201.9	7.4	0.0542847
15	63.9	1.2	0.0110867

HPV-G

Dose (Gy)	Mean	% S.F.	S.E.M.
0	37	100	1.186342
0.5	27.7	74.8	0.6654639
1	39	63.6	0.5462269
2.5	23.7	29.9	0.3382971
5	21.7	20.6	0.6010179
10	7.5	1.5	0.0442848
15	11.0	0.1	0.0117699

RTG-2

Dose (Gy)	Mean	% S.F.	S.E.M.
0	156.3	100	0.8402575
0.5	158.7	86.9	0.5780019
1	158.7	78.3	0.5122419
2.5	159.7	40.6	0.2730089
5	158.3	22.5	0.1380044
10	330	12.1	0.0157692
15	70.0	2.5	0.1308725

ZEB 2J

Dose (Gy)	Mean	% S.F.	S.E.M.
0	255.5	100	0.1304
0.5	251.2	98.3	0.1111387
1	341.8	89.2	0.1429243
2.5	384.6	75.3	0.0863585
5	426.5	66.8	0.0689314

Appendix V. Raw data from bystander experiments for all clonogenic cell lines (Figures 9 and 10).

RTG-2

Dose	Direct Mean	Direct %S.F.	S.E.M.	Recip Mean	Recip %S.F.	S.E.M.	HPV-G Recip	HPV-G %S.F.	S.E.M.
0	88.3	100	0.840257	95	100	1.0539164	24.8	100	0.6636215
0.5	76.7	86.9	0.578002	85.8	90.4	0.8053891	32.8	132.2	1.2265491
1	92.7	78.3	0.512242	86.3	90.9	0.8532245	28.3	114.1	0.9376738
2.5	59.8	40.6	0.273009	84.2	88.6	0.8986995	40.5	163.1	0.9707925
5	66	22.5	0.138004	114.3	120.4	0.937081	36.8	148.3	1.3319393

ZEB 2J

Dose	Direct Mean	Direct %S.F.	S.E.M.	Recip Mean	Recip %S.F.	S.E.M.	HPV-G Recip	HPV-G %S.F.	S.E.M.
0	255.5	100	0.1304	372.6	100	5.4325125	154.7	100	0.735004
0.5	251.2	98.3	0.111139	349.3	93.8	5.1151827	131	84.7	6.7753437
1	341.8	89.2	0.142924	331.5	89	4.2611007	165.7	107.1	2.1490618
2.5	384.6	75.3	0.086359	363.2	97.5	6.5808689	154	99.6	1.8156618
5	426.5	66.8	0.068931	316.3	84.9	11.505846	147.3	95.3	3.0161109

CHSE-214

Dose	Direct Mean	Direct %S.F.	S.E.M.	Recip Mean	Recip %S.F.	S.E.M.	HPV-G Recip	HPV-G %S.F.	S.E.M.
0	49.5	100	1.848629	51.2	100	2.0495918	64.8	100	1.6937878
0.5	70.1	106.2	1.924917	49.2	96.1	1.9452044	64.9	105.9417808	1.7046565
1	81.4	98.7	1.692168	50.2	98	1.9520402	67	113.293379	2.195571
2.5	106.3	85.9	1.465436	49.4	96.4	1.9819722	69.6	109.1818874	1.8033111
5	167.4	40.6	0.60822	46.9	91.7	1.8178254	65.3	103.2572298	1.9661095

RT-Gill
W1

Dose	Direct Mean	Direct %S.F.	S.E.M.	Recip Mean	Recip %S.F.	S.E.M.	HPV-G Recip	HPV-G %S.F.	S.E.M.
0	96.6	100	1.065515	60.9	100	0.3149099	76.7	100	4.0192438
0.5	81.8	84.7	0.928944	64.9	106.5	0.3788588	83.2	108.6	4.5554092
1	81.3	84.2	0.935246	66.8	109.7	0.313302	79.8	104.1	3.8793902
2.5	99.8	71.5	0.704333	68.8	112.9	0.2772375	86.6	112.9	4.3264566
5	124.8	53.9	0.496565	75	123.1	0.3080911	86.8	113.2	4.3499285

HPV-G

Dose	Direct Mean	Direct %S.F.	S.E.M.	Recip Mean	Recip %S.F.	S.E.M.
0	37	100	1.1127	26.3	100	0.8155314
0.5	27.7	85.3625	0.868	23	87.3	0.8679874
1	39	74.9	0.7725	19	72.2	0.6669507
2.5	23.7	45.0875	0.5267	16.7	63.3	0.4787538
5	21.7	21.4	0.4866	16.3	62	0.5455501

Appendix VI. Media only experiments for all clonogenic cell lines (n=3) (Figure 11).

CHSE

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	109.7	18.3	100	0.6994883
0.5	110.3	18.4	100.6	0.5365472
1	110.3	18.4	100.6	1.038051
2.5	109	18.2	99.4	0.6020376
5	110.3	18.4	100.6	0.5535913

RT-Gill

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	124.3	6.2	100	0.2059955
0.5	125.3	6.3	100.8	0.1832051
1	125.3	6.3	100.8	0.1533425
2.5	125.3	6.3	100.8	0.2836635
5	125.3	6.3	100.8	0.2557141

RTG-2

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	156.3	5.2	100	0.6259345
0.5	158.7	5.3	101.5	0.5512365
1	158.7	5.3	101.5	0.4530297
2.5	159.7	5.3	102.1	0.496561
5	158.3	5.3	101.3	0.454132

HPV-G

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	51.3	10.3	100	0.2494438
0.5	51	10.2	99.4	0.2098612
1	52	10.4	101.3	0.2127387
2.5	51	10.2	99.4	0.3882118
5	51	10.2	99.4	0.2898995

Appendix VII. Raw data for recipient non-clonogenic fish cell lines from bystander experiments (Figure 12).

EPC

Dose (Gy)	HPV-G Recip Mean	P.E.	% S.F.	S.E.M.
0	49.1	8.9	100	0.9804746
0.5	52.8	9.6	107.5	1.436217
1	54.1	9.8	110.2	1.1038368
2.5	55.1	10	112.2	1.2890733
5	48.8	8.9	99.3	1.1591219

RTS-pBk+

Dose (Gy)	HPV-G Recip Mean	P.E.	% S.F.	S.E.M.
0	49.3	9.9	100	0.7586538
0.5	52.4	10.5	106.3	1.1779492
1	50	10	101.4	0.8825766
2.5	46.6	9.3	94.4	0.664802
5	42.9	8.6	86.9	0.7181747

PBLE

Dose (Gy)	HPV-G Recip Mean	P.E.	% S.F.	S.E.M.
0	19.7	3.9	100	0.6377042
0.5	20.3	4.1	103.4	0.6434431
1	21.4	4.3	109	0.7488445
2.5	19.6	3.9	99.4	0.7386543
5	21.8	4.4	110.7	0.8179749

RTS-34st

Dose (Gy)	HPV-G Recip Mean	P.E.	% S.F.	S.E.M.
0	26.9	5.4	100	0.7340064
0.5	29.7	5.9	110.3	1.3577447
1	25.7	5.1	95.5	1.3131408
2.5	28.8	5.8	107	1.3053229
5	25.2	5	93.8	1.3614121

Appendix VIII. Initial Survival Fraction (n=3) and Progeny Survival Fraction (n=9) for RTG-2 cell line for the DCD/Lethal Mutation Experiment (Figure 13).

ISF

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	14.3	0.5	100	0.1224405
0.5	16.7	0.6	116.3	0.1285492
2.5	13.7	0.3	57.3	0.0728062
10	8.7	0.1	12.1	0.0157692

PSF

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	128.4	4.3	100	0.9818815
0.5	121.2	4	94.3	0.6829291
2.5	112.4	3.7	87.4	0.7221243
10	113.3	3.8	88	0.7731291

Appendix IX. Raw data of RTG-2 uncorrected growth curve cell counts for lethal mutation experiment at different doses (Figure 14).

0 Gy

Time (Days)	Cell Number	S.E.M
4	52826.667	173.422
8	118844	4935.158
12	403873.333	2375.906
16	1174414.667	16010.568
20	2530584	29419.156
24	3218880	39848.309
28	2791936	39182.868
32	2930648	45935.112
36	3220000	59680.137
40	3355408	10551.623

2.5 Gy

Time (Days)	Cell Number	S.E.M
4	52640	119.069
8	134456	1891.848
12	264548	1393.873
16	779576	7157.848
20	1967952	8833.479
24	4198293.333	66577.275
28	3039512	24775.067
32	3738658.667	40640.824
36	3636528	21984.595
40	2795688	43178.429

0.5Gy

Time (Days)	Cell Number	S.E.M
4	66042.667	135.815
8	176141.333	2370.891
12	278470.667	4367.546
16	1079344	7956.089
20	2702560	52225.942
24	3205968	18081.692
28	3158232	84830.274
32	2879128	70907.291
36	3295264	41119.656
40	3172960	16315.629

10 Gy

Time (Days)	Cell Number	S.E.M
4	37352	367.839
8	73920	1034.759
12	81685.333	747.376
16	145636	3308.093
20	259224	4313.763
24	759174.667	13090.318
28	1075928	7659.966
32	1507044	40104.218
36	2151128	19681.633
40	1700496	43171.102

Appendix X: Raw data of RTG-2 corrected for P.E. on the day of counting for the growth curve (Fig 14)

Days post initial plating	Uncorrected count	Control P.E.	Corrected count	S.E.M.
0Gy				
4	52830.67	2.9	1532.08937	8.7
8	118844	5	5942.2	6.9
12	403873.33	1.8	7269.71998	6.2
16	1174414.67	1.7	19965.0494	10.5
20	2527672	1.1	27804.392	3.7
24	3218880	2.2	70815.36	9.9
28	2791936	1.2	33503.232	3.9
32	2930648	1.2	35167.776	2.6
36	3220000	4.2	135240	7.3
40	3355408	5.5	184547.44	8
0.5Gy				
4	66042.67	1.6	1056.68269	9.2
8	176141.32	3.6	6341.08752	7.8
12	278470.67	2.1	5847.88403	4.3
16	1079344	2.1	22666.224	6.5
20	2697432	0.9	24276.888	4
24	3205968	0.9	28853.712	5
28	3158232	1.8	56848.176	4.4
32	2879128	1.9	54703.432	5.6
36	3295264	3.4	112038.976	5.6
40	3172960	5.2	164993.92	4.2
2.5Gy				
4	52640	1.4	736.96	4.7
8	134456	2.4	3226.944	4
12	264548	1.4	3703.672	4.9
16	779576	2.5	19489.4	9.6
20	1966664	1.2	23599.968	5.6
24	4200000	1.2	50400	4.6
28	3039512	2.4	72948.288	4.7
32	3738658.67	1.9	71034.5147	5.9
36	3636528	4.4	160007.232	5.9
40	2795688	5.1	142580.088	8.4
10Gy				
4	37352	0.1	37.352	0.6
8	73920	0.2	147.84	1.2
12	81685.32	0.4	326.74128	2.7
16	145636	0.7	1019.452	2.2
20	256144	1.1	2817.584	2.9
24	759174.67	1	7591.74668	2.9
28	1075928	1.7	18290.776	4.6
32	1507044	1.6	24112.704	4.5
36	2151128	2.9	62382.712	3
40	1700496	2.9	49314.384	9.4

Appendix XII. Cell Kinetics Raw Data (Figure 15).

Count 1 (Day 4)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	88	2.9	100	0.43947
0.5	48.6	1.6	55.3	0.36976
2.5	43.1	1.4	48.9	0.22576
10	3	0.1	3.4	0.02443

Count 2 (Day 8)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	150.9	5	100	0.34311
0.5	108.9	3.6	72.6	0.32511
2.5	72.2	2.4	48.3	0.18276
10	4.7	0.2	3.1	0.04403

Count 3 (Day 12)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	53	1.8	100	0.30814
0.5	62.1	2.1	116.8	0.29643
2.5	40.9	1.4	77.1	0.24213
10	12.3	0.4	23.3	0.10809

Count 4 (Day 16)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	49.7	1.7	100	0.52217
0.5	64.3	2.1	129.6	0.5308
2.5	74.2	2.5	149.5	0.64837
10	19.6	0.7	39.4	0.165

Count 5 (Day 20)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	34.1	1.1	100	0.18386
0.5	25.9	0.9	75.8	0.17229
2.5	37.6	1.2	110	0.24539
10	32	1.1	93.7	0.16008

Count 6 (Day 24)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	65.3	2.2	100	0.493006
0.5	28.2	0.9	43.3	0.23158
2.5	37.1	1.2	56.9	0.257218
10	31.1	1	47.7	0.194398

Count 7 (Day 28)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	82.7	1.2	100	0.084175
0.5	51.9	1.8	151.6	0.205257
2.5	72.6	2.4	201.6	0.205257
10	49.9	1.7	138.8	0.183676

Count 8 (Day 32)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	36.1	1.2	100	0.127215
0.5	56.2	1.9	156.3	0.244133
2.5	57.7	1.9	159.9	0.251853
10	49	1.6	136.1	0.202044

Count 9 (Day 36)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	126.2	4.2	100	0.366957
0.5	102.8	3.4	81.2	0.28939
2.5	131	4.4	103.7	0.339976
10	86.7	2.9	68.6	0.20756

Count 10 (Day 40)

Dose (Gy)	Mean	P.E.	% S.F.	S.E.M.
0	165.9	5.5	100	0.401252
0.5	155.9	5.2	93.8	0.300053
2.5	152.7	5.1	92	0.382159
10	88.2	2.9	53.3	0.347643