

ABSTRACT

EXPLORING THE BYSTANDER EFFECT FOLLOWING VERY LOW DOSE RADIATION (Under direction of Roberta M. Johnke, PhD) Department of Biology, July 2010.

Bystander effects are defined as the phenomenon in which unirradiated cells respond biologically when their neighbors are irradiated. The exact mechanisms of these cellular responses are still not known, especially in the region considered to be very low dose radiation (<20cGy). Many studies have shown cytokine levels are altered following exposure to radiation and may play a significant role in bringing about bystander responses.

A non-tumorigenic (MCF10A) and tumorigenic (A375) cell line were used to determine if chemical factors released from irradiated MCF10A cells will bring about bystander-induced proliferation on naïve (unirradiated) A375 or MCF10A cells using the Irradiated Conditioned Medium (ICM) model. A transfer of conditioned medium 5 hours post-irradiation appeared to bring about a marked bystander response in the naïve A375 cultures which were observed to be maximal between 2 and 6 cGy. This response was not observed at the 1 hour transfer of conditioned medium. Additionally, the data suggest that there is no temporal correlation between the proliferative bystander effects elicited and the levels of IL-6 within the conditioned medium. The data indicate that the MCF10A ICM IL-8 levels were statistically significant only at the 6 cGy dose at 1 hour. Although a significant proliferative bystander effect was observed in A375 cells when exposed to 5 hour MCF10A ICM, the data suggest that both IL-6 and IL-8 do not contribute significantly to this proliferative response.

©Copyright 2010

Sarah Elizabeth Hollis

EXPLORING THE BYSTANDER EFFECT FOLLOWING VERY LOW DOSE
RADIATION

A Thesis

Presented To

The Faculty of the Department of Biology
East Carolina University

In Partial Fulfillment

of the Requirements for the Degree

Master of Science in Molecular Biology and Biotechnology

by

Sarah Elizabeth Hollis

July, 2010

APPROVED BY:

DIRECTOR OF THESIS: _____
Roberta M. Johnke, PhD

COMMITTEE MEMBER: _____
C. Ruth Kempf, PhD

COMMITTEE MEMBER: _____
Baohong Zhang, PhD

COMMITTEE MEMBER: _____
Paul Hager, PhD

CHAIR OF THE DEPARTMENT OF BIOLOGY:

Jeff McKinnon, PhD

DEAN OF THE GRADUATE SCHOOL:

Paul J. Gemperline, PhD

Acknowledgements

There are so many people that have helped me along my journey through graduate school. First, I would like to thank my thesis advisor Dr. Roberta Johnke for all the guidance and direction that she has given me through this project. I would also like to Dr. C. Ruth Kempf for the generous support that she has provided.

To Jacquelyn Long, thank you for teaching me laboratory techniques and for sharing your expertise in the lab. I would also like to thank the members of my thesis committee: Dr. Baohong Zhang, Dr. Paul Hager and Dr. C. Ruth Kempf for all the valuable suggestions, guidance and feedback. Thank you to Linda S. Steelman for her valuable insight, and to everyone that has played any part in my journey, again thank you.

This work was supported by funds provided by the U.S. Army Research Office, Grant Title Superstructural Particle Evaluation and Characteristics with Targeted Reaction, C.R. Kempf, Principal Investigator. Grant # W911NF-09-1-0404.

In memory of my father, Luther E. Hollis, I dedicate this to you. Your encouragement and endearing love for me I remember every day. I know you would be proud.

TABLE OF CONTENTS

Acknowledgements.....	v
List of Figures.....	viii
Abbreviations.....	ix
 Chapter One.....	 1
Introduction.....	1
Background.....	4
Experimental Models.....	7
Hypothesis and Specific Aims.....	8
References.....	10
 Chapter Two.....	 16
Introduction.....	17
Materials and Methods.....	19
<i>Cell Lines</i>	19
<i>MCF10A Culture</i>	19
<i>A375 Culture</i>	20
<i>Determination of Bystander Effects</i>	20
<i>WST-1 Assay</i>	22
<i>Statistical Analysis</i>	23
Results.....	23
<i>1 hr. A375 Transfer to MCF10A</i>	23

5 hr. A375 Transfer to MCF10A.....	23
1 hr. MCF10A Transfer to MCF10A.....	24
5 hr. MCF10A Transfer to MCF10A.....	25
A375 Response to A375 Irradiated Conditioned Medium.....	25
Discussion.....	26
References.....	31
 Chapter 3.....	 43
Introduction.....	44
Materials and Methods.....	47
<i>Culture of MCF10A and A375 Cell Lines.....</i>	<i>47</i>
<i>Cytokines.....</i>	<i>47</i>
<i>Removal of Supernatant Post Irradiation.....</i>	<i>48</i>
<i>Enzyme-Linked-Immuno-Sorbent-Assay (ELISA).....</i>	<i>48</i>
<i>Statistical Analysis.....</i>	<i>49</i>
Results.....	49
<i>IL-6 Activity.....</i>	<i>49</i>
<i>IL-8 Activity.....</i>	<i>49</i>
Discussion.....	50
References.....	52

LIST OF FIGURES

Figure 1-1 Comparison of Old and New Paradigm.....	13
Figure 1-2 Molecular Pathways Affecting Bystander Responses.....	14
Figure 1-3 Key Aspects of Radiation Induced Bystander Responses.....	15
Figure 2-1 Schematic of WST-1 Mechanism.....	34
Figure 2-2 Results of 1 hr. Irradiated Conditioned Medium Transfers.....	35
Figure 2-3 Results of 5 hr. Irradiated Condition Medium Transfers.....	36
Figure 2-4 Change in Proliferation after 5 hr. ICM Transfer.....	37
Figure 2-5 Comparative Results between 1 and 5 hrs.....	38
Figure 2-6 MCF10A Control 1 hr.....	39
Figure 2-7 MCF10A Control 5 hrs.....	40
Figure 2-8 A375 Irradiated Conditioned Medium Transfers.....	41
Figure 2-9 Results of MCF10A at both 1 and 5 hrs.....	42
Figure 3-1 Pathways Regulating Radiation Induced Bystander Effects.....	55
Figure 3-2 IL-6 Pathway.....	56
Figure 3-3 Possible IL-8 Signaling Pathways.....	57
Figure 3-4 IL-6 Expression.....	58
Figure 3-5 IL-8 Expression.....	59

LIST OF ABBREVIATIONS

A375	Human Non- Metastatic Melanoma Cell Line
BE	Bystander Effects
cGy	Centigray
D	Dose in Gray
EGF	Epidermal Growth Factor
ELISA	Enzyme Linked Immuno-Sorbent Assay
FBS	Fetal Bovine Serum
GJIC	Gap Junction-Mediated Intercellular Communication
Gy	Gray
HFLIII	Human Diploid Primary Fibroblast Cell Line
ICM	Irradiated Conditioned Medium
IL-6	Interleukin Six
IL-8	Interleukin Eight
IR	Ionizing Radiation
LD	Low Dose

LQ	Linear Quadratic
MAPK	Mitogen-Activated Protein Kinase
MCF10A	Non-Tumorigenic Human Mammary Breast Epithelial Cell Line
NF- κ B	Nuclear Factor Kappa B
PBS	Phosphate Buffered Saline
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RIBE	Radiation Induced Bystander Effects
SF	Surviving Fraction
VLD	Very Low Dose

CHAPTER 1

Introduction

We are exposed to radiation throughout our lives, either by natural or artificial sources. Medical procedures, nuclear accidents, radon exposure and the increasing threat of nuclear terrorism give reason to understand more completely the effect that low dose radiation is having on the human population. In addition, underdeveloped countries are now gaining more access to medical procedures, including diagnostic and therapeutic radiation, resulting in increasing exposure to low doses of radiation throughout the world. It is important that we know the short and long term effects of these exposures, so that we may have a greater understanding of the physiological risks involved.

Historically, the belief has been that cellular responses after exposure to radiation followed a linear quadratic (LQ) model, $SF = \exp - (\alpha D + \beta D^2)$, where (SF) represents the surviving fraction, D is the dose in Gray (Gy), and α and β are constants. The linear quadratic model (LQ) was used to predict various detrimental effects observed after radiation. Some of these effects induced by irradiation include apoptosis, proliferation, double strand breaks, single strand breaks, DNA-DNA crosslinks, DNA-protein crosslinks, and several types of base damages (Prise, K., Schettino, G., Folkard, M., and Held, K. D. 2005; Rzeszowska-Wolny, J., Przybyszewski, W. M., and Widel, M., 2009; Burdak-Rothkamm, S. and Prise, Kevin M., 2009).

However, old concepts are being challenged, and there is now an evolving paradigm regarding cellular effects at low doses of radiation (< 100 cGy). Historically, in this low dose region of radiation, effects were extrapolated backward toward the origin using the LQ model, but recent findings do not agree with this mode of calculation. In the low dose region, indeed, there are many responses that do not follow the LQ model. These include bystander effects, hyper-radiosensitivity, and adaptive responses (Mothersill, C., Seymour, C., 2004; Prise et al., 2005; Little et al., 2002; Ermakov, Aleksei V., Konkova, Marina S., Kostyuk, Svetlana V., Egolina, Natalya A., Efremova, Liudmila V. and Veiko, Natalya N., 2009; Ballarini, F., Biaggi, M., Ottolenghi, A. and Sapora, O., 2002).

Bystander effects are defined as the phenomenon in which unirradiated cells respond biologically when their neighbors are irradiated (Mothersill et al., 2004; Yang, H., Assad, N., and Held, K., 2005; Huang, L., Kim, Perry M., Nickoloff, Jac A. and Morgan, William F., 2007; Prise et al., 2005; Ermakov et al., 2009; Little et al., 2002). In addition, adaptive responses are another low dose phenomenon that are defined as a radioprotective effect that occurs after high doses of radiation follow a previous low dose of radiation (Huang et al., 2007; De Toledo, Sonia M. and Azzam, Edouard I., 2006; Ermakov et al., 2009). Finally, as reported by Prise et al. (2005), hyper-radiosensitivity effects have been recorded in many different cell lines in the very low dose radiation region (<20 cGy). It has been suggested that bystander effects have many aspects in common with the adaptive responses, and, indeed, possibly represent a competing phenomenon (Ballarini, et al., 2002). Bystander effects, adaptive

responses and hyper-radiosensitivity appear to involve the same signaling pathways (Rzeszowska-Wolny, J. et al., 2009).

Previous studies reported by Rzeszowska-Wolny et al.,(2009) have shown increased survival of bystander cells when nearby cells were treated with a high irradiation dose (Mackonis et al., 2007) and a protective influence of the non-irradiated cells on those targeted by irradiation (Widel et al., 2008). The exact mechanisms of these cellular responses are still not known, especially in the region considered to be very low dose radiation (Fujimori, A., Okayasu, R., Ishihara, H., Yoshida, S., Eguchi-Kasai, K., Nojima, K., Ebisawa, S., and Takahashi, S., 2005). However, there has been increasing recognition of the importance of bystander effects and the impact they may have upon our understanding of radiation-induced biological response mechanisms and developing risk estimations (Yang, et al., 2005; Baverstock, 2000; Hall and Hei, 2003; Little, 2003; Mothersill and Seymour, 2003; Prise et al., 2003; Rzeszowska-Wolny et al., 2009). The need for better understanding of bystander effects, therefore, is becoming increasingly obvious. As a consequence, the objective of this study is to further investigate bystander effects at low dose radiation exposures (<100 cGy), particularly at very low dose radiation exposures (<20cGy). The following sections will summarize the current knowledge in this field.

Background

Bystander Effects

As stated before, the bystander effect refers to the phenomenon by which unirradiated cells respond biologically when their neighboring cells are irradiated. These responses were first identified clearly in 1992 when Nagasawa and Little recorded that, under conditions in which only 1% of the cell population had been traversed by a densely ionizing α particle, 30% of the population had chromosome damage in the form of sister chromatid exchanges (Little, J.B., Azzam, E. I., de Toledo, S. M., Nagasawa, H., (2002); Ballarini, B. et al. 2002). The responses include changes in gene expression (Iyer and Lehnert, 2000; Azzam et al., 2002) and protein expression (Little et al., 2002). These damaging signals were transmitted from the irradiated bystander cells by gap junction mediated intercellular communication. Similar effects have been found to occur *in vivo* (Koturbash, I., Boyko, A., Rodriguez-Juarez, R., McDonald, R.J., Tryndyak, V.P., Kovalchuk, I., Pogribny, I.P., and Kovalchuk, O., 2007; Emerit, I., Oganessian, N., Sarkisian, T., Arutyunyan, R., Pogosian, A., Asrian, K., Levy, A., and Cernjavski, L. (1995). Other genetic effects such as mutations (Zhou et al., 2000; Nagasawa et al., 2003), DNA damage (Azzam et al., 2001; Little et al., 2003), cell killing (Lyng et al., 2002) and malignant transformation (Sawant et al., 2001) have also been observed. As stated by Rzeszowska-Wolny et al.(2009), mutations and chromosomal aberrations induced in bystander cells may potentially lead to tumorigenesis. As well as cellular gene and protein expression changes, increases in cell survival due to the bystander effect following very low dose radiation have also been reported (Shao et al., 2005;

Fujimori et al., 2005). For example, Fujimori et al.(2005) demonstrated that three CXC chemokines were up-regulated in two human melanoma cell lines after being exposed to very low doses of x-rays (<20 cGy). The two cell lines, then in turn, exhibited growth advantages in the presence of these chemokines through their receptors.

To summarize, it is now widely accepted that the shape of the cell survival curve at low doses of radiation deviates significantly from that predicted by the linear quadratic model. Furthermore, phenomena such as induced radioresistance, hyper-radiosensitivity, adaptive responses, and bystander effects have all now been reported to occur in this low dose range of radiation exposure. Consequently, while it was once thought that abortive colonies and mutated cells resulting from direct damage to nuclear DNA were the major consequences of radiation exposure, a new paradigm is evolving regarding cellular response to low dose radiation which encompasses a host of autocrine and paracrine cell signaling responses (Figure 1-1).

Mechanisms of Bystander Effects

The exact mechanisms of bystander effects are not known, but evidence is constantly accumulating that multiple signal transduction pathways are involved (Figure 1-2). For example, changes in junction-mediated intercellular communication (GJIC) have been implicated, as has upregulation of the release of soluble factors that include lipid peroxidase products, inosine nucleotides and cytokines (Azzam et al., 2001; Mothersill and Seymour, 1997, 1998; Zhou et al., 2002; Narayanan et al., 1997; Lehnert and Godwin, 1997; Rzeszowska-Wolny, J. et al., 2009; Przybyszewski, Waldemar M.

and Widel, M., 2009; Fujimori, et al., 2005). While the precise mechanism is not known, many studies have shown that the mechanism underlying bystander effects may involve reactive oxygen species (ROS) such as superoxide radicals (Ballarini, F. et al., 2002; Kevin M Prise et al., 2005; Rzeszowska-Wolny, J. et al., 2009). Many studies have also shown cytokine levels are altered following exposure to radiation and may play a significant role in bringing about bystander responses (Fujimori et al., 2005; Narayanan P K, et al., 1999). As demonstrated by Beetz, A., et al. (1997) IL-8 is a significant factor in the proliferation in human melanoma cells. Other reports show that the inflammatory cytokines IL-6 and IL-8 are induced after exposure to ionizing radiation (Fujimori et al., 2005; Narayanan P K, et al., 1999). Specifically, CXCL8 (IL-8) is involved in response to inflammatory stresses and, as demonstrated by Fujimori et al. (2005) quantitative PCR procedure revealed that this cytokine increased by greater than 2-fold in the cells exposed to low-dose x-rays. To date, studies have suggested that mechanisms underlying bystander responses may vary from cell to cell. For example, Rzeszowska-Wolny et al. (2009) has postulated that the transmitted factors underlying bystander responses are diverse and may depend on cell line, cell type and/or on the physiological state of the cell. What is known, however, is that bystander responses may play a critical role in cellular response to radiation. Indeed, since it has been shown that the bystander effect is a saturable phenomenon (instead of increased responsiveness as the dose increases, the bystander response becomes saturated at relatively low doses - usually less than 100 cGy) Kevin M Prise et al. (2005) and its role in the area of low

dose radiation response could potentially be of significant clinical impact. (See Figure 1-3)

Experimental Models to Study Bystander Effects

There are two models now being used when studying bystander effects, the Irradiated Conditioned Medium transfer model and the Direct Irradiation model (Little et al., 2002; Ballarini, F. et al., 2002). With the Irradiated Conditioned Medium (ICM) transfer model, cells are irradiated and the medium is then transferred to nonirradiated cells. This allows for any signaling factors, growth factors, etc. that have been released into the medium to be transferred to cells that have not been exposed to radiation. It has been found that this transfer of signaling mediators leads to many different responses, such as proliferation, apoptosis and chromosomal aberrations, depending upon the cell type or experimental set up being used (Ballarini, F. et al., 2002). This ICM model allows for the investigation and characterization of the chemical mediators that may be involved in bystander responses *in vivo*.

The second model used for studying bystander effects is Direct Irradiation (Ballarini et al., 2002; Little et al., 2002). In this model, cells are plated, but only a portion of the cells are irradiated. Comparisons of both the irradiated and non-irradiated cells are then performed. Using this model, studies have demonstrated that the nonirradiated cells behave as if they had been directly irradiated (Ballarini, F. et al., 2002; Little et al., 2002).

Hypothesis and Specific Aims

Statement of Hypothesis

The intent of these studies is to better understand the cellular responses that can occur after exposure to low doses of radiation. As stated before, there is a concern throughout the scientific and health professions that an increasing number of the world's population will potentially be exposed to low doses of radiation through such events such as nuclear terrorism, nuclear accidents and the increasing use of diagnostic radiological assessment. This increasing exposure necessitates our gaining a better understanding of the physiological consequences of these events. The purpose of these studies, therefore, is to better understand the expression of cellular mediators that may play a role in radiation-induced bystander effects. It is my hypothesis that one of the bystander responses seen following low dose radiation is an increased proliferative response. This hypothesis is based upon previous findings reported by Fujimori et al. (2005) which show significant cell proliferation is exhibited when medium from healthy non-tumorigenic primary irradiated cell lines is transferred to non-irradiated cancerous cell lines. Using the ICM transfer model for investigation of bystander effects, experiments will investigate the bystander responses occurring in both a non-tumorigenic cell population (MCF10A breast epithelial cells) and a tumorigenic cell population (A375 melanoma cells) following transfer of MCF10A irradiated cell conditioned medium.

Specific Aim One: Use of Conditioned Medium From Irradiated MCF10A Cells to Investigate Proliferative Bystander Effects on the A375 Human Melanoma and the MCF10A Human Breast Epithelial Cells Lines (Chapter 2)

To determine whether a proliferative bystander effect exists in both a tumorigenic (A375 cells) and non-tumorigenic cell (MCF10A cells) model following the transfer of conditioned medium from non-tumorigenic MCF10A cells exposed to a series of very low dose radiation stresses (0, 2, 6, 10, 20 cGy). The Irradiated Conditioned Medium (ICM) transfer model will be used to perform these studies. Our endpoint for response will be determination of increased cellular proliferation using the WST-1 assay.

Specific Aim Two: Determination of Cytokine Levels In Conditioned Medium from MCF10A Cells Following a Series of Very Low Dose Radiation Exposures (Chapter 3)

In an attempt to elucidate possible soluble mediators involved in the bystander response, studies will be performed to measure if changes in the levels of IL-6 and IL-8 in MCF10A irradiated conditioned medium and determine whether they are correlated with a proliferative bystander effect. Determination of cytokine alterations in the irradiated conditioned MCF10A medium will be accomplished using Enzyme Linked-Immuno-Sorbent Assay (ELISA) procedures.

Literature Cited

- Azzam E. I., de Toledo, S. M., and Little, J. B., *Current Cancer Drug Targets* (2004); **4**:53-64.
- Ballarini, F., Biaggi, M., Ottolenghi, A., and Sapor, O., *Mutation Research* (2002); **501**: 1-12.
- Baskar, R., Balajee, Adayabalam S., Geard, Charles R., and Hande, M. Prakash, *The International Journal of Biochemistry and Cell Biology* (2008); **40**: 125-134.
- Beetz, A., Messer, G., Oppel, T., Van Beuningen, D., Peter, R.U., and Kind, P., *International Journal of Radiation Biology* (1997); **72** (1): 33-43.
- Biosource, Invitrogen, Immunoassay Kit, Protocol Booklet, (2007).
- Brenner, D.J., Hlatky, L.R., Hahnfeldt, P.J., Huang, Y., and Sachs, R.K., *Radiation Research* (1998); **150**: 83-91.
- Burdak-Rothkamm, S. and Prise, Kevin M., *European Journal of Pharmacology* (2009); **625**: 151-155.
- De Gonzalez, A. and Darby S., *The Lancet* (2004); **363**(9406): 345-351.
- De Toledo, Sonia, M. and Azzam, Edouard, I., *Dose-Response* (2006); **4** (4): 291-301.
- Emerit, I., Oganessian, N., Sarkisian, T., Arutyunyan, R., Pogolian, A., Asrian, K., Levy, A., and Cernjavski, L., *Radiation Research* (1995); **144**: 198–205.
- Enns, L., Bogen, Kenneth T., Wizniak, J., Murtha, Albert D., and Weinfeld, M., *Molecular Cancer Research* (2004); **2** (10): 557-566.
- Ermakov, Aleksei V., Konkova, Marina S., Kostyuk, Svetlana V., Egolina, Natalya A., Efremova, Liudmila V., and Veiko, Natalya N., *Mutation Research* (2009); **669**: 55-161.
- Fujimori, A., Okayasu, R., Ishihara, H., Yoshida, S., Eguchi-Kasai, K., Nojima, K., Ebisawa, S., and Takahashi, S., *Cancer Research* (2005); **65** ;(22):10159-10163.
- Hamada, N., Matsumoto, H., Hara, T., and Kobayashi, Y., *Radiation Research* (2007); **48**:87-95.
- Huang, L., Kim, Perry M., Nickoloff, Jac A., and Morgan, William F., *Cancer Research* 2007); **67**: 1099.

- Hu, B., Wu, L., Han, W., Zhang, L., Chen, S., Xu, A., Hei, Tom K., and Yu, Z., *Carcinogenesis* (2006); **27** (2): 245–251.
- Iyer R. and Lehnert B.E., *Archive Biochemistry Biophysics* (2000); **376**(1):14-25.
- Koturbash, I., Boyko, A., Rodriguez-Juarez, R., McDonald, R.J., Tryndyak, V.P., Kovalchuk, I., Pogribny, I.P., and Kovalchuk, O., *Carcinogenesis* (2007); **28**: 1831–1838.
- Libermann, Towia A. and Baltimore, D., *Molecular and Cellular Biology* (1990); **10** (5): 2327-2334.
- Little, J.B., Azzam, E. I., deToledo, S. M., and Nagasawa, H., *Radiation Protection Dosimetry* (2002); **99**:159-162.
- Mothersill, C. and Seymour, C., *Mutation Research* (2004); **568**:121-128.
- Norgauer, J., Metzner, B., and Schrtaufstätter, I., *Journal of Immunology* (1996); **156**: 1132-1137.
- Prise, K. and O’Sullivan, J., *Nature* (2009); **9**: 351-360.
- Prise, Kevin M., Schettino, G., Folkard, M., and Held, K., *Lancet Oncology*, (2005); **6**:520-528.
- Rzeszowska-Wolny, J., Herok, R., Widel, M., and Hancock, Ronald, *DNA Repair*, (2009); **8**: 732–738.
- Rzeszowska-Wolny, J., Przybyszewski, W. M., and Widel, M., *European Journal of Pharmacology*, (2009); **625**:156-164.
- Shao C, Furusawa Y, Aoki M, Matsumoto H and Ando K., *Int. J. Radiat. Biol* (2002); **78**, 837–844.
- Soule, Herbert D., Maloney, Terry M., Wolman, Sandra R., Peterson Jr., Ward D., Brenz, R., McGrath, Charles M., Russo, J., Pauley, Robert J., Jones, Richard F., and Brooks, S.C., *Cancer Research* (1990); **50**: 6075-6086.
- Tabata, C., Kubo, H., Tabata, R., Wada, M., Sakuma, K., Ichikawa, M., Fujita, S., Mio, T., and Mishima, M., *American Journal of Physiology- Lung Cell Mol Physiology* (2006); **290**: L597-L606.
- Tsai, Kevin K.C., Stuart, J., Chuang, Yao-Yu E., Little, John B. and Yuan, Z., *Radiation Research* (2009); **172**: 306-313.
- (United Nations Scientific Committee on the Effects of Atomic Radiation. *Sources and effects of ionizing radiation*. New York: United Nations, 2000.

Upadhyay, S., G. Li, et al, *Cancer Research* (1995) **55**(20): 4520-4.

Wu, M. et al, *Breast Cancer Research Treatment* (2004); **84**(1): 3-12.

Yang, H., Assad, N., and Held, K., *Oncogene* (2005); **24**:2096-2103.

FIGURES

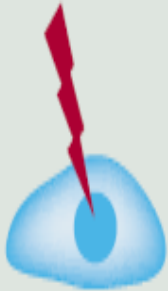
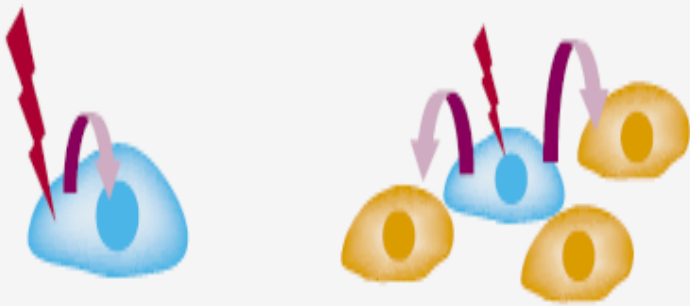
	Old paradigm	New paradigm	
Radiation target	Nuclear DNA	Membrane/cytoplasm	Few cells, any target
Signalling pathway			

Figure 1-1: Comparison of the Old and New Paradigm. Modified from Prise et al., 2005.

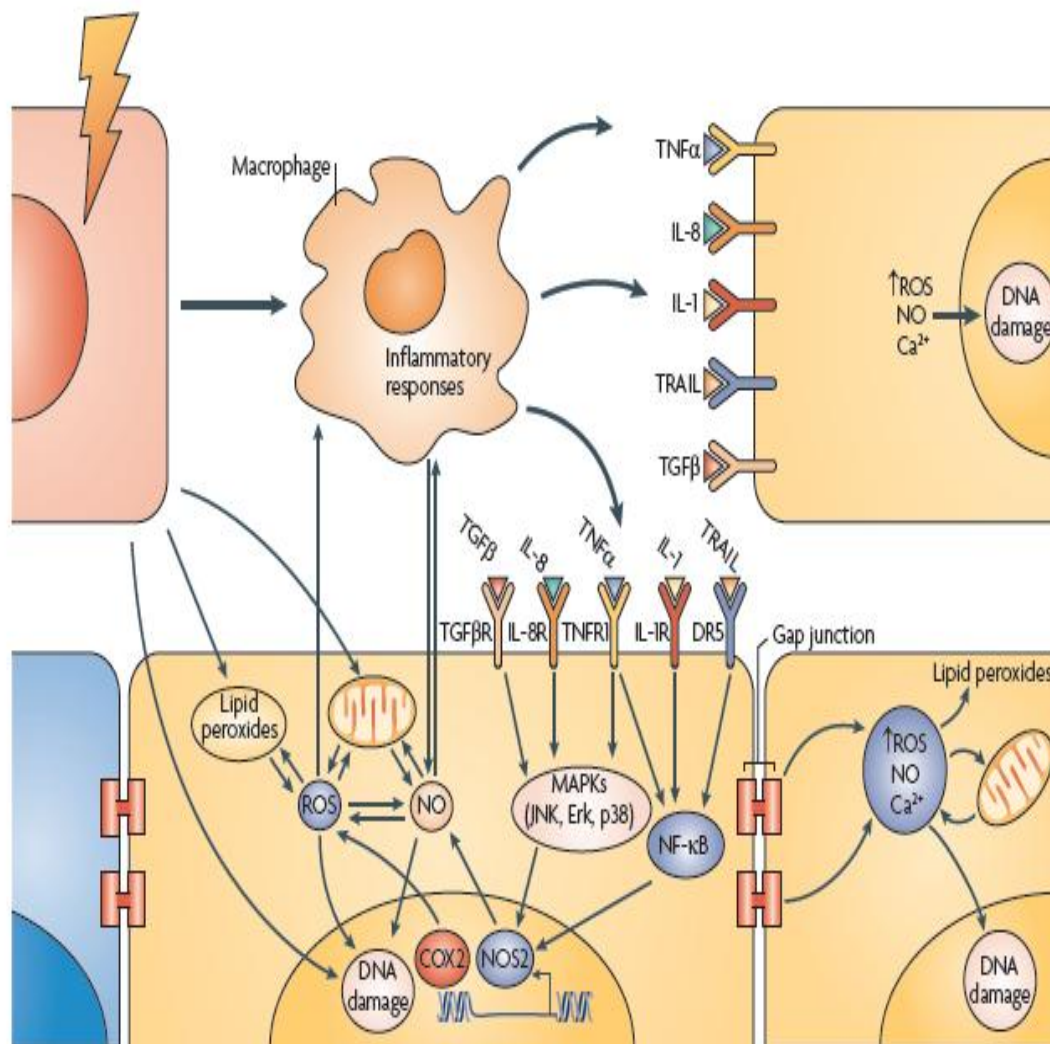


Figure 1-2: Molecular Pathways Affecting Bystander Responses (Prise et al., 2009).

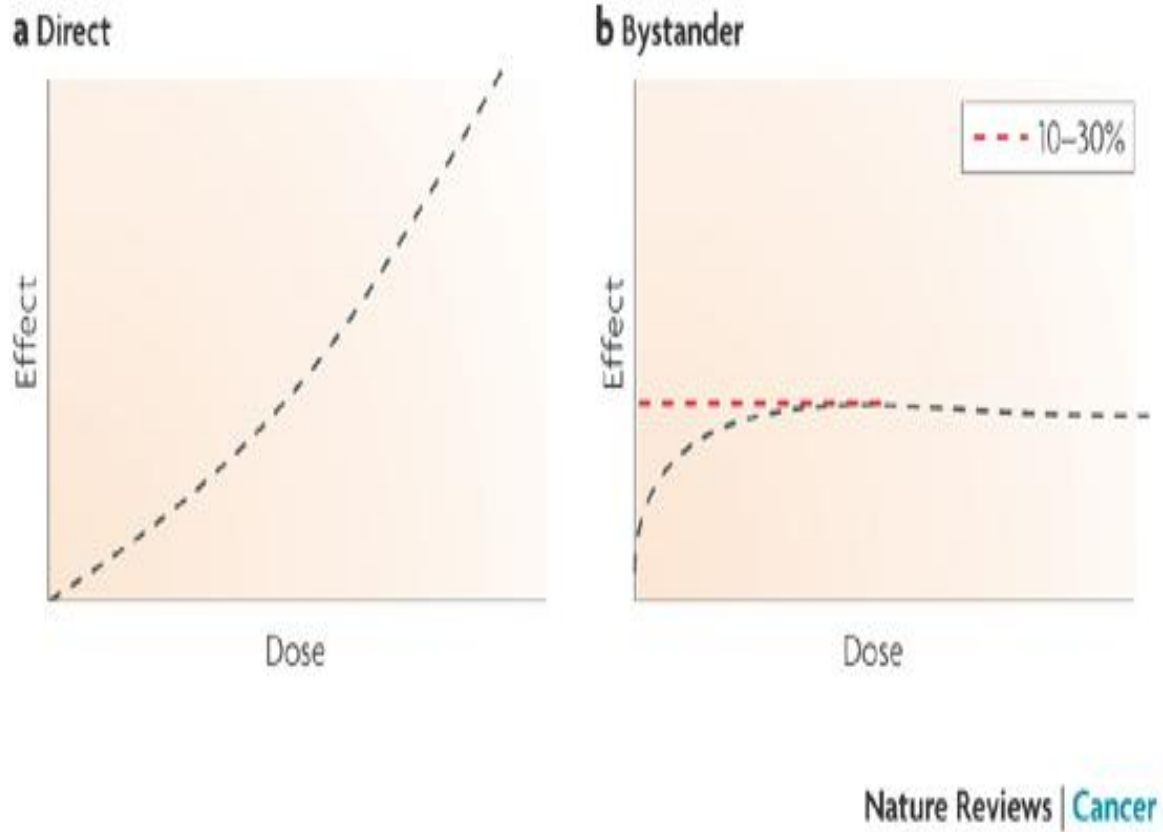


Figure 1-3: Key aspects of radiation-induced bystander responses. Typical dose response for direct (a) and bystander (b) responses are shown, highlighting the commonly observed saturation of response for bystander effects (Prise et al., 2009).

CHAPTER 2

Studies Using the Irradiated Conditioned Medium Transfer Model to Investigate Bystander Responses in the Tumorigenic A375 Cell Line and the Nontumorigenic MCF10A Cell Line

Introduction

Currently, there are very few studies that focus on the proliferative aspect of Bystander Effects (BE) following very low dose radiation. Research surrounding BE has primarily focused on other responses, such as cell killing, DNA lesions, and chromosomal changes. Nevertheless, cellular gene and protein expression changes and increases in cell survival due to the bystander effect following very low dose radiation have been reported. For example, Fujimori et al. (2005), demonstrated that three CXC chemokines were up-regulated in two human melanoma cell lines after being exposed to very low doses of x-rays (<20 cGy). The two cell lines, then in turn, exhibited growth advantages in the presence of these chemokines through their receptors. In addition, Rzeszowska-Wolny et al. (2009) have shown increased survival of bystander cells when nearby cells were treated with a high irradiation dose. Finally, increases in cell proliferation and micronuclei formation were reported by Shao et al. (2002) in human salivary gland neoplastic cells as a result of co-culturing with irradiated cells.

Experimental Models to Study Bystander Effects

There are two models used when studying bystander effects, the Irradiated Conditioned Medium transfer model and the Direct Irradiation model (Little et al., 2002; Ballarini, F. et al., 2002). With the Irradiated Conditioned Medium transfer model, cells are irradiated and the medium is then transferred to nonirradiated cells. This allows for

any signaling factors, growth factors, etc. that have been released into the medium to be transferred to cells that have not been exposed to radiation. It has been found that this transfer of signaling mediators leads to many different responses, such as proliferation, apoptosis and chromosomal aberrations, depending upon the cell type or experimental set up being used (Ballarini, F. et al., 2002). The second model used for studying bystander effects is the Direct Irradiation model (Ballarini, F. et al., 2002; Little et al., 2002). In this model, cells are plated, but only a portion of the cells are irradiated. Comparisons of both the irradiated and non-irradiated cells are then performed. Using this model, reports have demonstrated that the nonirradiated cells behave as if they had been directly irradiated (Ballarini, F. et al., 2002; Little et al., 2002).

Experimental Design

The purpose of these studies is to investigate a possible proliferative bystander response following very low dose exposure to Ionizing Radiation (IR). It is hoped that the data presented will add to the increasing body of knowledge that is developing regarding the contribution of bystander effects in the cellular response to very low dose radiation exposure. In these studies we have chosen to use the *in vitro* ICM transfer procedure as our experimental model for investigating the potential proliferative bystander response. *In vitro* studies were selected because it has been reported that they provide a way to better control the confounding factors (de Toledo et al., 2006), making it easier to interpret the cellular response observed following exposure to very low dose radiation. Nevertheless, although these are *in vitro* studies, it has been proposed that they are relevant to the bystander (BE) observed *in vivo* (de Toledo et al.,

2006; Mothersill et al., 2003 & 2004). The questions asked were: (1) What response would be seen if we irradiated nontumorigenic cultures of MCF10A cells and transferred the conditioned medium to tumorigenic cultures of naïve (unirradiated) A375 cells? (2) What response would be seen if we irradiated nontumorigenic cultures of MCF10A cells and transferred the conditioned medium to naïve nontumorigenic cultures of MCF10A cells? and (3) What response would be seen if we irradiated tumorigenic cultures of A375 cells and transferred it to naïve tumorigenic cultures of A375 cells?

Materials and Methods

Cell Lines

A non-tumorigenic (MCF10A) and tumorigenic (A375) cell line were used in these studies. The objective was to determine if chemical factors released from irradiated MCF10A cells will bring about bystander-induced proliferation on naïve (unirradiated) A375 or MCF10A cells using the ICM. Use of these two lines allows the study of bystander effects upon both similar versus non-similar histological cell types, and also upon non-tumorigenic versus tumorigenic cell populations.

MCF10A Cell Line and Culture

MCF10A is an immortalized human breast epithelial cell line that is non-tumorigenic and exhibits characteristics similar to normal, non-cancerous breast epithelial cells (Bell et al., 2006). The cell line was derived from a 36-year-old female.

Many studies have used MCF10A cells as a non-tumorigenic cell line control for tumorigenic cell models (Upadhyay et al., 1995; Wu et al., 2004). MCF10A cells were obtained from the Karmanos Cancer Institute (Detroit, MI). Under normal cell culture conditions, the cells were grown in Dulbecco's modified Eagle's Medium/Ham's F-12 nutrient mixture (DMEM/F12), containing 15 mM HEPES and 2.5 mM L-glutamine, supplemented with 5% heat inactivated equine serum, 500 ng/ml epidermal growth factor (EGF), 10µg/ml insulin, 100ng/ml gentamicin, and 10ng/ml cholera toxin (hereafter referred to as DMEM/F12 complete) at 37°C and in an atmosphere of 5% CO₂.

A-375 Cell Line and Culture

A375 is a human tumorigenic melanoma cell line derived from a 54-year-old female. It is rapidly growing in culture with a doubling time of 20-22 hours. It is an established model in immunosuppressed mice (Giard DJ, et al., 1973). Furthermore, A375 is a human tumorigenic melanoma cell line with a relatively large number of membrane receptors for CXC chemokines (Fujimori et al., 2005). A375 cells were obtained from ATCC, (Manassas, Va). A375 cells were cultured in DMEM medium with 10% FBS and maintained in 37° C with 5 % CO₂.

Determination of Proliferative Bystander Effects in A375 Cells or MCF10A Cells

These set of experiments were performed to determine if there was a proliferative BE when the ICM from irradiated MCF10A cells was transferred to naïve cell cultures. Specifically, the non-tumorigenic cell line MCF10A (breast epithelial) was

irradiated and the supernatant was transferred to the tumorigenic A375 (human melanoma) cell line and to naïve cell cultures of the same MCF10A cell line 1 hour and 5 hours following the irradiation stress. Briefly, this was performed by plating 80,000 MCF10A cells per 35 cm petri dish, transferring the dishes to an incubator, and maintaining them at 37° C with 5 % CO₂ for twenty four hours, and then irradiating the dishes at different radiation doses (0, 2, 6, 10 and 20 cGy). Cells were irradiated at room temperature using a Siemens Stabilipan x-irradiator (120 KeV, 2 mm Cu filtration, and an SSD of 51.5 inches). The controls were treated with the same experimental conditions, but were not exposed to radiation. One and five hours after irradiation, the conditioned medium from the irradiated cells was transferred to the naïve A375 or MCF10A cell cultures (seeded at 4000 cells per well in a 96 well plate 12 hrs prior to transfer of the ICM). The ICM was left on the naïve cultures for 48 hours and proliferation then analyzed using the WST-1 assay.

Determination of Proliferative Bystander Effects in the A375 Irradiated Conditioned Medium Transfer on A375 Cultures

These experiments were performed to examine the effects that an irradiated tumorigenic culture would have on a naïve tumorigenic cell cultures. Specifically, the carcinogenic cell line A375 was irradiated and the supernatant was transferred to previously plated naïve A375 cells. Similar to above, this was performed by plating 80,000 A375 cells per 35 cm petri dish, transferring the dishes to an incubator, maintaining them at 37° C with 5 % CO₂ for twenty four hours, and then irradiating the dishes at different radiation doses (0, 2, 6, 10 and 20 cGy). Cells were irradiated at

room temperature using a Siemens Stabilipan x-irradiator (SSD 51.5 inches, 120 KeV, 2 mm Cu filtration). The controls were treated with the same experimental conditions, but were not exposed to irradiation. Five hours after irradiation the conditioned medium from the irradiated cells (A375) was transferred to the naïve A375 cell cultures (seeded at 4000 cells per well in a 96 well plate 12 hrs prior to transfer of the ICM). The ICM was left on for 48 hours before determining proliferation using a WST-1 assay.

WST-1 Proliferation Assay

The WST-1 assay is for the quantification of cell proliferation and viability. This is a colorimetric assay based on the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases (Figure 2-1). The principle underlying this assay is that the expansion of viable cell numbers results in an increase in the overall activity of the mitochondrial dehydrogenases in the sample. This corresponds to an increase in formazan dye formation whose absorbance can be measured using a standard multiwell spectrophotometer. The assay has been routinely used in laboratories to determine cell proliferation in response to interactions with drugs, growth factors, cytokines, mitogens, and nutrients. Briefly, WST-1 assay kits were purchased from Takara Bio-Inc. (Otsushiga, Japan), and the assay was performed according to manufacturer's instructions. Briefly, 4000 cells were plated per well in 96 well plates in a final volume of 100 µl/well culture medium in a humidified atmosphere at 37° C, 5% CO₂. After the incubation period of 48 hrs, 10 µl/ well of Premix WST-1 was added to the cells. The absorbance was read at 450 nm after 30 minutes and again at 2 hours. Absorbance readings of samples were also taken at 690 nm to normalize for

background.

Statistical Analysis

Statistical significance was determined using the Student's t-Test. A p value of < 0.05 was used to assess significance.

Results

A375 Cellular Response to MCF10A Irradiated Conditioned Medium

1 hour ICM Transfer Studies

Studies were performed to test for a proliferative bystander effect on naïve A375 cells incubated in medium conditioned for one hour by irradiated MCF10A cells. The data of these 1 hour ICM transfer studies are presented in Figure 2-2, and reveal that no proliferative bystander effect was observed. The results demonstrate that there was no statistical significance found in growth (viability) of cells incubated with conditioned medium from unirradiated (control) and irradiated (0, 2, 6, 10, and 20 cGy) cultures, nor between cells incubated in conditioned medium from MCF210A cells exposed to any of the doses.

5 hour ICM Transfer Studies

Because it was of interest to determine if time was an important variable in elucidating a bystander response, studies were also performed to test for a proliferative bystander effect on naïve A375 cells incubated in medium conditioned for five hours by

irradiated MCF10A cells. As can be seen in Figure 2-3, in contrast to the 1 hr transfer, a 5 hr transfer of conditioned medium appeared to bring about a marked bystander response in the naïve A375 cultures which were observed to be maximal between 2 and 6 cGy. For example, as can be observed in Figure 2-4, which shows the percent changes in proliferation between control cultures and cultures incubated with conditioned medium from MCF10A cells exposed to the various various doses (2, 6, 10, 20 cGy), there was a 23.39% increase in proliferation between the control and 2 cGy, and a 19.66% increase between the control and 6cGy. At 10 cGy, however, the proliferative effect of the CM had returned to control levels, suggesting that this proliferative bystander effect is induced only by the MCF10A cells irradiated at very low doses (< 10 cGy). For ease of comparison of the results between the 1 and 5 hr MCF10A ICM transfer studies have been combined and summarized in Figure 2-5.

MCF10A Cellular Response to MCF10A Irradiated Conditioned Medium

1 hour ICM Transfer Studies

To determine the effect that ICM from MCF10A cells would have on cells of the same histological type, MCF10A cells were irradiated, conditioned medium harvested at 1 hour post irradiation, and transferred to naïve MCF10A cell cultures. The data from these studies are presented in Figure 2-6, and, similar, to the results for the 1 hour ICM transfer studies on A375 cultures, reveal that no proliferative bystander effect was observed. Briefly, the results demonstrated that there was no statistical significance

found in growth (viability) of cells incubated with conditioned medium from unirradiated (control) and irradiated (0, 2, 6, 10, and 20 cGy) cultures, nor between cells incubated in conditioned medium from MCF210A cells exposed to any of the doses.

5 hour ICM Transfer Studies

As stated above, to determine if time was an important variable in causing a bystander response, studies were also performed to test for a proliferative bystander effect on naïve MCF10A cells incubated in medium conditioned for five hours by irradiated MCF10A cells. Results of these studies are shown in Figure 2-7, and suggest that a similar trend was seen at 5 hrs in these studies as was observed in the MCF10A ICM transfer to A375 studies (Figure 2-3), in that an increased proliferative response appears to be present for cultures incubated with conditioned medium from MCF10A cells exposed to doses in the range of 2-10 cGy. However, unlike the studies on A375 cells, in this group of studies, while the trend was apparent, the individual increases did not prove to be statistically different from control.

A375 Cellular Response to A375 Irradiated Conditioned Medium

Bystander effects are often described as responses brought about by nontumorigenic cells such as would be represented by the MCF10A cell model. However, it was of interest to also ascertain if irradiated tumor cells could also elicit a proliferative bystander effect. To investigate this, these studies explored what effect that conditioned medium from irradiated A375 cells would have on naïve cultures of themselves. Only the 5 hour conditioned medium was studied, since this time point was

the one that proved to show positive proliferative results in the MCF10A ICM transfer studies. The results are presented in Figure 2-8. As can be observed, the data revealed that there was no statistical significance found in growth (viability) of cells incubated with conditioned medium from unirradiated (control) and irradiated (0, 2, 6, 10, and 20 cGy) cultures, suggesting that a proliferative bystander response was not induced.

Discussion

As stated previously, there have only been a few studies that focus on the proliferative aspect of Bystander Effects (BE) following very low dose radiation. Research surrounding BE has primarily focused on other responses, such as cell killing, DNA lesions, and chromosomal changes. However, Fujimori et al. (2005) has reported that three CXC chemokines were up-regulated in two human melanoma cell lines (A375 cells being one of them) after being exposed to very low doses of x-rays (<20 cGy) and led to growth advantages. In addition, Rzeszowska-Wolny et al. (2009) have shown increased survival of bystander cells when nearby cells were treated with a high irradiation dose, and, finally, increases in cell proliferation and micronuclei formation were reported by Shao et al. (2002) in human salivary gland neoplastic cells as a result of co-culturing with irradiated cells.

In this study, we attempted to add to the body of accumulating data characterizing possible proliferative bystander effects following low dose radiation exposures. To accomplish this, studies were designed to investigate bystander effects in both tumorigenic and nontumorigenic cell lines using the well established ICM

transfer model. Experiments were planned to ask what response would be seen if (1) we irradiated nontumorigenic cultures of MCF10A cells and transferred the conditioned medium to tumorigenic cultures of naïve (unirradiated) A375 cells?; (2) if we irradiated nontumorigenic cultures of MCF10A cells and transferred the conditioned medium to naïve nontumorigenic cultures of MCF10A cells?; and, finally, (3) if we irradiated tumorigenic cultures A375 cells and transferred the conditioned medium to naïve tumorigenic cultures of A375 cells?

Collectively, the data from these studies demonstrate the following: (1) MCF10 ICM transferred to A375 cultures 1 hr following irradiation did not influence cell growth at any of the radiation doses investigated. However, MCF10A ICM transferred to A375 cultures 5 hrs post irradiation significantly enhanced cell proliferation at doses less than 10 cGy when compared to control cultures, but not at the higher doses (10 & 20 cGy) studied in this investigation; (2) MCF10A ICM transferred to MCF10A cultures 1 hour post irradiation did not influence cell growth at any of the radiation doses investigated. However, although not statistically significant, results from the 5 hour ICM transfer studies did indicate the suggestion of a proliferative bystander response similar to that observed for the MCF10A ICM transfer to naïve A375 cells at 5 hrs post irradiation; and (3) A375 ICM transfer to A375 cultures 5 hrs post irradiation demonstrated there was no statistical significance found between the control and the individual doses, nor between any of the dose exposures.

Hence, in these studies, the proliferative bystander response appears to be time, dose and cell type dependent. Specifically, regarding cell type, only the A375 cells

appear to exhibit a strong proliferative bystander response (although there is suggestion that perhaps a small bystander response is occurring in the MCF10A cells). Moreover, this proliferative bystander response appears to be significant only when the A375 cells are incubated with the MCF10A conditioned medium which serves as our nontumorigenic (“normal”) cell model. No proliferative response was observed when conditioned medium from irradiated A375 cells was transferred to naïve cultures of themselves.

Secondly, in addition to cell type dependency, our data indicate a time dependency may be present for this proliferative bystander effect, since conditioned medium from irradiated cells transferred after one hour was not found to induce a proliferative effect for any of the experimental conditions we investigated. Only 5 hour conditioned medium resulted in stimulating our cell cultures. This result was somewhat surprising as it differed from the observations made by Fujimori et al. (2005) who reported a proliferative response in the A375 cell line after a 1 hour transfer of medium conditioned by cells irradiated at 2 and 6 cGy. It is unclear why the results in this study differed from Fujimori et al. (2005), but variations could be due to differences in the experimental cell models used. For example, a human diploid fibroblast cell line was used for irradiating and medium transfer in the work of Fujimori et al. (2005), while a human mammary epithelial cell line was used in this study.

Finally, the data from our studies demonstrate that the proliferative bystander effect is dose dependent, occurring only at exposures less than 10 cGy. Specifically, in agreement with Fujimori et al. (2005), we only observe a significant proliferative

bystander effect at 2 and 6 cGy. The exact reasons why this proliferative response is seen only in the extremely low dose range of radiation is not clear, but previous literature on several bystander effects has documented that this phenomenon is seen expressly at low doses and is not observed at higher doses. Indeed, Prise et al. (2009) have stated that a key characteristic of the bystander response, in contrast to direct radiation responses, is that the dose response curve for bystander effects becomes saturated at relatively low doses (See Figure 1-2 in Chapter 1 for a schematic of this dose dependency effect). Given reports that patterns of gene expression after low dose radiation exposures are different from those following high dose exposures (Amundsun et al, 2003; Prise et al, 2005), many of the responses observed in cells not directly targeted by radiation could be a consequence of differential gene expression, cell signaling or epigenetic changes predominating within the very low dose range of irradiation (Prise et al., 2005).

In conclusion, historically, studies involving cellular response to IR have generally focused more on the effects of higher doses ($>1\text{Gy}$), which are postulated to follow the Linear Quadratic (LQ) model. The new paradigm elucidating the responses of cells to lower doses ($<1\text{Gy}$), however, suggest that extrapolating data derived from high dose radiation studies back towards the origin using the LQ model does not adequately describe what is being observed in this low dose region. Phenomena such as bystander effects are increasingly being reported in cells following low doses of radiation, and there has been an increasing awareness of the need to better characterize and understand these effects and how they will affect estimations made

concerning radiation induced responses. Such understanding may change the way we view low dose radiation and the risks associated with what was once thought to be a relatively insignificant physiological stress.

Literature Cited

- Azzam E. I., de Toledo, S. M., and Little, J. B., *Current Cancer Drug Targets* (2004); **4**: 53-64.
- Ballarini, F., Biaggi, M., Ottolenghi, A., and Sapora, O., *Mutation Research* (2002); **501**: 1-12.
- Baskar, R., Balajee, Adayabalam S., Geard, Charles R., and Hande, M. Prakash, *The International Journal of Biochemistry and Cell Biology* (2008); **40**: 125-134.
- Baverstock K., *Mutation Research* (2000); **454**, 89–109.
- Beetz, A., Messer, G., Oppel, T., Van Beuningen, D., Peter, R.U., and Kind, P., *International Journal of Radiation Biology* (1997); **72** (1): 33-43.
- Biosource, Invitrogen, Immunoassay Kit, Protocol Booklet, (2007).
- Brenner, D.J., Hlatky, L.R., Hahnfeldt, P.J., Huang, Y., and Sachs, R.K., *Radiation Research* (1998); **150**: 83-91.
- Burdak-Rothkamm, S. and Prise, Kevin M., *European Journal of Pharmacology* (2009); **625**: 151-155.
- De Toledo, Sonia, M. and Azzam, Edouard, I., *Dose-Response* (2006); **4** (4): 291-301.
- Emerit, I., Oganessian, N., Sarkisian, T., Arutyunyan, R., Pogolian, A., Asrian, K., Levy, A., and Cernjavski, L., *Radiation Research* (1995); **144**: 198–205.
- Enns, L., Bogen, Kenneth T., Wizniak, J., Murtha, Albert D., and Weinfeld, M., *Molecular Cancer Research* (2004); **2** (10): 557-566.
- Ermakov, Aleksei V., Konkova, Marina S., Kostyuk, Svetlana V., Egoлина, Natalya A., Efremova, Liudmila V., and Veiko, Natalya N., *Mutation Research* (2009); **669**:155-161.
- Fujimori, A., Okayasu, R., Ishihara, H., Yoshida, S., Eguchi-Kasai, K., Nojima, K., Ebisawa, S., and Takahashi, S., *Cancer Research* (2005); **65** ;(22):10159-10163.
- Hall EJ and Hei TK.(2003). *Oncogene*, **22**, 7034–7042.
- Hamada, N., Matsumoto, H., Hara, T., and Kobayashi, Y., *Radiation Research* (2007);**48**:87-95.
- Huang, L., Kim, Perry M., Nickoloff, Jac A., and Morgan, William F., *Cancer Research* (2007); **67**: 1099.

- Hu, B., Wu, L., Han, W., Zhang, L., Chen, S., Xu, A., Hei, Tom K., and Yu, Z., *Carcinogenesis* (2006); **27** (2): 245–251.
- Iyer R. and Lehnert B.E., *Archive Biochemistry Biophysics* (2000); **376**(1):14-25.
- Ivanov, V, Zhou, T., Ghandhi, S., Karasic, T., Yaghoubian, B., Amundson, S., Hei, T., *Cellular Signalling* (2010); **22**: 1076–1087.
- Koturbash, I., Boyko, A., Rodriguez-Juarez, R., McDonald, R.J., Tryndyak, V.P., Kovalchuk, I., Pogribny, I.P., and Kovalchuk, O., *Carcinogenesis* (2007); **28**: 1831–1838.
- Libermann, Towia A. and Baltimore, D., *Molecular and Cellular Biology* (1990); **10** (5): 2327-2334.
- Little, J.B., Azzam, E. I., deToledo, S. M., and Nagasawa, H., *Radiation Protection Dosimetry* (2002); **99**:159-162.
- Little JB, Nagasawa, H, Li, GC and Chen, DJ. *Radiat. Res* (2003); **159**, 262–267.
- Mothersill C and Seymour C., *Oncogene* (2003); **22**, 7028–7033.
- Mothersill, C. and Seymour, C., *Mutation Research* (2004); **568**:121-128.
- Norgauer, J., Metzner, B., and Schrtaufstätter, I., *Journal of Immunology* (1996); **156**: 1132-1137.
- Prise, K. and O’Sullivan, J., *Nature* (2009); **9**: 351-360.
- Prise K. Folkard M and Michael BD, *Oncogene*(2003); **22**,7043–7049.
- Prise, K., Schettino, G., Folkard, M., and Held, K., *Lancet Oncology*, (2005); **6**:520-528.
- Rzeszowska-Wolny, J., Herok, R., Widel, M., and Hancock, Ronald, *DNA Repair*, (2009); **8**: 732–738.
- Rzeszowska-Wolny, J., Przybyszewski, W. M., and Widel, M., *European Journal of Pharmacology*, (2009); **625**:156-164.
- Shao C, Furusawa Y, Aoki M, Matsumoto H and Ando K., *Int. J. Radiat. Biol* (2002); **78**, 837–844.
- Soule, Herbert D., Maloney, Terry M., Wolman, Sandra R., Peterson Jr., Ward D., Brenz, R., McGrath, Charles M., Russo, J., Pauley, Robert J., Jones, Richard F., and Brooks, S.C., *Cancer Research* (1990); **50**: 6075-6086.

Tabata, C., Kubo, H., Tabata, R., Wada, M., Sakuma, K., Ichikawa, M., Fujita, S., Mio, T., and Mishima, M., *American Journal of Physiology- Lung Cell Mol Physiology* (2006); **290**: L597-L606.

Tsai, Kevin K.C., Stuart, J., Chuang, Yao-Yu E., Little, John B. and Yuan, Z., *Radiation Research* (2009); **172**: 306-313.

(United Nations Scientific Committee on the Effects of Atomic Radiation. *Sources and effects of ionizing radiation*. New York: United Nations, 2000.

Upadhyay, S., G. Li, et al, *Cancer Research* (1995) **55**(20): 4520-4.

Wu, M. et al, *Breast Cancer Research Treatment* (2004); **84**(1): 3-12.

Yang, H., Assad, N., and Held, K., *Oncogene* (2005); **24**:2096-2103.

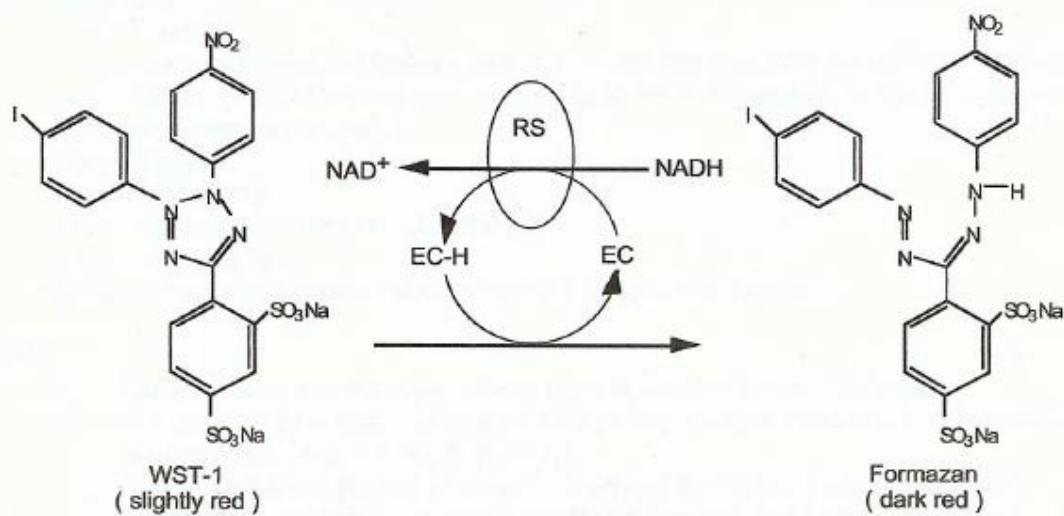
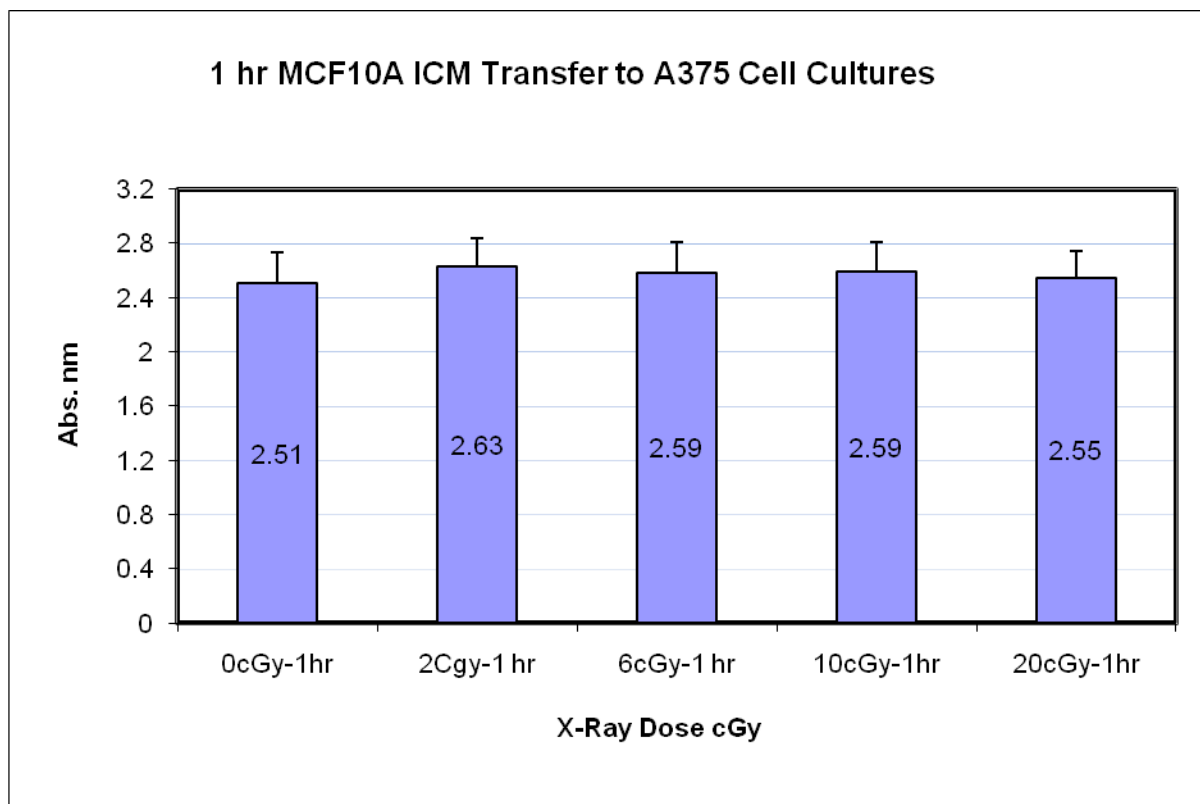


Fig.1 Cleavage of the tetrazolium salt (WST-1) to formazan.
 (EC = electron coupling reagent, RS = mitochondrial succinate-tetrazolium-reductase system)

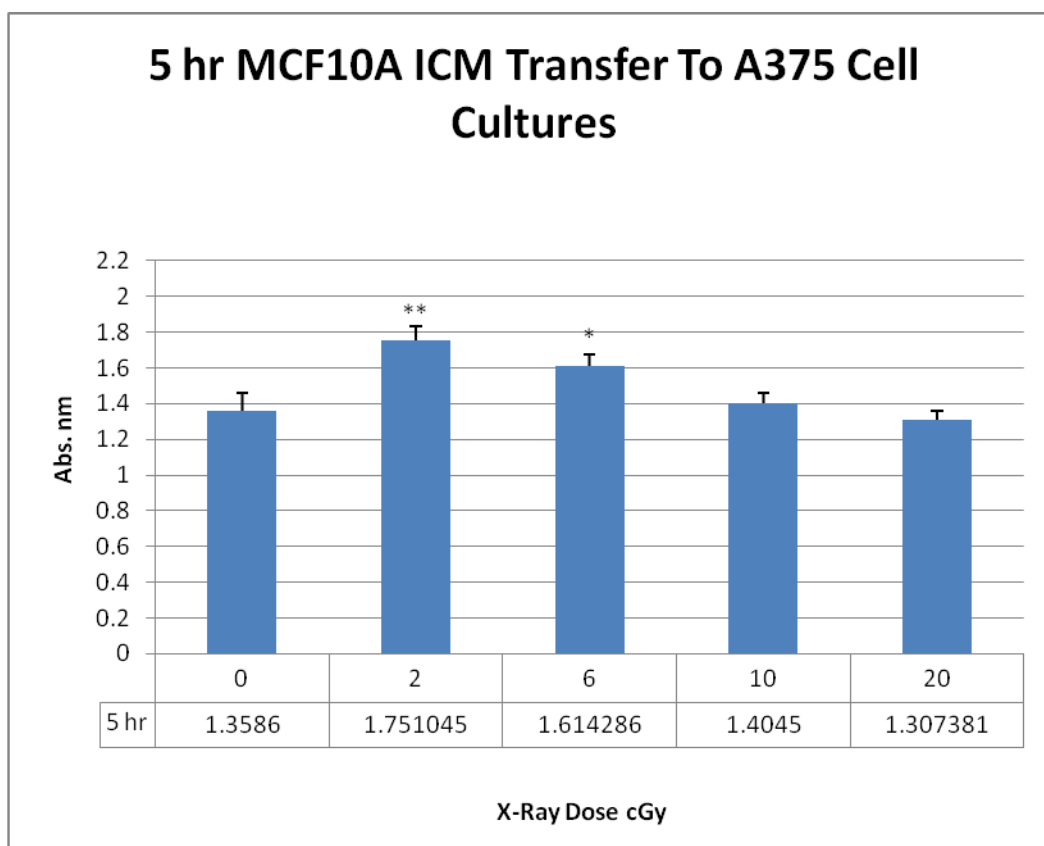
Figure 2-1: Schematic of WST-1 assay mechanism (Takara Bio-Inc., 2008).



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

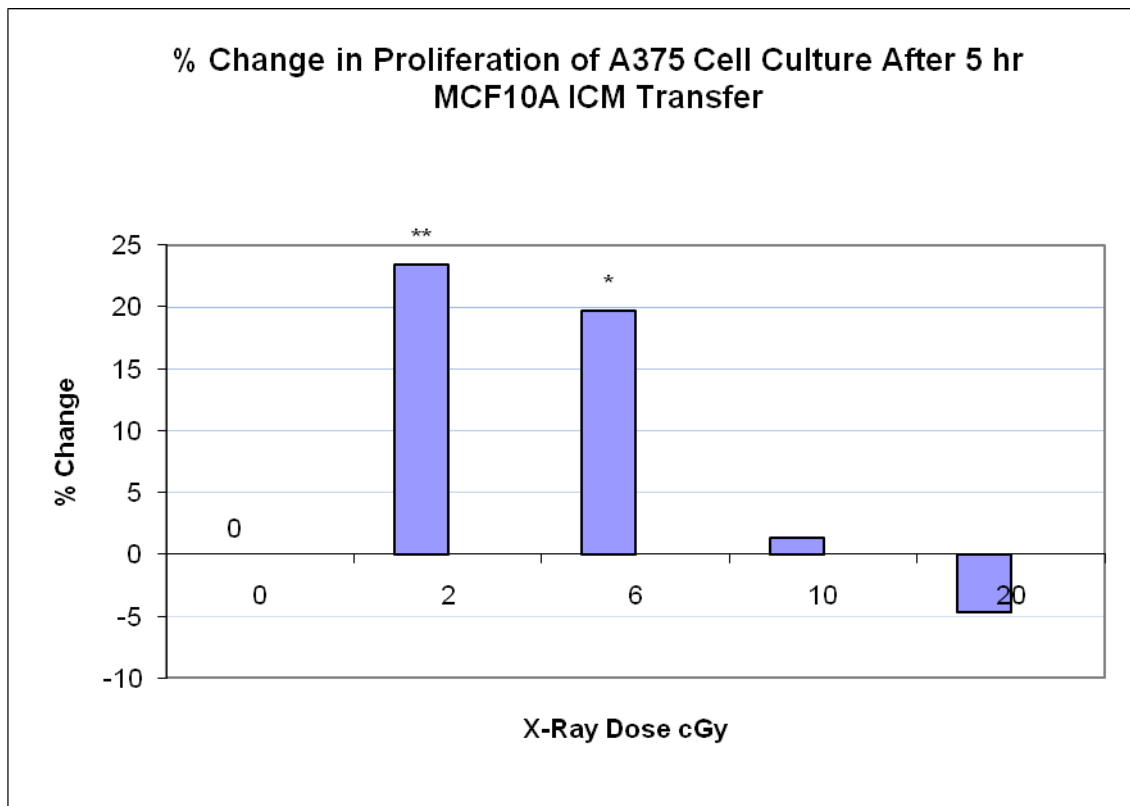
Figure 2-2: Results for WST-1 Assay for 1 hr ICM transfers. Assay was performed 48 hrs post- irradiation medium transfer. Study performed in triplicates, $n=4$.



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

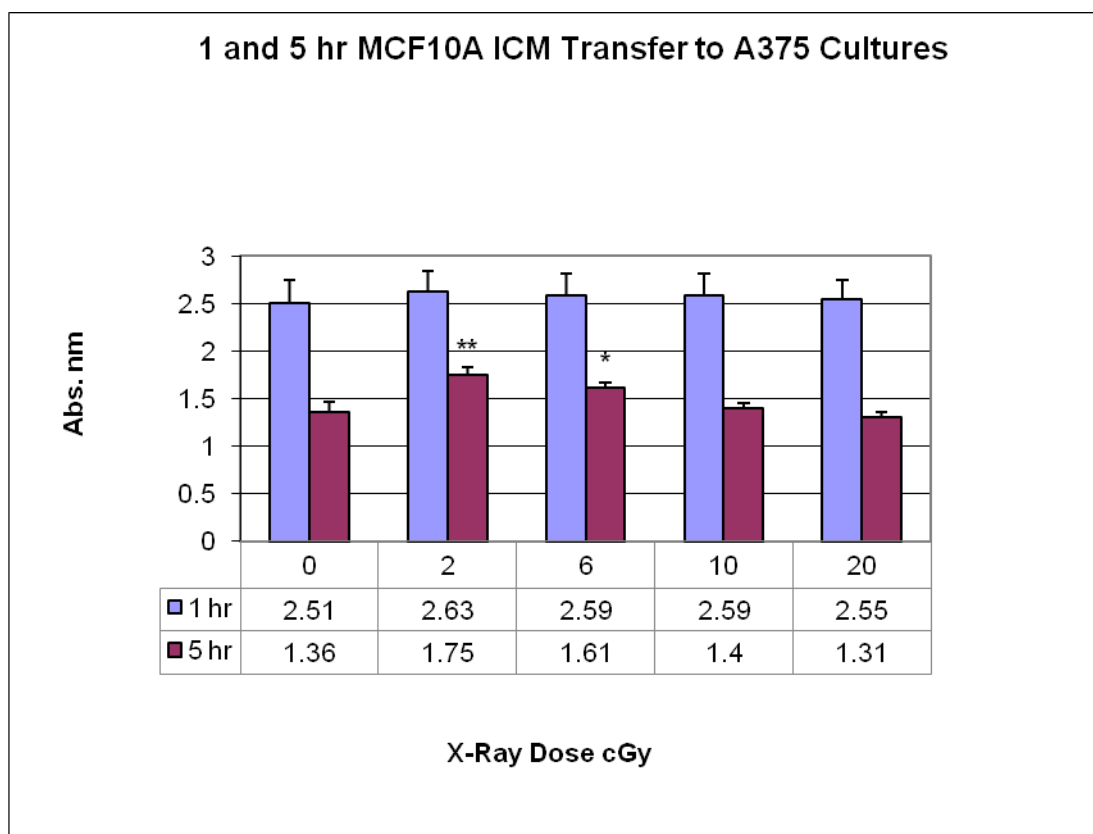
Figure 2-3: Results for WST-1 Assay for 5 hr ICM transfers. Assay was performed 48 hrs post- irradiation medium transfer. Study performed in triplicates, $n=3$. Statistical Significance was found between the control and 2 cGy ($p < 0.01$) and also between control and 6 cGy ($p < 0.05$). At 10cGy and 20cGy data were not statistically different from control.



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

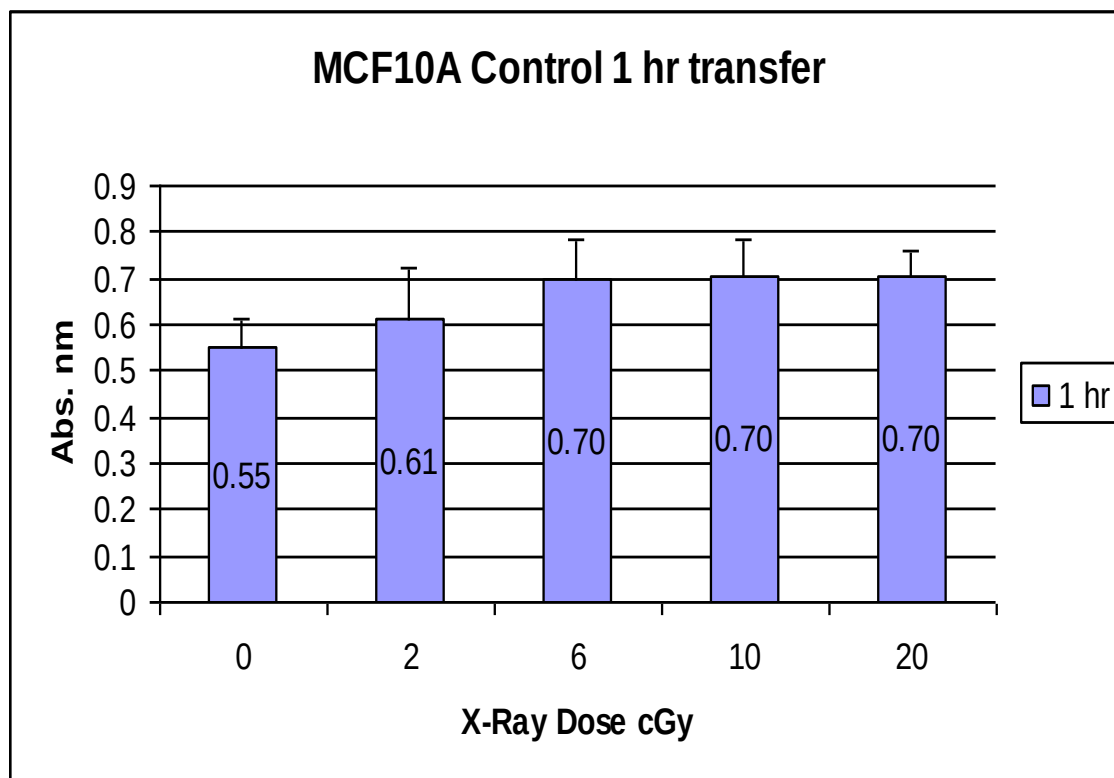
Figure 2-4: The Change in Proliferation after Irradiated Conditioned Medium (ICM) from MCF10A Cell Line has been transferred to A375 Cell Line 5 hours post irradiation. All percentages were calculated by comparing treatments values to untreated control values.



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

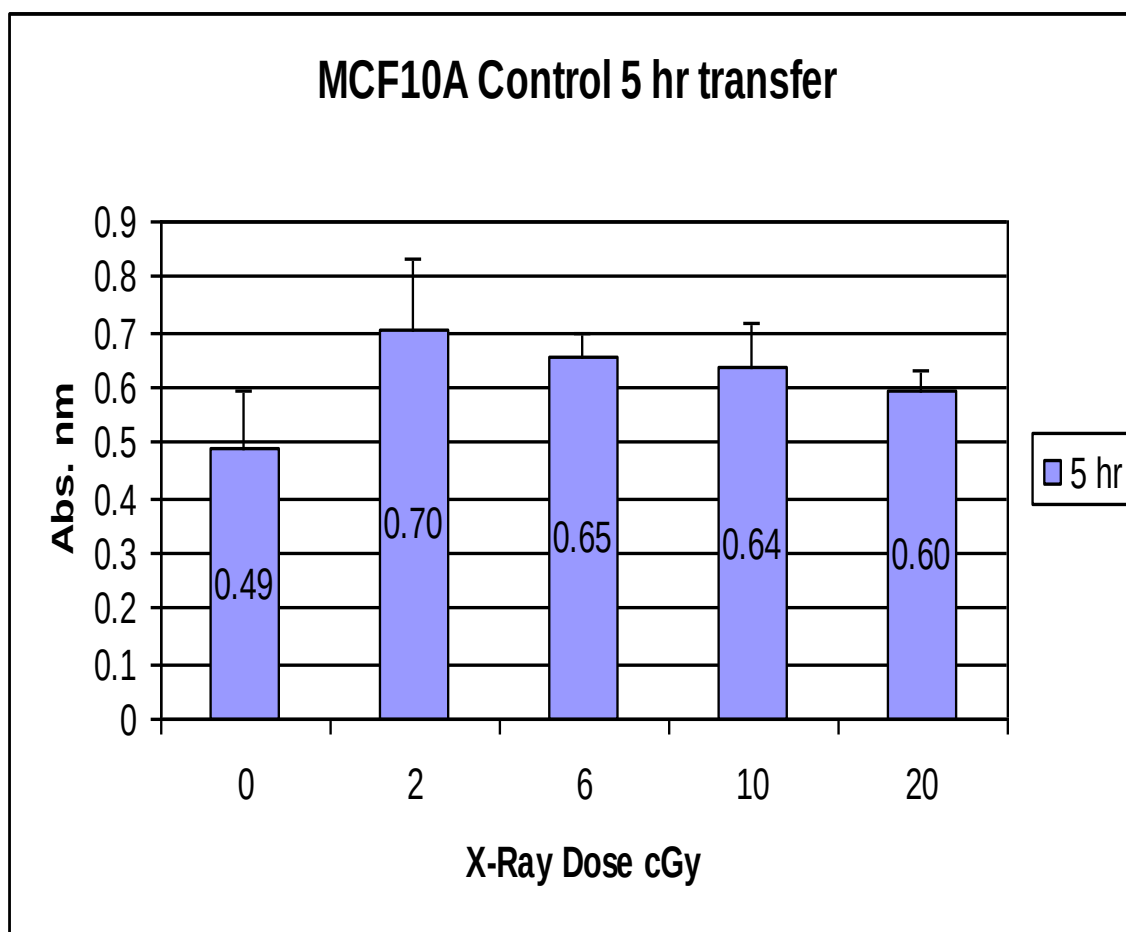
Figure 2-5: Results for WST-1 Assay for 1 and 5 hr ICM transfers. Assay was performed 48 hrs post- irradiation medium transfer. Statistical Significance was found at the 5 hr transfer between the control and 2 cGy ($p < 0.01$) and also between control and 6 cGy ($p < 0.05$). At 10cGy and 20cGy cultures returned to control levels.



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

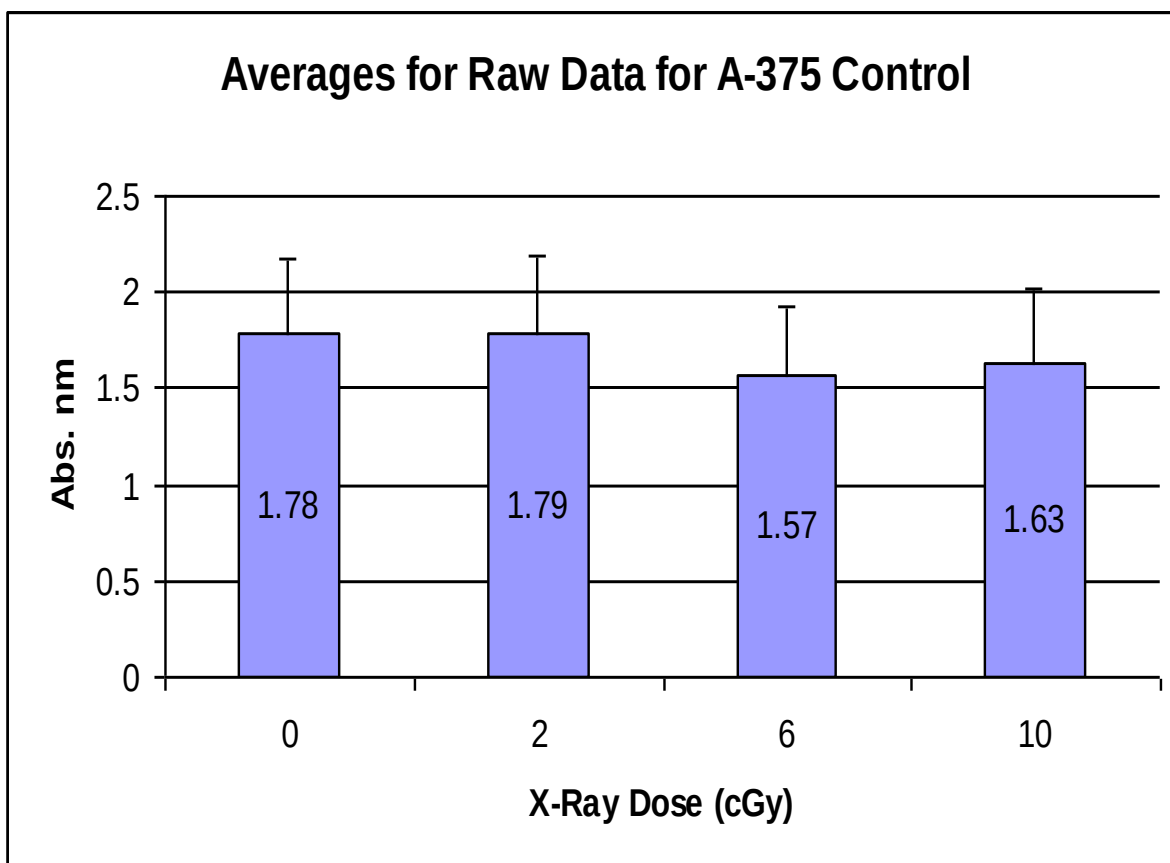
Figure 2-6: Results of MCF10A cell line after ICM 1 hr transfer from MCF10A cell line. Study performed in triplicates, $n=3$. This was performed using WST-1 assay 48 hrs post- irradiation. No Statistical Significance was found.



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

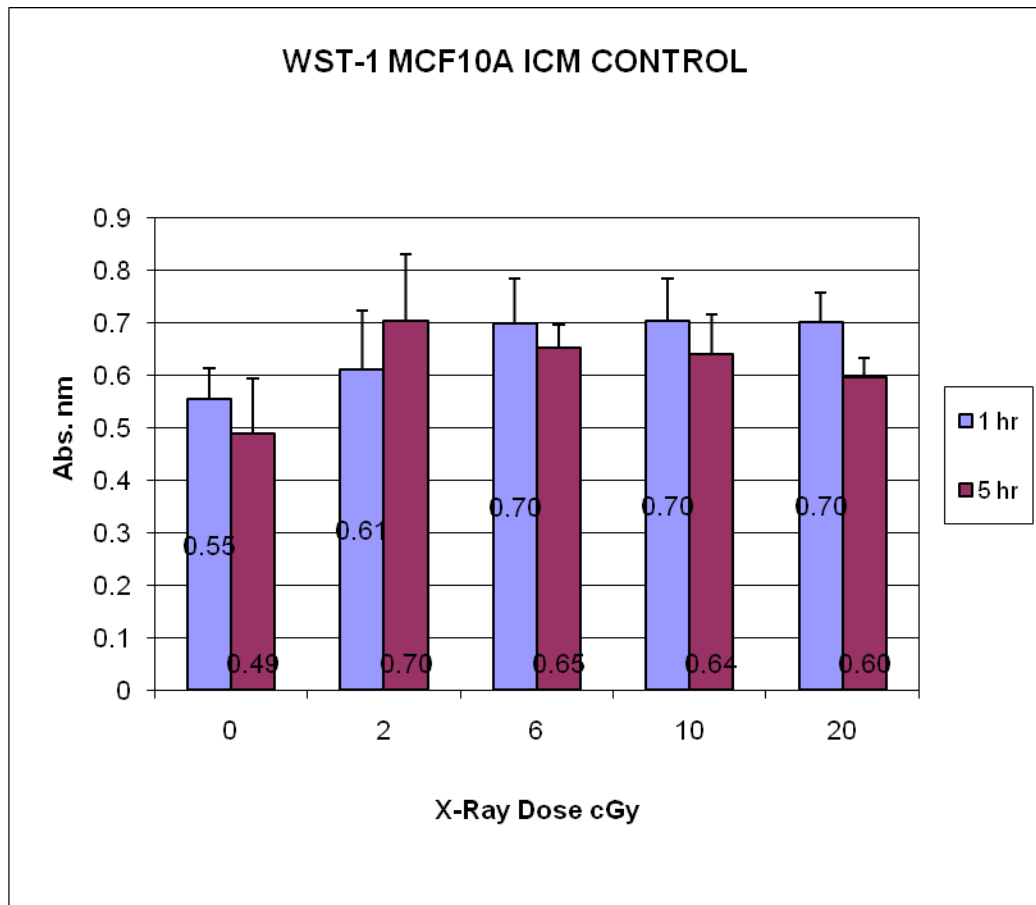
Figure 2-7: Results of MCF10A cell line after ICM 5 hr transfer from MCF10A cell line. Study performed in triplicates, $n=3$. This was performed using WST-1 assay 48 hrs post- irradiation. No Statistical Significance was found, although there is a similar trend in proliferation as seen in the MCF10A ICM transfer to A375 cell cultures.



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

Figure 2-8: Results of A375 cell line after ICM transfer from A375 cell line. Study performed in triplicate, $n=3$. This was performed using WST-1 assay 48 hrs post-irradiation. No Statistical Significance was found.



* Denotes Statistical Significance $p < 0.05$.

** Denotes Statistical Significance $p < 0.01$.

Figure 2-9: Results of MCF10A cell line after ICM transfer from MCF10A cell line.

This was performed using WST-1 assay 48 hrs post- irradiation. This graph represents the 1 and 5 hr MCF10A ICM transfer collectively.

CHAPTER 3

Determination of Cytokine Levels Following Very Low Dose Radiation Exposure

Introduction

In Chapter 2 we demonstrated that a proliferative bystander effect (BE) existed using the Irradiated Conditioned Medium (ICM) transfer model. In this chapter we attempt to delineate possible factors that may be involved with the proliferative BE observed. To accomplish this, we investigated the levels of two cytokines (IL-6, IL-8) present in 1 hour and 5 hour conditioned medium (CM) derived from MCF10A cells receiving doses of 0, 2, 6, 10 or 20 cGy. Both IL-6 and IL-8 are well known pro-inflammatory cytokines whose cellular upregulation and release is strongly associated with environmental stressors such as radiation (Narayanan P K, et al., 1999) and are two of the factors whose gene expression is known to be significantly upregulated following activation of NF κ B - a suggested mechanism underlying bystander responses (Tabata, 2005; Ivanov, 2010). These factors were chosen because of published reports implicating their possible involvement in the signaling pathways regulating the Radiation-Induced Bystander Effect (RIBE; Fujimori, 2005, Ivanov, 2010). A schematic illustrating the role of IL-6 and IL-8 in bystander responses is presented in Figure 3-1.

Mechanisms of Bystander Effects

The exact mechanisms of bystander effects are not known, but evidence is accumulating that multiple signal transduction pathways are involved (Figure 1-3). In fact, Ivanov et al (2010) has recently postulated that there are at least five mechanistic categories underlying the initiation and development of bystander responses: i) radiation-induced stress reactions of exposed cells, which could be accompanied by

release of pre-existing signal transmitters with nuclear localization, such as interleukin-1 α (IL-1 α , followed by initiation of autocrine and paracrine stimulation of cells by these ligands via the corresponding receptors ; ii) activation of DNA damage sensing mechanisms such as the ATM-p53 and ATM-NF- κ B signaling pathways followed by the stimulation of NF- κ B-dependent gene expression of factors; iii) the activation and stimulation of bystander cells via a paracrine mechanism using cytokine or growth factor interactions with the correspondent receptors and an induction of cell signaling pathways in bystander cells; iv) translocation of reactive oxygen species (ROS) and nitric oxide (NO) from directly irradiated cells to bystander cells through gap junction channels ; v) induction of cell survival mechanisms through shifts in the balance between survival and apoptotic signaling at the late stages of bystander response.

Reports in the literature indicate that many studies have suggested that changes in gap junction-mediated intercellular communication (GJIC) underlie bystander responses. In contrast, several reports link upregulation of the release of soluble factors, including lipid peroxidase products, inosine nucleotides and growth factors to bystander effects (Azzam et al., 2001; Mothersill and Seymour, 1997, 1998; Zhou et al., 2002; Narayanan et al., 1997; Lehnert and Godwin, 1997; Rzeszowska-Wolny, J. et al., 2009; Przybylski, Waldemar M. and Widel, M., 2009; Fujimori, et al., 2005). Additional reports on investigation of bystander mechanisms implicate reactive oxygen species (ROS) such as superoxide radicals (Ballarini, F. et. al 2002; Kevin M Prise et al, 2005; Rzeszowska-Wolny, J. et al., 2009). Finally, several studies have also shown cytokine levels are altered following exposure to radiation and may play a significant

role in bringing about bystander responses (Fujimori et al., 2005; Narayanan P K, et al., 1999). As demonstrated by Beetz, A., et al. (1997) IL-8 is a significant factor in the proliferation in human melanoma cells. Other reports show that the inflammatory cytokines IL-6 and IL-8 are induced after exposure to ionizing radiation (Fujimori et al., 2005; Narayanan P K, et al., 1999). Specifically, CXCL8 (IL-8) is involved in response to inflammatory stresses and, as demonstrated by Fujimori et al., quantitative PCR procedure revealed that this cytokine increased by greater than 2-fold in the cells exposed to low-dose x-rays.

Perhaps the most apparent message obtained to date regarding bystander mechanisms is that they vary from cell to cell (i.e. they are cell type specific) and probably involve multiple cell signaling pathways. For example, activation of mitogen-activated protein kinase (MAPK) signaling (known to be linked with cell survival and cell proliferation; Kamohara et al., 2007; Brew et al., 2000; Takamori et al., 2000; Luppi et al., 2007; Venkatakrishnan et al., 2000; MacManus et al., 2007, Waugh et al., 2008) has been reported to be a mechanism underlying the bystander effects observed in several cell lines (reviewed in Hu et al, 2006; Ivanov, 2010). Additionally, Ballarini et al (2006) postulates that mechanisms involved in proliferative bystander effects (BE) include the activation of the transcription factor Nuclear Factor kappa B (NF- κ B) by cytokines or tumor promoters. More recently, in support of the role of NF- κ B, Ivanov et al.(2010), after a global gene expression investigation in directly radiated and bystander cells, has postulated that activation of NF- κ B is a dominant signaling hub in bystander response.

Materials and Methods

Culture of MCF10A and A375 Cell Lines

MCF10A cells were obtained from the Karmanos Cancer Institute (Detroit, MI). Under normal cell culture conditions, the cells were grown in Dulbecco's modified Eagle's Medium/Ham's F-12 nutrient mixture (DMEM/F12), containing 15 mM HEPES and 2.5 mM L-glutamine, supplemented with 5% heat inactivated equine serum, 500 ng/ml epidermal growth factor (EGF), 10µg/ml insulin, 100ng/ml gentamicin, and 10ng/ml cholera toxin (hereafter referred to as DMEM/F12 complete) at 37° C and in an atmosphere of 5% CO₂. A375 cells were obtained from ATCC. They were cultured in DMEM media with 10% Fetal Bovine Serum (FBS) and maintained in 37° C with 5 % CO₂.

Cytokines

Cytokines consist of a large family of secreted proteins; including pro-inflammatory agents and growth hormones. Cytokines influence cell function by binding to specific cell-surface receptors and use phosphorylation and other intracellular mechanisms to trigger signals that produce cellular changes. Cytokines act in a complex, intermingled network in which the production of one cytokine can induce production of many other cytokines (Ballarini et al., 2002)

The cytokine interleukin-6 (IL-6) is a well known pro-inflammatory cytokine involved in many physiological processes (Figure 3-2). The IL-6 signal transduction occurs via the JAK/ STAT pathway, i.e. tyrosine kinases of the Janus family activate

signal transducers and activators of transcription.

Interleukin -8 is also known as CXCL8 and is a proinflammatory CXC chemokine (Figure 3-3). There are many well-defined signaling pathways that are induced downstream of IL-8 receptors and demonstrate the significance of IL-8 in promoting proliferation and cell survival (Waugh et al., 2008). IL-8 is a chemo-attractant for neutrophils, and this interleukin is produced after inflammatory stimuli such as radiation or chemotherapy.

Removal of Supernatant Post Irradiation

Cells were plated with 80,000 MCF10A cells per 35 cm dish 24 hours before irradiation. Immediately prior to irradiation the medium was removed, and the cells were washed with PBS and 2 mL of DMEM with 1% serum was added to each dish. 24 hours after incubation, cell line MCF10A was irradiated at different radiation doses (2, 6, 10, and 20 cGy). Cells were irradiated using a Sieman's Stabilipan x-irradiator (SSD 51.5 inches, 120 KeV, 2 mm Cu filtration). The controls were treated with the same experimental conditions, but were not exposed to irradiation. One and five hours after irradiation, the supernatant of the irradiated cells (MCF10A) was collected and stored at -80° C until ELISAs could be performed.

Enzyme Linked-Immuno-Sorbent Assay (ELISA)

Levels of IL-6 and IL-8 in ICM were determined 1 and 5 hrs after MCF10A cells were irradiated at doses of 0, 2, 6, 10, and 20 cGy. This was performed using ELISA kits that were ordered from Invitrogen (Carlsbad, CA) and performed following the

manufacturer's instructions included within the kit.

Statistical Analysis

Statistical significance was determined using the Student's t-Test. A p value of < 0.05 was used to assess significance.

Results

IL-6 Expression Levels in MCF10A ICM 1 and 5 Hours Following VLD Radiation

Results from the ELISA analysis of the ICM for the presence of the IL-6 are presented for the 1 and 5 hour MCF10A ICM in Figure 3-4. As can be seen, there appears to be no significant elevation of IL-6 levels at any of the doses measured when compared to controls. Furthermore, the data suggest that there is no temporal correlation between the proliferative bystander effects described in Chapter 2 (Figure 2-4) and the levels of IL-6 within the conditioned medium. Briefly, Figure 3-5 demonstrates that, although not significant, MCF10 ICM IL-6 levels in the 1 hr conditioned medium were slightly above control concentrations at the 10 and 20 cGy doses. For the 5 hr time point data, only the 10 cGy dose levels were slightly elevated.

IL-8 Expression Levels in MCF10A ICM 1 and 5 Hours Following VLD Radiation

Results from the ELISA analysis of the ICM for the presence of the IL-8 are presented for the 1 and 5 hour conditioned medium post irradiation in Figure 3-6. The data indicate that the MCF10A ICM IL-8 levels for the 1 hour conditioned medium were moderately above control concentrations at all radiation doses tested (2, 6, 10 and

20cGy), but this elevation was statistically significant only at the 6 cGy dose. However, the results indicate that IL-8 levels in ICM from the 5 hour post irradiation studies do not rise above control concentrations except following 2 cGy, and this elevation was not found to be significant. Therefore, although a significant proliferative bystander effect was observed in A375 cells when exposed to 5 hour MCF10A ICM (Chapter 2, Figure 2-4), these data suggest that IL-8 does not contribute significantly to this proliferative response.

Discussion

Bystander effects on cells following a radiation stress are now a well established phenomenon. However, the mechanisms underlying this phenomenon are not clearly understood. In this study, the possible correlation with radiation-induced elevated levels of IL-6 and IL-8 in MCF10A ICM and the proliferative bystander effect the ICM has on naïve cultures of A375 cells (Figure 2-4) has been studied. Collectively, the data indicated the MCF10 ICM IL-6 and IL-8 levels in the 1 hr and 5 hour conditioned medium showed little to no elevation when compared to control (the exception being the significantly increased IL-8 levels seen at 6 cGy in the 1 hour ICM), suggesting no correlation with the proliferative bystander effect described in the previous chapter. Ivanov et al (2010) have speculated that the upregulation of IL-6, IL-8, and IL-33 after radiation-induced activation of NF κ B are likely mediators for propagation of bystander signals, and Fujimori et al (2005) reported a strong correlation between proliferative bystander responses in A375 cells and increases in IL-8 gene expression, but our results do not substantiate these reports. It is unclear why this is so, but may be due to

variances in experimental design. Both Fujimori et al (2005) and Ivanov et al (2010) measured cytokine variations at the gene expression level, while we measured cytokine levels in conditioned medium using ELISA techniques. Obviously, more studies are necessary to elucidate the mechanism underlying the proliferative bystander response we observe in the A375 cells following incubation in 5 hour MCF10A ICM. Because several other factors have been suggested to potentially induce bystander responses, including tumor necrosis factor alpha (TNF α), cyclooxygenase-2 (COX2), interleukin 33 (IL-33), transforming growth factor beta (TGF- β), interleukin 1 (IL-1), and TNF α -related apoptosis inducing ligand (TRAIL; Prise et al 2009; Ivanov et al 2010), studies concentrating on the role that these factors potentially play should perhaps serve as the base for future studies.

Literature Cited

- Azzam E. I., de Toledo, S. M., and Little, J. B., *Current Cancer Drug Targets* (2004); **4**:53-64.
- Ballarini, F., Biaggi, M., Ottolenghi, A., and Sapor, O., *Mutation Research* (2002); **501**: 1-12.
- Baskar, R., Balajee, Adayabalam S., Geard, Charles R., and Hande, M. Prakash, *The International Journal of Biochemistry and Cell Biology* (2008); **40**: 125-134.
- Beetz, A., Messer, G., Oppel, T., Van Beuningen, D., Peter, R.U., and Kind, P., *International Journal of Radiation Biology* (1997); **72** (1): 33-43.
- Biosource, Invitrogen, Immunoassay Kit, Protocol Booklet, (2007).
- Brenner, D.J., Hlatky, L.R., Hahnfeldt, P.J., Huang, Y., and Sachs, R.K., *Radiation Research* (1998); **150**: 83-91.
- Brew R, Erikson JS, West DC, Kinsella AR, Slavin J, Christmas SE, *Cytokine* (2000);**12**:78-85.
- Burdak-Rothkamm, S. and Prise, Kevin M., *European Journal of Pharmacology* (2009); **625**: 151-155.
- De Gonzalez, A. and Darby S., *The Lancet* (2004); **363**(9406): 345-351.
- De Toledo, Sonia, M. and Azzam, Edouard, I., *Dose-Response* (2006); **4** (4): 291-301.
- Emerit, I., Oganessian, N., Sarkisian, T., Arutyunyan, R., Pogolian, A., Asrian, K., Levy, A., and Cernjavski, L., *Radiation Research* (1995); **144**: 198–205.
- Enns, L., Bogen, Kenneth T., Wizniak, J., Murtha, Albert D., and Weinfeld, M., *Molecular Cancer Research* (2004); **2** (10): 557-566.
- Ermakov, Aleksei V., Konkova, Marina S., Kostyuk, Svetlana V., Egoлина, Natalya A., Efremova, Liudmila V., and Veiko, Natalya N., *Mutation Research* (2009); **669**: 55-161.
- Fujimori, A., Okayasu, R., Ishihara, H., Yoshida, S., Eguchi-Kasai, K., Nojima, K., Ebisawa, S., and Takahashi, S., *Cancer Research* (2005); **65** ;(22):10159-10163.
- Hamada, N., Matsumoto, H., Hara, T., and Kobayashi, Y., *Radiation Research* (2007); **48**:87-95.

- Huang, L., Kim, Perry M., Nickoloff, Jac A., and Morgan, William F., *Cancer Research* (2007); **67**: 1099.
- Hu, B., Wu, L., Han, W., Zhang, L., Chen, S., Xu, A., Hei, Tom K., and Yu, Z., *Carcinogenesis* (2006); **27** (2): 245–251.
- Iyer R. and Lehnert B.E., *Archive Biochemistry Biophysics* (2000); 376(1):14-25.
- Kamohara H, Takahashi M, Ishiko T, Ogawa M, Baba H., *Int J Oncology* (2007); **31**:627-632.
- Koturbash, I., Boyko, A., Rodriguez-Juarez, R., McDonald, R.J., Tryndyak, V.P., Kovalchuk, I., Pogribny, I.P., and Kovalchuk, O., *Carcinogenesis* (2007); **28**: 1831–1838.
- Libermann, Towia A. and Baltimore, D., *Molecular and Cellular Biology* (1990); **10** (5): 2327-2334.
- Little, J.B., Azzam, E. I., deToledo, S. M., and Nagasawa, H., *Radiation Protection Dosimetry* (2002); **99**:159-162.
- Luppi F, Longo AM, de Boer WI, Rabe KF, Hiemstra PS., *Lung Cancer* (2007); **56**:25-33.
- MacManus CF, Pettigrew J, Seaton A, et al., *Mol Cancer Res* (2007); **5**:737-48.
- Mothersill, C. and Seymour, C., *Mutation Research* (2004); **568**:121-128.
- Norgauer, J., Metzner, B., and Schraufstatter, I., *Journal of Immunology* (1996); **156**: 1132-1137.
- Prise, K. and O'Sullivan, J., *Nature* (2009); **9**: 351-360.
- Prise, Kevin M., Schettino, G., Folkard, M., and Held, K., *Lancet Oncology*, (2005); **6**:520-528.
- Rzeszowska-Wolny, J., Herok, R., Widel, M., and Hancock, Ronald, *DNA Repair*, (2009); **8**: 732–738.
- Rzeszowska-Wolny, J., Przybyszewski, W. M., and Widel, M., *European Journal of Pharmacology*, (2009); **625**:156-164.
- Soule, Herbert D., Maloney, Terry M., Wolman, Sandra R., Peterson Jr., Ward D., Brenz, R., McGrath, Charles M., Russo, J., Pauley, Robert J., Jones, Richard F., and Brooks, S.C., *Cancer Research* (1990); **50**: 6075-6086.

- Tabata, C., Kubo, H., Tabata, R., Wada, M., Sakuma, K., Ichikawa, M., Fujita, S., Mio, T., and Mishima, M., *American Journal of Physiology- Lung Cell Mol Physiology* (2006); **290**: L597-L606.
- Takamori H, Oades ZG, Hoch OC, Burger M, Schraufstatter IU, *Pancreas* (2000);**21**:52-56.
- Tsai, Kevin K.C., Stuart, J., Chuang, Yao-Yu E., Little, John B. and Yuan, Z., *Radiation Research* (2009); **172**: 306-313.
- Upadhyay, S., G. Li, et al, *Cancer Research* (1995); **55**(20): 4520-4.
- Venkatakrisnan G, Salgia R, Groopman J E, *J Biol Chem* (2000);**275**: 6868-6875.
- Waugh, David J.J. and Wilson, Catherine, *Clinical Cancer Research* (2008); **14**(21): 6735-6741.
- Wu, M. et al, *Breast Cancer Research Treatment* (2004); **84**(1): 3-12.
- Yang, H., Assad, N., and Held, K., *Oncogene* (2005); **24**: 2096-2103.

FIGURES

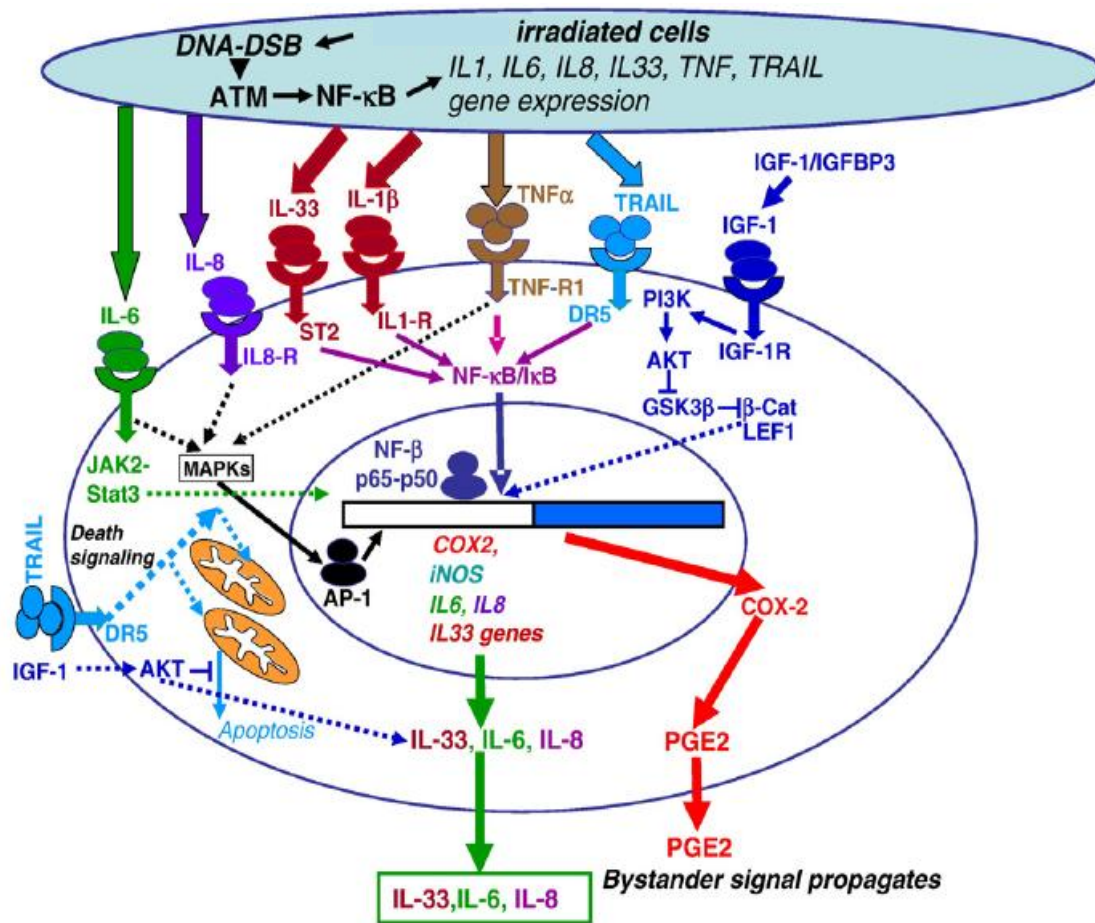


Figure 3-1: Signaling pathways regulating radiation induced bystander effects. Modified from Ivanov et al (2010).

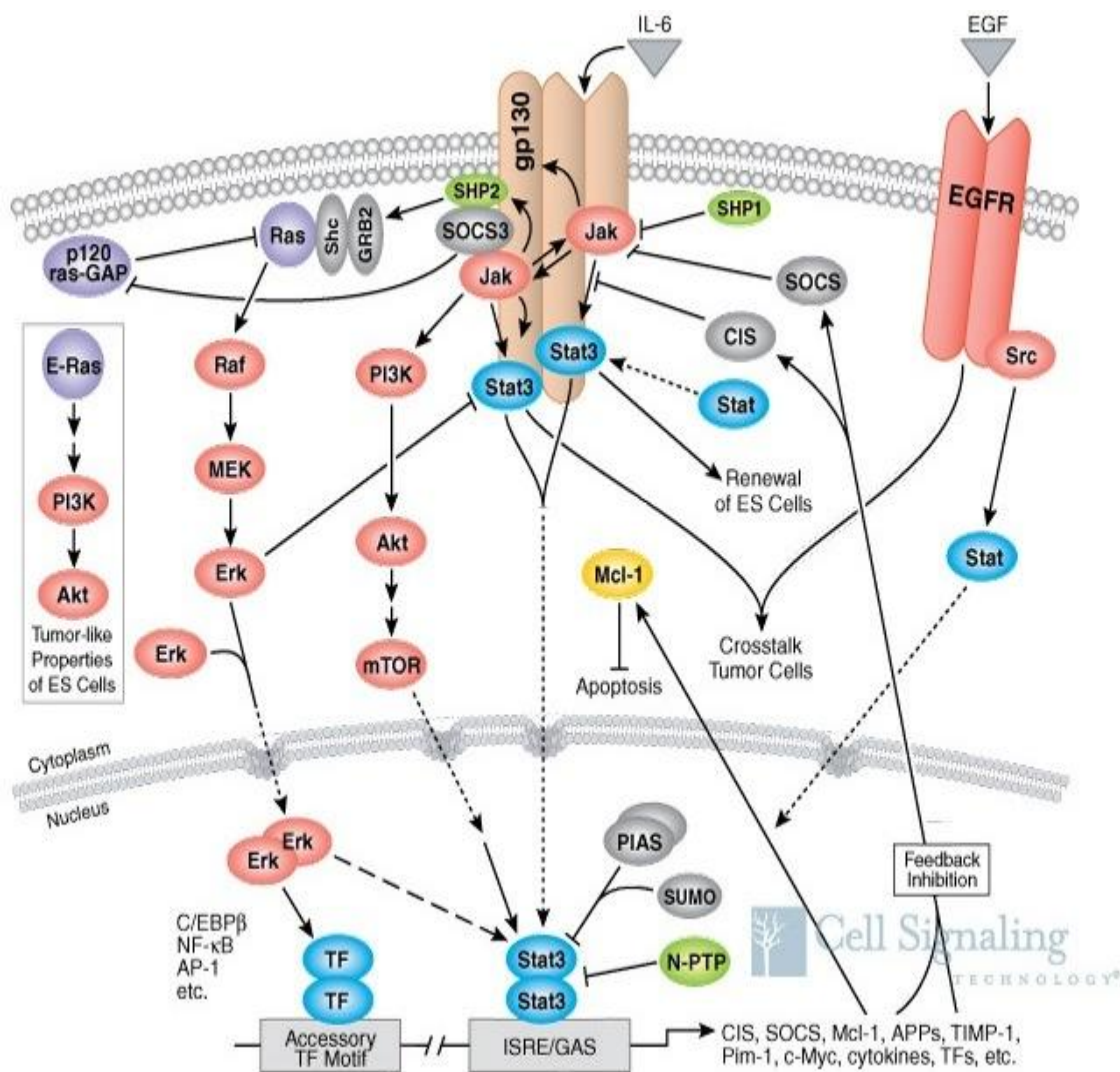


Figure 3-2: IL-6 Signaling Pathway (Cell Signaling, 2010).

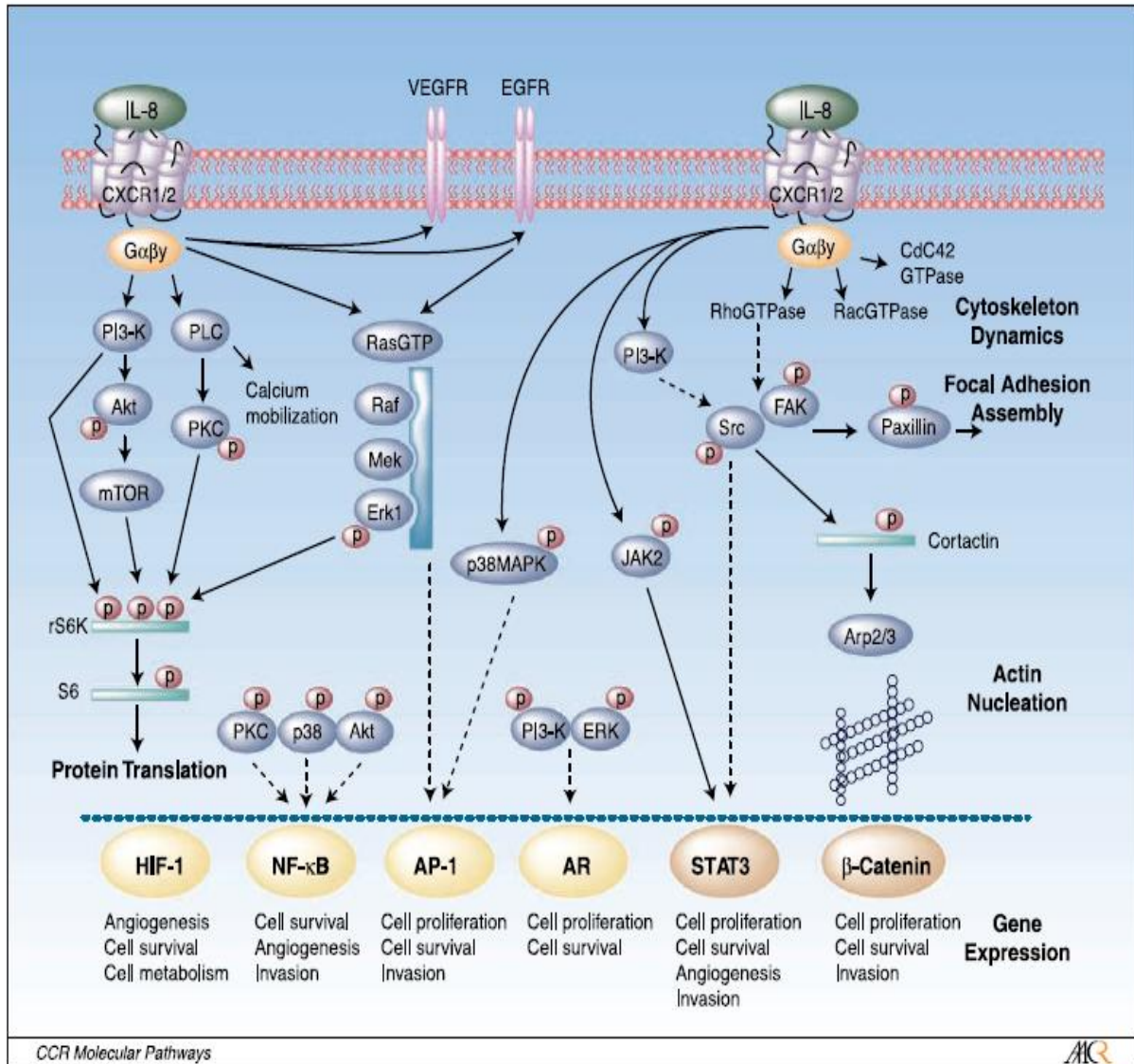
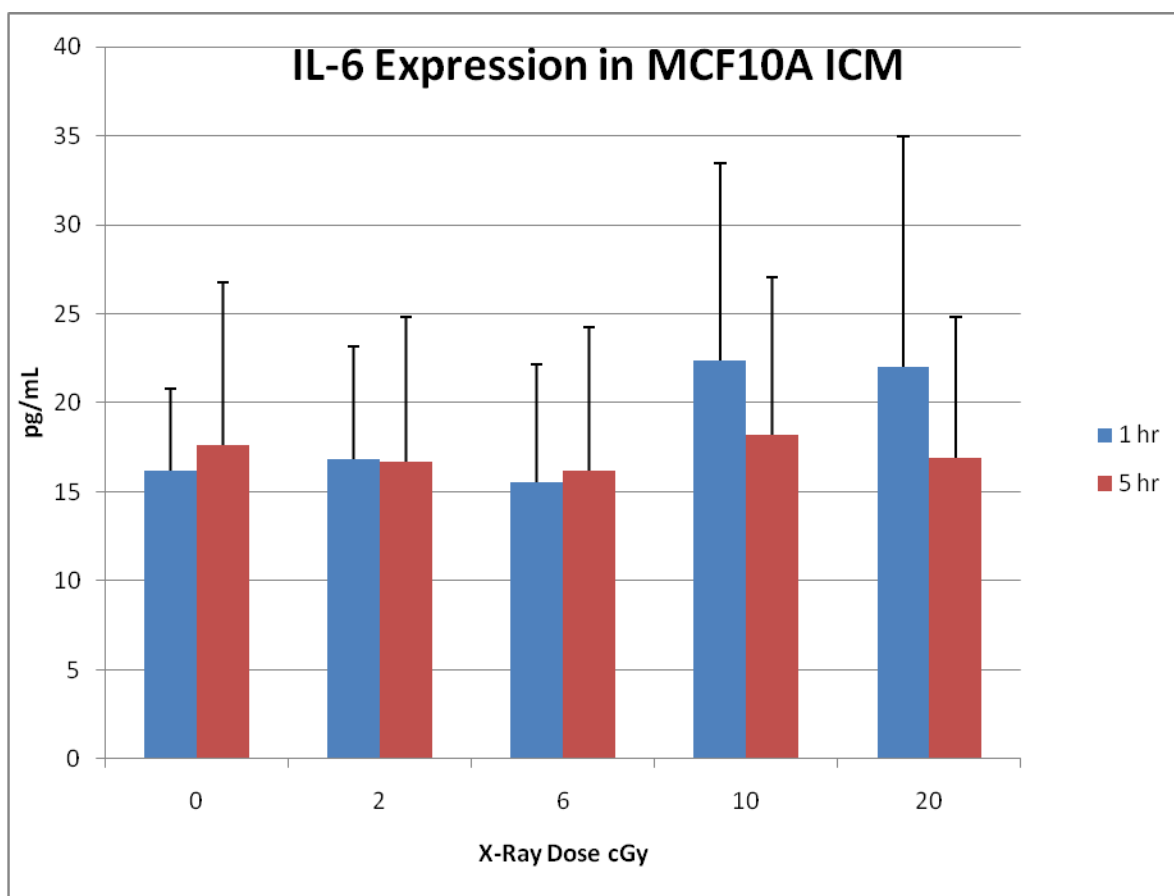
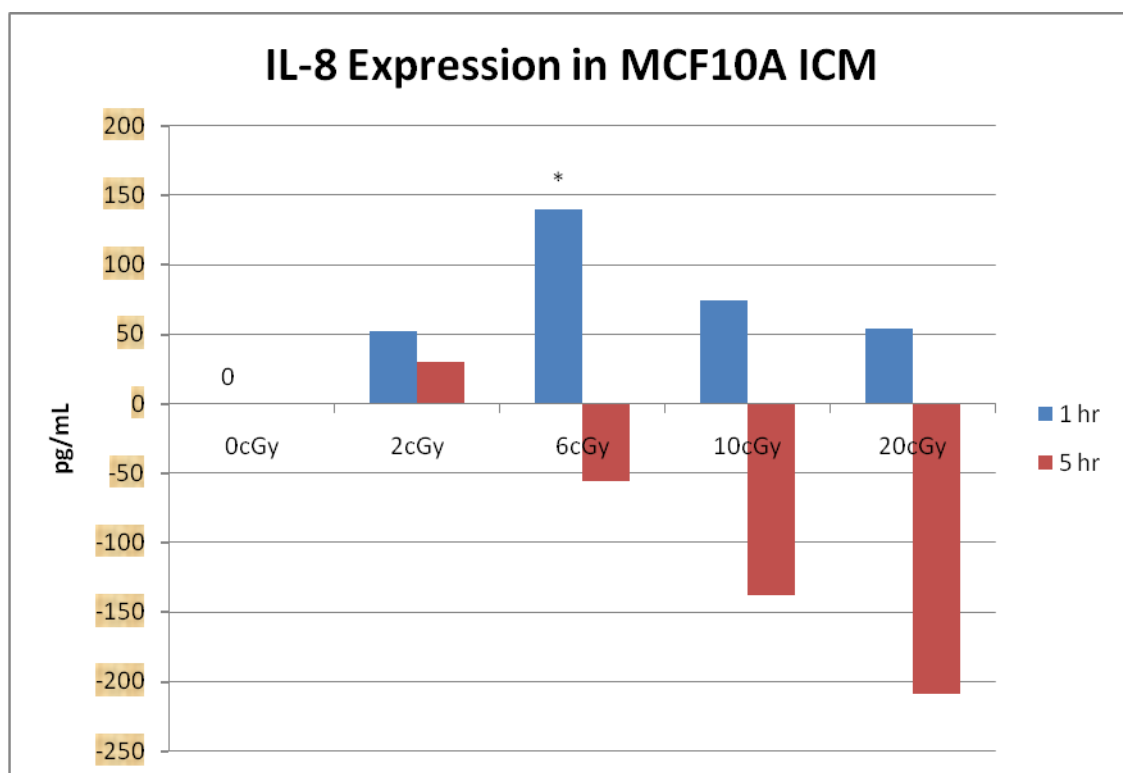


Figure 3-3: Possible IL-8 Signaling Pathways (Waugh and Wilson, 2008).



* Denotes Statistical Significance $p < 0.05$

Figure 3-4: IL-6 Expression in MCF10A ICM at 1 and 5 hrs post irradiation. Studies performed in triplicates. N=4. No statistical significance was observed.



* Denotes Statistical Significance $p < 0.05$

Figure 3-5: IL-8 Expression in MCF10A ICM 1 and 5 hrs post irradiation. The highest levels of IL-8 are observed at 1 hr. Studies were performed in triplicates. N=4.

