RADIOBIOLOGICAL BRAIN STUDIES FOR SYNCHROTRON IRRADIATION

PRE-CLINICAL RADIOBIOLOGICAL STUDIES OF MURINE BRAIN AND BRAIN CANCER CELLS TO SYNCHROTRON X-RAYS AND GAMMA IRRADIATION

By

CRISTIAN FERNANDEZ-PALOMO, MSc.

A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy

McMaster University

DOCTOR OF PHILOSOPHY (2015) McMaster University

Medical Physics and Applied Radiation Sciences Hamilton, ON

(Radiobiology)

TITLE: Pre-Clinical Radiobiological Studies Of Murine Brain And Brain Cancer Cells To Synchrotron X-Rays And Gamma Irradiation

AUTHOR: Cristian Fernandez-Palomo

SUPERVISOR: Prof. Carmel Mothersill

NUMBER OF PAGES: xix, 302

Abstract

This thesis demonstrates the relevance of bystander effect mechanisms after exposure to two Synchrotron modalities – Microbeam Radiation Therapy and Pencilbeam – that are currently at the preclinical stage but aim to treat brain tumours. We elucidate the relationship between the hyper-radiosensitivity phenomenon and radiation-induced bystander effects by studying the dose response of three glioma cell lines. The relevance of these low-dose effects for both Synchrotron modalities is because the tissue exposed to low valley-doses is predicted to be where hyper-radiosensitivity and bystander effects might be expected to predominate.

In vivo experiments were conducted in the European Synchrotron radiation Facility in Grenoble, France and also in the University of Freiburg's Hospital in Freiburg, Germany. Experiments conducted *in vitro* were performed at McMaster University.

The most relevant results of this thesis revealed that the low-dose hyper-radiosensitivity phenomenon can coexist with radiation-induced bystander effects and evidence points towards bystander signalling mechanisms as the primary cause of cell killing during hyper-radiosensitivity. Bystander and abscopal effects

can occur in rats and even in immune-compromised nude mice after exposure to Synchrotron Microbeam Radiation and Pencilbeam. Bystander effects can be communicated from irradiated rats to healthy unirradiated cage mate rats and the presence of a tumour modulates both the bystander and abscopal responses. The γ-H2AX biomarker can successfully be used for the detection of DNA damage in the brain of rodents after Synchrotron Radiation.

In conclusion, this thesis considerably expands the understanding of the role of bystander effects in cells lines, tissues, and animals exposed to Synchrotron radiation. It is suggested that further exploration of the role of bystander effects and hyper-radiosensitivity during Synchrotron treatments could identify new targets leading to better tumour control.

Dedication

To Sarahí.

"*Sleep is the best meditation" – Dalai Lama*

Rest in peace, my dear friend. You'll always be loved.

Acknowledgements

This doctoral thesis would not have been possible to accomplish without the constant guidance and support of my supervisors, as well as the encouraging words from my friends and family.

First and foremost, I would like to give special thanks to my supervisor Prof. Carmel Mothersill. Carmel: I am more than grateful for having you as my mentor. Thank you for your patience, words of encouragement, constant support and trust. Thank you for replying to my dozens of daily email – especially during the last stages of the writing process. I truly believe I am one of the luckiest for having you as my supervisor.

I would like to extend my sincere thanks to my co-supervisor Dr. MD. Elisabeth Schültke. Elisabeth: thank you for your support, trust, friendship and for treating me as an equal from the very beginning. My research trips to Freiburg have definitely left a beautiful mark. Thank you for inviting me and for introducing me to the field of Synchrotron Radiation.

I would like to thank my wonderful colleagues Elke Bräuer-Krisch and Jean Laissue. Besides being extremely talented professionals, both of you are wonderful people. Thank you for making me feel so welcomed during my first experiments in Grenoble and for all the nice conversations about science and life. I hope to see you again soon.

To my committee members Dr. MD. Sheila Singh, Prof. Colin Seymour and Dr. Tom Farrell, thank you for your support and guidance and for making of my committee meetings a wonderful conversation.

To my friends/lab-mates/psychologists Christine and Michelle, it has been wonderful to share so many years with you. I would like to give special thanks to Christine, who always showed me support during the stressing times of the PhD research.

To my friend/sister Alejandra Salinas, who has supported me unconditionally since my move to Canada more than six years ago: “Gracias por ser la mejor amiga que uno podría desear”.

I would like to thank my amazing family for their support during all these years that I have been away from Chile.

Finally, I would like to thank my partner in crime and in life. Norman, thank you for your constant encouragement, love and for putting up with mood swings. ☺ One of the best things of finishing my PhD is that I can share moments like this with you.

Table of Content

List of Figures

List of Tables

List of Equations

Chapter 1

INTRODUCTION

In the last twenty years, numerous studies regarding the effects of ionising radiation have revealed that non-targeted biological effects occur in addition to known effects resulting from direct DNA damage (E. Azzam, de Toledo, & Little, 2004; Burdak-Rothkamm & Prise, 2009; Eric J. Hall & Giaccia, 2006; M. A. Kadhim & Hill, 2015; Mothersill & Seymour, 2001, 2015; Kevin M Prise & O'Sullivan, 2009). In fact, a great body of literature has shown that radiation can induce complex biological responses, such as genomic instability (M. A. Kadhim et al., 1992; C. B. Seymour, Mothersill, & Alper, 1986), bystander effects (E I Azzam, de Toledo, Gooding, & Little, 1998; Mothersill & Seymour, 1997; Nagasawa & Little, 1992) and adaptive responses (Maguire, Mothersill, McClean, Seymour, & Lyng, 2007; Olivieri, Bodycote, & Wolff, 1984; Ryan, Seymour, Joiner, & Mothersill, 2009). These effects are exhibited both by cells traversed by radiation and by unirradiated cells, although with certain variations depending mostly on the type of cell or organism and its genetic background (Mothersill, Seymour, & Joiner, 2002; Ryan et al., 2009; Singh et al., 2011), the dose of radiation delivered (Gow, Seymour, Byun, & Mothersill, 2008; Mothersill & Seymour, 2002) and whether the cell was directly hit (E I Azzam et al., 1998; Nagasawa & Little, 1992) or received a signal from irradiated cells (Maguire et al.,

2007). In cells that are directly hit, chemical bonds break leading to structural and DNA damage. Non-targeted effects are observed in unirradiated cells that receive a signal from irradiated populations (Mothersill & Seymour, 1997) and in the progeny of irradiated cells (Mothersill & Seymour, 2001; K. M. Prise, 1998). The mechanisms behind these processes are complex and require a more detailed description, which is presented below.

1.1 TARGETED EFFECTS - DIRECT RADIATION DAMAGE

For the sake of exposition, I have grouped the outcomes that can occur after ionising radiation has traversed a cell into three categories. First, the damage to the cell is so large that it causes loss of function and death (Dewey, Ling, & Meyn, 1995; Fei & El-Deiry, 2003; Eric J. Hall & Giaccia, 2006; Kevin M Prise & O'Sullivan, 2009). Second, the cell survives but loses its capacity to replicate (Eric J. Hall & Giaccia, 2006; Korystov YuN, 1992). Third, the cell suffers mutations but not enough damage to stop it from replicating, leading to either genomic instability (M. A. Kadhim et al., 1992; C. B. Seymour et al., 1986) or cancerous transformation (Fei & El-Deiry, 2003; Eric J. Hall & Giaccia, 2006).

Reproductive cell death and apoptosis are two of the most studied mechanisms by which cells exposed to radiation die (Kevin M Prise, Schettino, Folkard, & Held, 2005; Puck & Marcus, 1956). Other mechanisms of cell death

include autophagy, necrosis, necroptosis and senescence (Lauber, Ernst, Orth, Herrmann, & Belka, 2012).

1.1.1 Reproductive cell death (Mitotic Catastrophe)

Reproductive cell death describes a form of delayed cell death due to radiation which is measured by loss of clonogenic survival (Puck & Marcus, 1956). It believed to occur due to radiation-induced DNA double strand breaks (DSBs), which remain unrepaired and lead to the accumulation of mutations and chromosomal aberrations in every consecutive division (Elkind & Whitmore, 1967; Weidhaas, Eisenmann, Holub, & Nallur, 2006). The accumulation of chromosomal aberrations will lead to cell death in the first few divisions post-irradiation. In contrast, apoptosis takes place soon after the radiation insult and before division (Brown & Wilson, 2003).

1.1.2 Apoptosis

Apoptosis is a normal process that regulates the number of cells within a cell population. It responds either to tissue homeostasis or to cell injury, and unlike necrosis it does not involve an inflammatory response (Kevin M Prise et al., 2005). The concept of apoptosis was first proposed by Kerr et al. (1972) to explain a mode of cell death that was different from necrosis and autophagy.

Apoptosis is characterized by increased cytoplasmic granularity, cell shrinkage, chromatin condensation, and membrane blebbing (Kerr et al., 1972; Strasser, O'Connor, & Dixit, 2000; Willingham, 1999). Although these morphological features are characteristic of apoptosis, there are three cellular apoptotic pathways, of which two are relevant to radiation induced cell death: the extrinsic and the intrinsic pathways. In vertebrates, these pathways lead to the formation of apoptotic bodies and their clearance by phagocytes (Elmore, 2007). The extrinsic pathway is characterized by activation of a death receptor while the intrinsic pathway responds to stimulation of the mitochondria (Elmore, 2007). Although both signalling cascades have a different initiating event, they converge at the same apoptotic step where caspases 3, 6 and 7 execute the final death process (Elmore, 2007).

1.1.3 Necrosis and Necroptosis

Necrosis is described as an unregulated and uncontrolled cell death in response to extreme non-physiological and exogenous cell damage (Muñoz, Lauber, Schiller, Manfredi, & Herrmann, 2010; Vandenabeele, Galluzzi, Vanden Berghe, & Kroemer, 2010). Necrosis is characterized by a rapid deterioration of the integrity of the cell membrane, leading to swelling and leakage of cytoplasmic components into the extracellular space (Silva, 2010). Simultaneously, necrotic cells release endogenous signals that alert the innate immune system of the

danger (Li, Ambade, & Re, 2009). Alternatively, a secondary necrosis process can occur as a consequence of the absence of phagocytosis after the full apoptotic program is completed (Silva, 2010). This secondary process becomes relevant in the context of radiotherapy, when phagocytic cells are overwhelmed due to the elevated induction of apoptosis (Lauber et al., 2012).

Recent evidence has revealed that necrosis can also occur in a *regulated* manner which has been named “necroptosis” (Degterev et al., 2005). Necroptotic cells are characterized as having necrotic morphology but showing activation of autophagy (Degterev et al., 2005). Autophagy, and thus necroptosis, as well, is initiated by activation of death receptors, such as tumour necrosis factor receptor 1, and other cellular pathways that lead to production of reactive oxygen species (ROS), lipid peroxidation, active disintegration of the plasma, mitochondrial and lysosomal membranes, and release of intracellular contents (Lauber et al., 2012; Vandenabeele et al., 2010). Necroptosis also shows the characteristic activation of the innate immune system observed during necrosis (Vandenabeele et al., 2010). Overall, while necrosis is caused by extrinsic, non-physiological factors, necroptosis is induced by cellular activation.

1.1.4 Senescence (Permanent Cell Cycle arrest)

Cellular senescence is also a mechanism that limits the proliferation of cells exposed to radiation, but unlike mitotic catastrophe the cells are not able to initiate DNA replication (Rodier & Campisi, 2011). This type of cell death can

occur as a result of aging or tissue repair, or in order to escape tumour transformation (Rodier & Campisi, 2011). Cells undergoing senescence are characterized by becoming flattened, enriched with vacuoles, having an acidic pH, heterochromatinization, and formation of autolysosomes (Rodier & Campisi, 2011).

1.2 NON-TARGETED EFFECTS OF IONISING RADIATION

Non-targeted effects (NTE) refer to the radiation effects observed in cell populations, tissues, and organs that are not caused by direct radiation exposure (Koturbash et al., 2008; F M Lyng, Seymour, & Mothersill, 2000; Mothersill & Seymour, 1998; Mothersill et al., 2005; K. M. Prise, 1998)(M. Kadhim et al., 2013). The term includes Genomic Instability, abscopal and bystander effects (M. Kadhim et al., 2013; Mothersill & Seymour, 2015).

1.2.1 Genomic Instability

Genomic instability is a concept that describes delayed genetic alterations observed in the progeny of the exposed cells many generations after the initial radiation insult (M. A. Kadhim et al., 1992; W. F. Morgan, 2003; Mothersill & Seymour, 2012; Mothersill & Seymour, 2015; C. B. Seymour et al., 1986). The importance of the discovery of genomic instability lies in the recognition that cell

populations surviving radiation exposure and their progeny which show no evidence of mutations or altered fitness will not necessarily behave normally. Up to the nineteen eighties, the central dogma in radiobiology was that all the damage was put into the cell by the ionising radiation. If the cell was able to repair the damage and undergo successful mitosis - at least 5 times - then it (and its progeny) carried no residual damage and the population was as fit as if never irradiated (Elkind & Whitmore, 1967). The key paradigm changing finding which came from several independent studies was that the progeny of irradiated cells which have survived according to the above criteria, show evidence of de novo, non-clonal effects meaning that the damage was not induced by the energy deposition from radiation.

One of the first publications suggesting that the progeny of irradiated cells might also be at risk was published by Seymour et al. (1986). The team observed lethal mutations in the distant progeny of cells exposed to photons from a Cobalt-60 source. They suggested that the appearance of lethal mutations might require many successful generations to be expressed. Those investigations were reinforced by reports from Pampfer and Streffer (1989), which showed chromosomal aberrations at the second mitosis after X-irradiating a two-cell mouse embryo. Streffer's group subsequently showed that 29% of the aberrations were carried from the first to the second mitosis (Weissenborn & Streffer, 1989). Similar data to those presented by Seymour et al. and Streffer et al. were published by Mendonca and colleagues (1989) where delayed cell death

started after 10 successful divisions; and by Kadhim and colleagues (1992) although they compared the effect of X-rays against alpha particles instead. These studies were performed in bone marrow stem cells – which meant clonal lineages could be followed – and revealed that alpha particles were more effective at inducing non-clonal aberrations than X-rays. Later studies revealed persistent levels of reactive oxygen species in cells showing genomic instability, suggesting that oxidative stress plays a role in perpetuating the insult (Clutton, Townsend, Walker, Ansell, & Wright, 1996; Limoli et al., 1998; Limoli, Kaplan, Giedzinski, & Morgan, 2001).

Although genomic instability occurs as consequence of direct radiation exposure, there are several reports indicating it might also involve indirect radiation effects because the damage in the progeny is non-clonal and therefore cannot be attributed to DNA mutations only (Chang & Little, 1992; M. A. Kadhim et al., 1992; C. Seymour & Mothersill, 1992)

1.2.2 Abscopal and Bystander Effects

Another broad category of non-targeted effects are bystander effects. They can occur in vivo and in vitro, and refer to horizontal transmission of radiation damage – as opposed to the vertical transmission seen in genomic instability experiments. When bystander effects are described in vivo, they are generally referred to as abscopal effects. Therefore, the term of abscopal – or

out-of-field – describes all those biological responses observed in unirradiated organs or tissues following irradiation of cells in a distant location of the same organism. Abscopal effects are known to occur after exposure to high or low doses of ionising radiation and other stressors in vivo and are commonly detected following high doses of partial-body targeted radiotherapy (Kaminski et al., 2005; Zeng, Harris, Lim, Drake, & Tran, 2013). The communication of the effect is attributed to systemic factors such as blood or the endocrine system (Blyth & Sykes, 2011; M Mancuso et al., 2012; Munro, 2009; Tomita & Maeda, 2014; G. Yang et al., 2008). The immune system is also thought to play an important role. Experiments show that high levels of macrophage activation and neutrophil infiltration in mice are a consequence of the detection and clearance of apoptotic cells induced by radiation (Lorimore, Coates, Scobie, Milne, & Wright, 2001).

1.2.2.1 History of Abscopal effects

Murphy and Morton (1915) removed tumours from mice, X-irradiated the animals, and returned the tumours to their hosts. In a control group, mice were irradiated without their tumours being removed. Murphy and Morton observed that the unirradiated tumour grafts shrank by fifty percent and the animals lived for roughly five weeks, whereas members of the control group died shortly after one week. Murphy and Morton had discovered an association between exposure

of normal tissues to radiation and tumour growth, although the casual mechanism remained unknown for many years.

Parsons et al. (1954) published a study showing reduction of cells in the sternal bone marrow of patients with chronic granulocytic leukemia who had been treated with X-radiation in another part of the body. This study reinforced the earlier finding of a radiation-induced effect observed in tissue distant from the site of exposure.

Over the next fifty years, several studies reported similar findings: Brooks et al. (1974) showed that irradiation of the liver of rats produced cytotoxic effects in the unirradiated distant tissue; Poncy et al. (1979) showed that exposure of rats to radon inhalation induced sister chromatid exchanges in bone marrow cells; and a series of studied from 1995 to 1999 reported bilateral pneumonitis after performing exclusively unilateral lung irradiation (Arbetter, Prakash, Tazelaar, & Douglas, 1999; C. Martin et al., 1999; G. W. Morgan & Breit, 1995).

More recently, Khan et al. (1998; 2003) observed abscopal effects in the shielded lungs of mice whose contralateral lungs had been irradiated. The shielded lung showed damage through an increase in micronuclei, which was attenuated by the addition of radical scavengers. An interesting paper by Camphausen et. al. (2003) reported that irradiating the tumour-free left legs of mice greatly reduced tumour growth in the unirradiated right legs. The authors also revealed that abscopal effects were absent in p53 null animals. There are

other reports by Mancuso (2008) where abscopal effects increased the incidence of meduloblastoma in shielded heads compared to animals receiving whole-body irradiation. Our group has also demonstrated abscopal responses after synchrotron radiation in Wistar rats (Fernandez-Palomo et al., 2013), showing that the effects in the directly irradiated rats became weaker as the distance from the targeted area increased. Overall, the early studies concerning abscopal effects show non-targeted radiation changes induced in tumours, organs or cells in the same organism.

1.2.3 Radiation-induced bystander effects

Radiation-induced bystander effects are radiation-like responses observed in unirradiated cells or organisms after receiving signals from irradiated populations either by direct cell-to-cell contact, exposure to conditioned medium or signals from irradiated cells (Hall, 2003; Edouard I Azzam, de Toledo, & Little, 2003; K. M. Prise, 1998; Mothersill & Seymour, 1997). The bystander effect terminology was borrowed from the field of experimental gene therapy (Mothersill & Seymour, 2001), where the use of antivirals to treat tumours transfected with viral DNA caused the *collateral* death of cells that did not incorporate the gene (Freeman et al., 1993). A recent publication has identified that reproductive cell death predominates in a human epithelial cell (HaCat) line exposed to a medium conditioned by irradiated cells (Kishore Kumar Jella, Garcia, McClean, Byrne, &

Lyng, 2013). The concept of conditioned medium refers to spent media harvested from cultured cells, which in this case contains all signals secreted by the cells exposed to ionising radiation.

Nagasawa and Little (1992) published the first modern report of radiation-induced bystander effects. Their data showed that alpha particles directed to one percent of a particular cell group induced sister chromatid exchanges in thirty percent of that population. The authors supported their findings with statistical data, which showed that the ratio of cells damaged by a single alpha particle traversal was lower than the number of cells displaying sister chromatid exchanges. Two studies reinforced Nagasawa and Little's findings. Hickman et al. (1994) reported that the exposure of cells to low doses of alpha radiation induced higher p53 protein levels than predicted by the numbers of cells traversed. Deshpande et al. (1996) found that twenty three percent of cells showed sister chromatid exchanges while only 2.7% of cells had been hit by 0.4 cGy of alpha particles.

Until the introduction of particle microbeams, studies of bystander effect were unable to determine which cellular structures were involved in the production of the signals, although DNA damage was believed to be an important factor (Azzam, de Toledo, & Little, 2001; Nagasawa & Little, 1992). Since the application of particle microbeams, researchers have shown that damage to the

DNA is not a *necessary* condition for the production of bystander signals (Shao, Folkard, Michael, & Prise, 2004; Sawant et al., 2002; Sokolov et al., 2005).

Although bystander effects are commonly observed as decreases in cell clonogenicity, several reports indicate that bystander responses may not be harmful (Hideki Matsumoto, Takahashi, & Ohnishi, 2004; Mothersill & Seymour, 2006, 2010). Two different studies looking at the bystander proteome indicated that bystander signals may confer beneficial effects by overexpressing protective proteins in rainbow trout exposed to bystander signals (Smith, Wang, Bucking, Mothersill, & Seymour, 2007) and in the unirradiated left brain hemisphere of healthy Wistar rats (Smith et al., 2013). Moreover, earlier reports of protective bystander effects *in vitro* include work by Azzam and colleagues (Azzam, de Toledo, Raaphorst, & Mitchel, 1996) where they showed that bystander signals induced reduction of neoplastic transformations. There is also evidence of bystander effects inducing adaptive responses which are discussed by several authors (Matsumoto et al., 2004; Mothersill & Seymour, 2010, 2006)

Bystander signal communication is understood to occur through direct cell-to-cell contact (Azzam et al., 2001, 1998) and by extracellular factors (Mothersill & Seymour, 1997, 1998). These mechanisms will be described in more detail below.

1.2.3.1 Past History of Radiation-induced bystander effects

While Nagasawa and Little published the first modern report about radiation-induced bystander effects, several earlier reports describing similar phenomena remained unnoticed for years until Mothersill and Seymour (2001) reviewed them. Nagasawa and Little's study was *in vitro*, but studies that pre-date their work pointed towards the production of bystander signals *in vivo* – both animals and humans. These earlier studies provide important data indicating that bystander effects are not localized to the vicinity of the irradiated area or to the same organism, unlike the abscopal effect, which although not localized to the vicinity of the irradiated area is only observed *within* the same organism.

The first *in vivo* bystander response identified in the literature was reported by Souto (1962). The data showed that female rats developed tumours after being injected with plasma from lethally irradiated rats and sheep. Hollowell and Littlefield (1967) showed that chromosomal damage in cultured lymphocytes occurred when they were exposed to plasma from irradiated patients. Although the effect was observed *in vitro*, the signal was produced from irradiated human patients, showing that the effect of the signal is not isolated to the same organism or cellular type.

Similarly, Goh and Sumner (1968) observed chromosomal breaks in unirradiated lymphocytes from healthy individuals that had been exposed to the plasma of humans who had received whole-body irradiation. In the same year,

Hollowell and Littlefield (1968) delved deeper into their study of plasma-mediated bystander effects. They found that plasma from irradiated patients induced chromosomal breaks and dicentric chromosomes in cultured lymphocytes from healthy patients. A few years later, Demoise and Conrad (1972) found that the number of chromosomal aberrations in lymphocytes that had been exposed to plasma from irradiated individuals was double the number of aberrations in the controls. Goh (1975) showed unirradiated leukocytes exhibited chromosomal breaks after being cultured with that plasma from whole body irradiated patients.

The following set of historical data shows that the bystander signals remain active in the organism for long periods of time, even years. An interesting study by Pant and Kamada (1977) showed that plasma from atomic bomb survivors (who were exposed over 30 years earlier) induced chromosomal breaks in 10.9% of leukocytes compared to the 3.7% observed in the controls, which were administered plasma from unirradiated humans. This is the first study demonstrating that bystander factors remain active for at least 30 years after radiation exposure. Faguet et al. (1984) showed that chromosomal breaks persisted in the circulation for at least ten weeks in whole-body irradiated rats and that plasma alone irradiated in vitro was not clastogenic, meaning it did not induce chromosomal damage. Factors which had the ability to induce chromosomal breaks were later shown to persist in plasma from 9 to 20 years in the blood of Chernobyl survivors (Emerit et al., 1994; Marozik, Mothersill,

Seymour, Mosse, & Melnov, 2007). Around this time, research had begun to focus on the communication of bystander effects, which we turn to now.

1.2.3.2 Communication of the Bystander Effects in Cells

Researchers in the field are in agreement that the induction of bystander effects is mediated by signals communicated from the irradiated cell or organism to the unexposed cell or organism (Kadhim et al., 2013; Mothersill & Seymour, 2015). Bystander effects are known to be communicated in two ways, both of which were discovered around the same time by separate researchers. The first is by gap-junction intercellular communication (GJIC) (Azzam et al., 1998), and the second is by extracellular soluble factors (Mothersill & Seymour, 1997) . These mechanism were the only known ways of communication for several years until it was recently discovered that UV photons from irradiated cells can also generate bystander responses (Ahmad et al., 2013; Le, McNeill, Seymour, Rainbow, & Mothersill, 2015).

The involvement of GJIC was first reported by Azzam et al. in 1998 (Azzam et al., 1998) and then confirmed in 2001 (Azzam et al., 2001). In the first series of experiments, the team blocked the gap junctions using Lindane, which prevented the production of bystander effects. Lindane is known to reduce membrane permeability, gap junction number and/or connexin-43 expression (Guan, Bonney, & Ruch, 1995). In the second set of experiments, the authors

found that bystander effects were not induced in human epithelial cells that lacked gap junctions, whereas effects were observed in epithelial cells with normal GJIC. In a follow up study, the authors reported that low doses of alpha particles stimulated the expression of connexin-43, which is one of the proteins required for gap junction formation (Azzam, de Toledo, & Little, 2003). Altogether, these studies suggested that gap junctions play a crucial role in the communication of bystander effects.

Mothersill and Seymour reported the involvement of intracellular soluble factors - as mean of bystander communication – around the same time the GJIC findings were published (Mothersill & Seymour, 1997). In their experiments, they showed that exposure of cells to the culture medium from irradiated epithelial cells reduced the clonogenic survival of unirradiated cells. The following year (Mothersill & Seymour, 1998), the researchers reported that cell-to-cell contact among the irradiated cells was not required for the release of bystander signals into the medium, and that the exposure of cells to the conditioned medium for thirty minutes was sufficient to induce the bystander effect.

Vast amounts of literature have since confirmed the involvement of both GJIC and intercellular soluble factors in the communication of bystander effects and they are regarded as independent mechanisms, which result in a similar effect. (Autsavapromporn et al., 2013; Edouard I Azzam et al., 2003; Fiona M Lyng, Seymour, & Mothersill, 2002; Mothersill & Seymour, 2002; Mothersill et al.,

2006; Kevin M Prise et al., 2005; Schettino et al., 2003). Accordingly, it is not surprising that we now find a large volume of publications aimed at identifying "the bystander molecule". So far, research has identified several molecules that participate in the signal production and response processes, although candidates for the triggering factor remain elusive.

1.2.3.3 Factors causing the bystander effects

The molecules that participate in the signalling process can be subdivided into extracellular mediators and intracellular pathways, a classification that I employ in the interest of clarity.

Extracellular Mediators

The first two bystander factors discovered to induce DNA damage in unirradiated cells were the reactive oxygen species (ROS) and plasma bound NAD(P)H oxidase (Narayanan, Goodwin, & Lehnert, 1997). Interleukin-8 (IL-8) was later found to be associated with increases in ROS (Narayanan, LaRue, Goodwin, & Lehnert, 1999), and further confirmation of the ROS involvement was provided by Azzam et al. (2001; 2003; 2004). In addition to ROS and NAD(P)H oxidase, the transforming growth factor- β1 (TGF-β1) and the tumour necrosis factor-α (TNF-α) were also found to be involved in the bystander signalling process (Iyer & Lehnert, 2000).

Nitric oxide was found to play a role in the bystander signalling process when cellular proliferation was induced in neoplastic human salivary gland cells (Shao, Furusawa, Aoki, Matsumoto, & Ando, 2002; Chunlin Shao, Stewart, Folkard, Michael, & Prise, 2003). Increased radioresistance in p53 wild-type glioblastoma cells (Matsumoto et al., 2001) was observed after exposure to conditioned media containing nitric oxide.

Finally, cyclooxygenase-2 (COX2) (Zhou et al., 2005) and Serotonin (Poon, Agnihotri, Seymour, & Mothersill, 2007) were found to be involved in the bystander signaling process, the latter having been corroborated by several publications (Fazzari, Mersov, Smith, Seymour, & Mothersill, 2012; Fiona M. Lyng, Desplanques, Jella, Garcia, & McClean, 2012; Mothersill, Saroya, Smith, Singh, & Seymour, 2010; Saroya, Smith, Seymour, & Mothersill, 2009). Recent research has shown that long-noncoding RNAs contained in exosomes are bystander candidates (Al-Mayah, Irons, Pink, Carter, & Kadhim, 2012; K Kumar Jella et al., 2014; O'Leary et al., 2015; Xu et al., 2015) while UV photons represent the only physical factor proven to induce bystander effects (Ahmad et al., 2013; Le et al., 2015).

Intracellular Pathways

The extracellular factors presented above communicate the bystander stress by interacting with the plasma membrane and/or the nuclear membrane receptors.

Upon activation of the receptors, it has been shown that mitogen-activated protein kinases (MAPKs) and the NF-κB transcription factor stimulate, either individually or together, the expression of COX2 and Nitric Oxide Synthase (NOS) in the nucleus (Iyer & Lehnert, 2000; Kevin M Prise & O'Sullivan, 2009).

Research has shown that ROS have an active intracellular bystander role in addition to the extracellular participation. ROS can induce bystander micronucleus formation mediated by the production of Super Oxide and Hydrogen Peroxide (Azzam, De Toledo, Spitz, & Little, 2002); as well as inducing intracellular calcium fluxes in bystander cells (Lyng et al., 2002); and regulation of the expression and activity of stress-inducible proteins in the p53 pathways ($p21^{Waf1}$, MDM2, $p34^{cdc2}$) and the MAPK (ERK ½, JNK, p38, Raf1) pathways (Azzam et al., 2004; Edouard I Azzam et al., 2002; F. M. Lyng, Maguire, McClean, Seymour, & Mothersill, 2006; Mothersill & Seymour, 2004).

Other investigations show that blocking the calcium channels abolishes the bystander response (Shao, Lyng, Folkard, & Prise, 2006; F. M. Lyng et al., 2006; Hamada, Matsumoto, Hara, & Kobayashi, 2007) and that external calcium

ions coming into the cell – instead of calcium ions released from intracellular stores – initiated the “bystander pathway” (Lyng et al., 2006). Reduction in the number of mitochondria significantly affects the response to bystander signals, suggesting an important functional role (Hei et al., 2008; Tartier, Gilchrist, Burdak-Rothkamm, Folkard, & Prise, 2007). Earlier studies had also suggested an important role for mitochondria showing that bystander signals induce loss of mitochondrial membrane potential (Lyng, Seymour, & Mothersill, 2001), damage mitochondrial DNA (Murphy, Nugent, Seymour, & Mothersill, 2005) and impair mitochondrial function (Zhou, Ivanov, Lien, Davidson, & Hei, 2008). Protein Kinase C has been found to be significantly elevated in bystander fibroblast compared to directly irradiated controls (Baskar, Balajee, Geard, & Hande, 2008) and blocking the expression of protein kinase C has been found to significantly reduce the number of micronuclei in bystander cells (Hu, Shen, Su, Geard, & Balajee, 2009).

1.2.3.4 Inter-animal Communication of Bystander Signals

Most published studies involving bystander effects in animals are commonly grouped together with abscopal effects and therefore describe biological effects observed in distant unirradiated tissues. However this section aims to review the published data describing the communication of bystander signals from one irradiated animal to another unirradiated animal. In this way,

true bystander mechanisms can be studied in vivo without the complication of blood, neural or endocrine factors.

Surinov et al. (1997)[a] published the first observation of inter-animal communication of bystander effects, although it was not described as such until later. In their experiments, the team placed unirradiated rats and mice in the same cage with irradiated animals for one to two weeks. The researchers observed that the peripheral blood from the unirradiated animals exhibited a marked decrease in leukocyte and nuclear neutrophils – a bystander effect. As further evidence of inter-animal communication, the researchers found that the cage mate unirradiated animals also exhibited decreases in immune reactivity.

The following year, the team showed that the bystander signals were contained in the urine of irradiated mice and rats, and that the immune response of the bystander animals also decreased (Surinov, Karpova, Isaeva, & Kulish, 1998)[b]. The researchers took the bedding from the cage that contained the irradiated animals and placed it beneath the cage of the unirradiated mice and

---

[a,b,c,d] These studies were published in Russian and only their abstracts were available in English.

[e] Their first publication in English.

rats. Since there was no physical contact, the bystander signal must have travelled via volatiles.

In another set of publications, it was suggested that immunosuppression is gender dependent (Surinov, Karpova, Isaeva, & Kulish, 2000)[c] and that the signal involves volatiles that are not species specific (Surinov, Isaeva, & Tokarev, 2001)[d]. If bystander-induced immunosuppression is found to be gender dependent in humans, as well, then it may lead to a more personalized care post-radiotherapy. Surinov et al. (Surinov, Isaeva, & Dukhova, 2004)[e] showed that the immune response of unirradiated C57B1/6 mice was decreased after exposure to volatiles contained in the urine of irradiated mice. Confirmation of these findings were published the following year (Surinov, Isaeva, & Dukhova, 2005), showing that unirradiated animals seemed to be attracted to the bystander signals, and that the immune disturbances could be passed to a second group of unirradiated animals from the bystander non-irradiated group.

Mothersill et al. (2006) published the first report about bystander communication between fish. In these experiments, unirradiated rainbow trout exhibited bystander effects after swimming together with trout that had been exposed to radiation. Further confirmation of bystander effects between fish was conducted in Zebrafish (Mothersill, Smith, Agnihotri, & Seymour, 2007) and by examining the bystander proteome in rainbow trout (Smith et al., 2007). Bystander responses were also found to be persistent during the fish's life span

after receiving radiation in the early life stages (Mothersill, Smith, et al., 2010). Bystander studies in amphibians have shown that unirradiated bullfrog tadpoles swimming with tadpoles previously housed in water containing tritium exhibited an increased resistance to high dose of radiation (Audette-Stuart & Yankovich, 2011). These bystander responses observed in aquatic environments become relevant in areas surrounding CANDU nuclear reactors, where the use of heavy water (deuterium) as cooling system leads to the build up of tritium (Robertson, 1978). Although water purification systems are in place, reports indicate that 1% of tritium escapes through the ventilation system and that lower amounts of heavy water – containing tritium – are released to neighbouring waterways due to malfunction the moderator or cooling system ("The Canadian Nuclear FAQ - Section D: Safety and Liability").

Our group at McMaster University was the first to use Synchrotron radiation in the study of *in vivo* bystander effects. We repeated Surinov's experiments but using Wistar rats and found that irradiated animals communicated bystander signals to unirradiated rats in as few as forty-eight hours (Mothersill et al., 2014). The short animal pairing time in our study contrasted with the much longer one to two week period used by Surinov. In a subsequent study, we conducted a follow up experiment using a different animal model, Fischer rats, and increasing the number of animals (Fernandez-Palomo et al., 2015). We employed two techniques to test for abscopal and bystander mechanisms: first, we cultured tissue explants from animals receiving targeted

irradiation to the right brain and exposed reporter cells to the conditioned medium harvested from these explants; and second, we placed unirradiated animals in the same cage as the irradiated animals for forty-eight hours. We found both that both abscopal within the irradiated animal and bystander effects in the cage mates were produced.

Although there is no similar research involving humans, the investigations presented above may generate concern about the effects of radiotherapy treatments in terms of bystander effect signalling. This concern is described below.

1.3 THE INFLUENCE OF BYSTANDER EFFECTS IN RADIOTHERAPY

For more than a century ionizing radiation has been an essential tool in cancer treatment because of its capacity to kill tumour cells by inducing direct DNA damage (Burdak-Rothkamm & Prise, 2009; Rzeszowska-Wolny, Przybyszewski, & Widel, 2009). Although studies have shown that non-targeted effects play an important role in modulating the damage to normal tissue, conventional radiotherapy treatment remains focused on targeting the DNA (Gamulin, Garaj-Vrhovac, & Kopjar, 2007; Lomax, Folkes, & O'Neill, 2013).

Our laboratory at McMaster University has focused on trying to elucidate the cellular mechanisms and the importance that non-targeted effects have to

humans and non-human biota health (Mothersill & Seymour, 2004). Some authors have suggested that bystander effects could perhaps be modulated to increase tumour cell killing (Kevin M Prise & O'Sullivan, 2009), which is supported by data showing reduction in neoplastic transformations (Azzam et al., 1996). Additionally, bystander effects could potentially affect clinical therapies as a consequence of dose-gradients, which are often observed during intensity-modulated radiotherapy (Kevin M Prise & O'Sullivan, 2009), and are a fundamental part during Synchrotron Microbeam Radiation treatments (Bräuer-Krisch et al., 2010). Nevertheless, the relevance of bystander effects in radiotherapy remains an area requiring further investigation.

This doctoral thesis attempts to shed light on the subject by providing experimental data from preclinical brain radiotherapy studies after exposure to Synchrotron Microbeam Radiation.

1.4 SYNCHROTRON MICROBEAM RADIATION THERAPY

The use of Synchrotron microbeams for radiotherapy was initiated by The Brookhaven National laboratory in Upton, New York (Slatkin, Spanne, Dilmanian, & Sandborg, 1992). Researchers at the laboratory took the concept of GRID therapy – which spatially fractionated the dose to reduce the normal tissue damage – from the centimeter to the micrometer range. This reduction in the beam array allowed for the creation of Microbeam Radiation Therapy (MRT),

which is in a pre-clinical stage and aims to treat brain tumours. The MRT is formed by an array of micro-sized (<100 μm) quasi-parallel rectangular beamlets created by inserting a multi-slit collimator into the path of the high-flux Synchrotron X-rays (Mohiuddin et al., 1999; Trapp et al., 2004). This unique spatial distribution allows the delivery of peak and valley doses. The former is directly deposited on the tissue by the microbeam while the latter is deposited in the tissue between the microbeams by the scattered photons (Blattmann et al., 2005). The combination of peak and valley doses has successfully increased the therapeutic index compared to the broad beam, as will be described.

1.4.1 Benefits of Using Synchrotron Microbeams

There are several advantages of using MRT over conventional radiotherapy.

First, sparing of the normal tissue can be highly achieved by MRT particularly in the central nervous system. Pre-clinical studies have demonstrated this advantage in several animal models, such as weanling piglets (Laissue et al., 2001), duck embryos (Dilmanian et al., 2001), and suckling and adult rats (Bouchet et al., 2014; F Avraham Dilmanian et al., 2003; F. a Dilmanian et al., 2005; F.A. Dilmanian et al., 2002; J. A. Laissue et al., 1999; J. a Laissue et al., 2013; Serduc et al., 2009; D N Slatkin, Spanne, Dilmanian, Gebbers, & Laissue, 1995). The skin has also been shown to tolerate very well, doses of 835-1335

Gy, which are far above of those used in pre-clinical studies (350 Gy) (Zhong, Morris, Bacarian, Rosen, & Avraham Dilmanian, 2003). Moreover, the acute effects on skin produced by high MRT doses were similar to the effects of low doses of broad beam (Priyadarshika, Crosbie, Kumar, & Rogers, 2011). Thus, the organ tolerance, particularly of the normal brain, could allow re-irradiation of the tumour.

Second, since irradiation takes only a few seconds, the treatment requires a very short time. Allowing the MRT to be given in about 1 or 2 days instead of weeks. This is supported by current animals studies showing that imagining can be performed right before irradiation, and the change between one to the other takes around a minute (Serduc, Berruyer, Brochard, Renier, & Nemoz, 2010).

Finally, the delivery of higher doses to tumours may allow the possibility to impair local growth rates. Increasing the palliation of brain malignancies in children who at present cannot be safely and efficiently palliated by existing radiotherapies.

Normal tissues show remarkable tolerance while tumours display high sensitivity to the Synchrotron treatment. This has been attributed to the combination of valley doses reinforced by the peak doses (Bräuer-Krisch et al., 2010). Specifically, the combination of peak and valley doses appears to be high enough to damage the tortuous tumour microvasculature while causing relatively low harm to normal tissues. Research has also revealed that MRT seems to

modulate the immune system by regulating the expression of growth factors, cytokines and lymphokines (Bouchet et al., 2013) and the recruitment of tumour-associated immune cells (Y. Yang et al., 2014) – responses that are also associated with abscopal effects. The question of why or how Synchrotron radiation achieves these responses may lie in the combination of peak and valley doses, which expose the volume of tissue to a range of low and very high doses that may become relevant for the induction of bystander effects. This relevance is described in the next section.

1.4.2 Bystander Effects induced by Synchrotron Radiation

Bystander Effects are relevant to Synchrotron radiation because the tissue exposed to valley doses will also receive the signals emitted by cells in the peak dose areas. Research on the importance of these bystander interactions to potential Synchrotron radiotherapy had, at the time this thesis project was conceived, a traceable history of no more than four years.

The first study suggesting that bystander effects play an important role during Synchrotron microbeam therapy was published by Dilmanian et al. (2007). In their experiments, the team irradiated bovine aortic endothelial cells and rats' spinal cords with MRT. The results revealed that the elimination of apoptotic cells and the repair processes occurred faster than expected. This finding suggested the involvement of "beneficial" bystander factors leading to tissue restoration by

promoting proliferation, migration and differentiation of progenitor cells in the spinal cord. There are earlier studies however, that show increases in cell proliferation (Zhong et al., 2003) and radiation-induced changes in the immune response of local immune cells and tumours (Smilowitz et al., 2006), but these studies did not make any links to bystander effects.

Kashino et. al. (2009) provided further evidence of bystander effects by studying the response of the C6 glioma cell line to Synchrotron microbeams. The authors observed a higher number of DNA DSBs in cells adjacent to the irradiated area than expected to be produced by scatter radiation. They also found cellular migration into the zones irradiated with peak doses. The team corroborated the role of bystander effects by exposing unirradiated cells to the conditioned medium and observing marked increases in the 53BP1 foci.

Tomita, Maeda et al. (2010) found that normal human fibroblast WI-38 cells that had been exposed to Synchrotron microbeams induced cell death in unirradiated cell. The authors corroborated their result by treating WI-38 cells with aminoguanidine (an inhibitor of nitric oxide) prior to irradiation, which abolished the bystander effects and thus the cell death. Another study published by the same group (Maeda, Tomita, Usami, & Kobayashi, 2010) showed that Synchrotron-induced bystander effects were also observed in V79 Chinese hamster lung cells, and they were also abolished by using nitric oxide inhibitors.

The investigations by Tomita, Maeda and team (Maeda et al., 2010; Tomita et al., 2010) also showed a bystander clonogenic survival characterized by high cell killing at low doses, which was followed by a marked escalation in survival as the dose increased. The authors described this response as a parabolic enhancement of bystander cell death. However, this is a well-known response in Radiation Biology known as low-dose Hyper-Radiosensitivity (HRS) and Increased Radioresistance (IRR). Our laboratory at McMaster University has a history of studying HRS and IRR, which was one of the driving factors motivating the investigation of the role of HRS/IRR in Synchrotron radiation as part of this PhD thesis.

1.5 UNDERSTANDING THE LOW-DOSE HYPER-RADIOSENSITIVITY AND INDUCED-RADIORESISTANT PHENOMENA

HRS and IRR are related phenomena that describe a radiation dose response, which is characterised by hypersensitivity to doses below 0.5 Gy followed by a switch to a more radioresistant response. This non-linear response differs from that predicted by a linear-quadratic model (Joiner, Marples, Lambin, Short, & Turesson, 2001; Marples, Wouters, Collis, Chalmers, & Joiner, 2004; K. A. Skov, 1994).

The first modern HRS/IRR study was published by Joiner and Denekamp (1986) and involved a study of the effect of low doses of ionising radiation on the

skin of mice. The results revealed that the cell survival was lower than what the Linear Quadratic (LQ) Model predicted for doses below 1 Gy. Further confirmation of HRS/IRR was published by Marples and Joiner (1993) where Chinese hamster V79-379A cells showed a survival lower than what the LQ Model predicted for doses of X-rays below 0.6 Gy. Those investigations led to the development of the Induced Resistance (IR) Model that better represented this cellular behaviour (Lambin, Marples, Fertil, Malaise, & Joiner, 1993). Interestingly, similar biphasic responses had been reported in the late sixties in protozoa (Calkins, 1967) and in insect cell lines in the eighties (Koval, 1984), but had gone unnoticed for several years.

Although detailed mechanistic processes are provided in Chapter 3 of this thesis, it is important to highlight that HRS seems to be the result of inefficient DNA DSB repair pathways instead of a failure to recognize DNA damage (Wykes, Piasentin, Joiner, Wilson, & Marples, 2006; Yuan, Chang, & Lee, 2003). Consequently, HRS has been reported to be more frequent during the G2 phase of the cell cycle (Short, Bourne, Martindale, Woodcock, & Jackson, 2005; Short, Woodcock, Marples, & Joiner, 2003), but it also occurs in G1 and the early S phase (Kass & Jasin, 2010). Reports suggest that HRS may occurs due to inefficient or delayed homologous recombination, which is mostly activated in the G2 phase (Short et al., 2005) and due to mismatch repair deficiency (Krueger, Collis, Joiner, Wilson, & Marples, 2007; L. Martin et al., 2009). IRR seems to be linked with Non-Homologous End Joining (K. Skov, Marples, Matthews, Joiner, &

Zhou, 1994), which is the primary DSB repair mechanism in mammalian cells during G1 and early S phase (Kass & Jasin, 2010).

While members of our laboratory have reported a direct relationship between cells showing HRS and adaptive responses (Ryan et al., 2009), our findings concerning the relationship between HRS and bystander effects (Mothersill et al., 2002; Ryan et al., 2009) contradict those of others (Heuskin, Wéra, Riquier, Michiels, & Lucas, 2013; Nuta & Darroudi, 2008). This thesis uses glioma cell lines to re-examine the link between bystander effects and HRS.

1.6 THESIS OVERVIEW

1.6.1 Problem Description

Radiation-induced bystander effects are now widely accepted as alternative phenomena to direct DNA damage (Autsavapromporn et al., 2013; Blyth & Sykes, 2011; M. A. Kadhim & Hill, 2015; M. Kadhim et al., 2013; Mothersill & Seymour, 2015). Although an accumulation of research shows great advances in elucidating the bystander cellular mechanisms, these investigations mostly involve the use of conventional sources of ionising radiation (i.e. alpha particles, gamma and X-Ray photons).

Pre-clinical studies show that MRT achieves superior tumour control compared to conventional radiotherapy but the cellular and physical mechanisms

that allow such results remain unknown. Bystander effects have been shown to play an important role at modulating the response of normal tissue after synchrotron exposure. However, the means by which Synchrotron radiation modulates the bystander signal production is poorly understood, and does not take into consideration other types of radiobiological responses such as HRS and IRR.

1.6.2 Proposed Approach

The author proposes to approach the problem from two angles. First, using a set of experiments investigating the direct effects of Synchrotron radiation in the brain of Wistar and Fischer rats and in nude mice, proceeding to determine whether abscopal and bystander effects were able to occur in the tumour-free and tumour-bearing animals in vivo.

The second approach is to examine the response in vitro of the F98 and C6 glioma cell lines used during the animals studies mentioned above, and to compare them to the well-established human T98G glioma cell line. The focus is on elucidating a link between HRS/IRR and bystander effect after exposures to Synchrotron radiation and Gamma photons. Allowing us to better understand the mechanisms involved in the production of bystander signals using these potentially important therapeutic approaches.

1.6.3 General Objective

The general objective of this thesis is to provide new relevant data to the field of Synchrotron Microbeam Radiation Therapy and Radiobiology by studying the production of radiation-induced bystander effects and their relationship with HRS and IRR in vivo and in vitro.

1.6.4 Description of the chapters

The first experimental data are presented in chapter two. The chapter presents results showing how the classical HRS/IRR response occurs in the T98G glioma cell line after exposure to gamma photons. The results also link the HRS phenomenon to bystander effects. The aim of this chapter was to study the mechanisms that provide the foundations for the HRS and IRR responses, which occur in many glioma cell lines and thus are highly relevant since synchrotron MRT is being investigated as a new therapy for glioma multiforme.

The experiments in chapter three were designed to study the HRS and IRR responses of glioma C6 and F98 cell lines after exposure to both Synchrotron and gamma irradiation. This experimental chapter aimed to provide data about the radiation response of these two glioma cell lines, which were employed as tumour models during the animal studies described in chapter four and five.

The research conducted in chapter four looked at the induction of abscopal and bystander effects in Fischer rats harbouring the F98 glioma. Although only the right brain hemisphere of the animals was treated with Synchrotron Radiation, two experiments were performed to distinguish between abscopal and bystander effects. The aim of this research was to validate previous studies showing that inter-animal bystander communication can occur in mammals after exposure to Synchrotron radiation, and to study whether abscopal responses were similar.

Chapter five focused on studying whether abscopal effects can occur in immuno-compromised mice after exposures to Synchrotron Radiation. This work built on chapter four and aimed to elucidate whether the manifestation of abscopal responses required a healthy immune system. It also aimed to study whether the presence of the F98 glioma in the brain of the rats can, in any way, interfere with the abscopal signalling in the animal.

The experiments in Chapter six and seven focused on studying the DNA damage in the brain of Wistar rats and mice after Synchrotron Microbeam Radiation and pencilbeam therapy respectively. Although the endpoint measured was the same in both studies, the chapters had different objectives. Chapter six aimed to increase the current understanding of Synchrotron Microbeams inducing direct DNA damage in the brain of rats, while chapter seven looked to study for

the first time the dynamics of DNA damage after whole-brain pencil beam irradiation.

1.7 REFERENCES

Ahmad, S. B., McNeill, F. E., Byun, S. H., Prestwich, W. V, Mothersill, C., Seymour, C., … Fernandez, C. (2013). Ultra-Violet Light Emission from HPV-G Cells Irradiated with Low Let Radiation From (90)Y; Consequences for Radiation Induced Bystander Effects. *Dose-Response : A Publication of International Hormesis Society*, *11*, 498–516. doi:10.2203/dose-response.12-048.Ahmad

Al-Mayah, A. H. J., Irons, S. L., Pink, R. C., Carter, D. R. F., & Kadhim, M. A. (2012). Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. *Radiation Research*, *177*(5), 539–45. doi:10.1667/RR2868.1

Arbetter, K. R., Prakash, U. B., Tazelaar, H. D., & Douglas, W. W. (1999). Radiation-induced pneumonitis in the “nonirradiated” lung. *Mayo Clinic Proceedings. Mayo Clinic*, *74*(1), 27–36. doi:10.4065/74.1.27

Audette-Stuart, M., & Yankovich, T. (2011). Bystander effects in bullfrog tadpoles. *Radioprotection*, *46*(6), S497–S497. doi:http://dx.doi.org/10.1051/radiopro/20116770

Autsavapromporn, N., Suzuki, M., Funayama, T., Usami, N., Plante, I., Yokota, Y., … Murakami, T. (2013). Gap junction communication and the propagation of bystander effects induced by microbeam irradiation in human fibroblast cultures: the impact of radiation quality. *Radiation Research*, *180*(4), 367–75. doi:10.1667/RR3111.1

Azzam, E., de Toledo, S., & Little, J. (2004). Stress Signaling from Irradiated to Non-Irradiated Cells. *Current Cancer Drug Targets*, *4*(1), 53–64. doi:10.2174/1568009043481641

Azzam, E. I., de Toledo, S. M., Gooding, T., & 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*(5), 497–504. doi:10.2307/3579865

Azzam, E. I., de Toledo, S. M., & Little, J. B. (2001). Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha -particle irradiated to nonirradiated cells. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(2), 473–8. doi:10.1073/pnas.011417098

Azzam, E. I., de Toledo, S. M., & Little, J. B. (2003). Expression of CONNEXIN43 Is Highly Sensitive to Ionizing Radiation and Other Environmental Stresses. *Cancer Res.*, *63*(21), 7128–7135.

Azzam, E. I., de Toledo, S. M., & Little, J. B. (2003). Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. *Oncogene*, *22*(45), 7050–7. doi:10.1038/sj.onc.1206961

Azzam, E. I., de Toledo, S. M., Raaphorst, G. P., & Mitchel, R. E. (1996). Low-dose ionizing radiation decreases the frequency of neoplastic transformation to a level below the spontaneous rate in C3H 10T1/2 cells. *Radiation Research*, *146*(4), 369–373. doi:10.2307/3579298

Azzam, E. I., De Toledo, S. M., Spitz, D. R., & Little, J. B. (2002). Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from ??-particle-irradiated normal human fibroblast cultures. *Cancer Research*, *62*(19), 5436–5442.

Baskar, R., Balajee, A. S., Geard, C. R., & Hande, M. P. (2008). Isoform-specific activation of protein kinase c in irradiated human fibroblasts and their bystander cells. *The International Journal of Biochemistry & Cell Biology*, *40*(1), 125–34. doi:10.1016/j.biocel.2007.07.002

Blattmann, H., Gebbers, J.-O., Bräuer-Krisch, E., Bravin, A., Le Duc, G., Burkard, W., … Laissue, J. a. (2005). Applications of synchrotron X-rays to radiotherapy. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 17–22. doi:10.1016/j.nima.2005.03.060

Blyth, B. J., & Sykes, P. J. (2011). Radiation-induced bystander effects: what are they, and how relevant are they to human radiation exposures? *Radiation Research*, *176*(2), 139–57. doi:10.1667/RR2548.1

Bouchet, A., Bidart, M., Miladi, I., Le Clec'h, C., Serduc, R., Coutton, C., … Le Duc, G. (2014). Characterization of the 9L gliosarcoma implanted in the Fischer rat: an orthotopic model for a grade IV brain tumor. *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *35*(7), 6221–33. doi:10.1007/s13277-014-1783-6

Bouchet, A., Sakakini, N., El Atifi, M., Le Clec'h, C., Brauer, E., Moisan, A., … Pelletier, L. (2013). Early gene expression analysis in 9L orthotopic tumor-bearing rats identifies immune modulation in molecular response to synchrotron microbeam radiation therapy. *PloS One*, *8*(12), e81874. doi:10.1371/journal.pone.0081874

Bräuer-Krisch, E., Serduc, R., Siegbahn, E. a, Le Duc, G., Prezado, Y., Bravin, A., … Laissue, J. A. (2010). Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. *Mutation Research*, *704*(1-3), 160–6. doi:10.1016/j.mrrev.2009.12.003

Brooks, A., Retherford, J. C., & McClellan, R. (1974). Effect of239 PuO2 Particle Number and Size on the Frequency and Distribution of Chromosome Aberrations in the Liver of the Chinese Hamster. *Radiation Research*, *59*(3), 693–709.

Brown, J. M., & Wilson, G. (2003). Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? *Cancer Biology & Therapy*, *2*(5), 477–490.

Burdak-Rothkamm, S., & Prise, K. M. (2009). New molecular targets in radiotherapy: DNA damage signalling and repair in targeted and non-targeted cells. *European Journal of Pharmacology*, *625*(1-3), 151–5. doi:10.1016/j.ejphar.2009.09.068

Calkins, J. (1967). An unusual form of response in x-irradiated protozoa and a hypothesis as to its origin. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry, and Medicine*, *12*(3), 297–301.

Camphausen, K., Moses, M. a., Ménard, C., Sproull, M., Beecken, W. D., Folkman, J., & O'Reilly, M. S. (2003). Radiation abscopal antitumor effect is mediated through p53. *Cancer Research*, *63*(8), 1990–1993. doi:10.1016/S0360-3016(02)03449-1

Chang, W. P., & Little, J. B. (1992). Persistently elevated frequency of spontaneous mutations in progeny of CHO clones surviving X-irradiation: Association with delayed reproductive death phenotype. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *270*(2), 191–199. doi:10.1016/0027-5107(92)90130-T

Clutton, S. M., Townsend, K. M., Walker, C., Ansell, J. D., & Wright, E. G. (1996). Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. *Carcinogenesis*, *17*(8), 1633–1639. doi:10.1093/carcin/17.8.1633

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., … Yuan, J. (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. *Nature Chemical Biology*, *1*(2), 112–119. doi:10.1038/nchembio711

Demoise, C. F., & Conrad, R. A. (1972). Effects of age and radiation exposure on chromosomes in a Marshall Island population. *Journal of Gerontology*, *27*(2), 197–201. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5060721

Deshpande, a, Goodwin, E. H., Bailey, S. M., Marrone, B. L., & Lehnert, B. E. (1996). Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. *Radiation Research*, *145*(3), 260–267. doi:10.2307/3578980

Dewey, W. C., Ling, C. C., & Meyn, R. E. (1995). Radiation-induced apoptosis: Relevance to radiotherapy. *International Journal of Radiation Oncology Biology Physics*, *33*(4), 781–796. doi:10.1016/0360-3016(95)00214-8

Dilmanian, F. a, Qu, Y., Liu, S., Cool, C. D., Gilbert, J., Hainfeld, J. F., … McDonald, J. W. (2005). X-ray microbeams: Tumor therapy and central nervous system research. *Nuclear Instruments & Methods in Physics Research. Section A, Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 30–37. doi:10.1016/j.nima.2005.03.062

Dilmanian, F. A., Button, T. M., Le Duc, G., Zhong, N., Peña, L. A., Smith, J. A. L., … Ren, B. (2002). Response of rat intracranial 9L gliosarcoma to microbeam radiation therapy. *Neuro-Oncology*, *4*(1), 26. doi:10.1016/j.ejrad.2008.04.055

Dilmanian, F. A., Morris, G. M., Le Duc, G., Huang, X., Ren, B., Bacarian, T., … Shivaprasad, H. L. (2001). Response of avian embryonic brain to spatially segmented x-ray microbeams. *Cellular and Molecular Biology (Noisy-Le-Grand, France)*, *47*(3), 485–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11441956

Dilmanian, F. A., Morris, G. M., Zhong, N., Bacarian, T., Hainfeld, J. F., Kalef-Ezra, J., … Rosen, E. M. (2003). Murine EMT-6 carcinoma: high therapeutic efficacy of microbeam radiation therapy. *Radiation Research*, *159*(5), 632–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12710874

Dilmanian, F. A., Qu, Y., Feinendegen, L. E., Peña, L. A., Bacarian, T., Henn, F. A., … McDonald, J. W. (2007). Tissue-sparing effect of x-ray microplanar beams particularly in the CNS: is a bystander effect involved? *Experimental Hematology*, *35*(4 Suppl 1), 69–77. doi:10.1016/j.exphem.2007.01.014

Elkind, M. M., & Whitmore, G. F. (1967). *The radiobiology of cultured mammalian cells*. Gordon and Breach. Retrieved from https://books.google.com/books?id=UQe2AAAAIAAJ&pgis=1

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic Pathology*, *35*(4), 495–516. doi:10.1080/01926230701320337

Emerit, I., Levy, a, Cernjavski, L., Arutyunyan, R., Oganesyan, N., Pogosian, a, … Quastel, M. (1994). Transferable clastogenic activity in plasma from persons exposed as salvage personnel of the Chernobyl reactor. *Journal of Cancer Research and Clinical Oncology*, *120*(9), 558–61. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8045922

Faguet, G. B., Reichard, S. M., & Welter, D. a. (1984). Radiation-induced clastogenic plasma factors. *Cancer Genetics and Cytogenetics*, *12*(1), 73–83. doi:10.1016/0165-4608(84)90010-4

Fazzari, J., Mersov, A., Smith, R., Seymour, C., & Mothersill, C. (2012). Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect. *International Journal of Radiation Biology*, *88*(10), 786–90. doi:10.3109/09553002.2012.714519

Fei, P., & El-Deiry, W. S. (2003). P53 and radiation responses. *Oncogene*, *22*(37), 5774–5783. doi:10.1038/sj.onc.1206677

Fernandez-Palomo, C., Bräuer-Krisch, E., Laissue, J., Vukmirovic, D., Blattmann, H., Seymour, C., … Mothersill, C. (2015). Use of synchrotron medical microbeam irradiation to investigate radiation-induced bystander and abscopal effects in vivo. *Physica Medica : PM : An International Journal Devoted to the Applications of Physics to Medicine and Biology : Official Journal of the Italian Association of Biomedical Physics (AIFB)*, 1–12. doi:10.1016/j.ejmp.2015.03.004

Fernandez-Palomo, C., Schültke, E., Smith, R., Bräuer-Krisch, E., Laissue, J., Schroll, C., … Mothersill, C. (2013). Bystander effects in tumor-free and tumor-bearing rat brains following irradiation by synchrotron X-rays. *International Journal of Radiation Biology*, *89*(6), 445–53. doi:10.3109/09553002.2013.766770

Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., & Abraham, G. N. (1993). The “bystander effect”: Tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Research*, *53*(21), 5274–5283.

Gamulin, M., Garaj-Vrhovac, V., & Kopjar, N. (2007). Evaluation of DNA damage in radiotherapy-treated cancer patients using the alkaline comet assay. *Collegium Antropologicum*, *31*(3), 837–845.

Goh K-O. (1975). Total-body irradiation and human chromosomes. V. Additional evidence of a transferable substance in the plasma of irradiated persons to induce chromosomal breakages. *Journal of Medicine*, *6*(1), 51–60.

Goh, K., & 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*(1), 171–181.

Gow, M. D., Seymour, C. B., Byun, S.-H., & Mothersill, C. E. (2008). Effect of dose rate on the radiation-induced bystander response. *Physics in Medicine and Biology*, *53*(1), 119–32. doi:10.1088/0031-9155/53/1/008

Guan, X., Bonney, W. J., & Ruch, R. J. (1995). Changes in gap junction permeability, gap junction number, and connexin43 expression in lindane-treated rat liver epithelial cells. *Toxicology and Applied Pharmacology*, *130*(1), 79–86. doi:10.1006/taap.1995.1011

Hall, E. J. (2003). THE BYSTANDER EFFECT. *Health Physics*, *85*(1), 31. Retrieved from http://journals.lww.com/health-physics/Abstract/2003/07000/The_Bystander_Effect.8.aspx

Hall, E. J., & Giaccia, A. J. (2006). *Radiobiology for the Radiologist*. Lippincott Williams & Wilkins. Retrieved from http://books.google.ca/books/about/Radiobiology_for_the_Radiologist.html?id=6HhjwRyqBzgC&pgis=1

Hamada, N., Matsumoto, H., Hara, T., & Kobayashi, Y. (2007). Intercellular and Intracellular Signaling Pathways Mediating Ionizing Radiation-Induced Bystander Effects. *Journal of Radiation Research*, *48*(2), 87–95. doi:10.1269/jrr.06084

Hei, T. K., Zhou, H., Ivanov, V. N., Hong, M., Lieberman, H. B., Brenner, D. J., … Geard, C. R. (2008). Mechanism of radiation-induced bystander effects: a unifying model. *The Journal of Pharmacy and Pharmacology*, *60*(8), 943–50. doi:10.1211/jpp.60.8.0001

Heuskin, A.-C., Wéra, A.-C., Riquier, H., Michiels, C., & Lucas, S. (2013). Low-Dose Hypersensitivity and Bystander Effect are Not Mutually Exclusive in A549 Lung Carcinoma Cells after Irradiation with Charged Particles. *Radiation Research*, *180*(5), 491–8. doi:10.1667/RR13358.1

Hickman, a W., Jaramillo, R. J., Lechner, J. F., & Johnson, N. F. (1994). Alpha-particle-induced p53 protein expression in a rat lung epithelial cell strain. *Cancer Research*, *54*(22), 5797–5800.

Hollowell, J. G. J., & Littlefield, L. G. (1967). CHROMOSOME ABERRATIONS INDUCED BY PLASMA FROM IRRADIATED PATIENTS: A BRIEF REPORT. *J. S. C. Med. Ass., 63: 437 442(Dec. 19, 1967).* Retrieved from http://www.osti.gov/scitech/biblio/4798922

Hollowell, J. G., & Littlefield, L. G. (1968). Chromosome damage induced by plasma of x-rayed patients: an indirect effect of x-ray. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.)*, *129*(1), 240–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5686521

Hu, B., Shen, B., Su, Y., Geard, C. R., & Balajee, A. S. (2009). Protein kinase C epsilon is involved in ionizing radiation induced bystander response in human cells. *The International Journal of Biochemistry & Cell Biology*, *41*(12), 2413–21. doi:10.1016/j.biocel.2009.06.012

Iyer, R., & Lehnert, B. E. (2000). Factors underlying the cell growth-related bystander responses to ?? particles. *Cancer Research*, *60*(5), 1290–1298. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10728689

Jella, K. K., Garcia, A., McClean, B., Byrne, H. J., & Lyng, F. M. (2013). Cell death pathways in directly irradiated cells and cells exposed to medium from irradiated cells. *International Journal of Radiation Biology*, *89*(3), 182–90. doi:10.3109/09553002.2013.734942

Jella, K. K., Rani, S., O'Driscoll, L., McClean, B., Byrne, H. J., & Lyng, F. M. (2014). Exosomes are involved in mediating radiation induced bystander signaling in human keratinocyte cells. *Radiation Research*, *181*(2), 138–45. doi:10.1667/RR13337.1

Joiner, M. C., & Denekamp, J. (1986). The effect of small radiation doses on mouse skin. *The British Journal of Cancer. Supplement*, *7*, 63–6. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2149747&tool=pmcentrez&rendertype=abstract

Joiner, M. C., Marples, B., Lambin, P., Short, S. C., & Turesson, I. (2001). Low-dose hypersensitivity: current status and possible mechanisms. *International Journal of Radiation Oncology, Biology, Physics*, *49*(2), 379–89. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11173131

Kadhim, M. A., & Hill, M. A. (2015). NON-TARGETED EFFECTS OF RADIATION EXPOSURE: RECENT ADVANCES AND IMPLICATIONS. *Radiation Protection Dosimetry*, 1–7. doi:10.1093/rpd/ncv167

Kadhim, M. A., Macdonald, D. A., Goodhead, D. T., Lorimore, S. A., Marsden, S. J., & Wright, E. G. (1992). Transmission of chromosomal instability after plutonium alpha-particle irradiation. *Nature*, *355*(6362), 738–740. doi:10.1038/355738a0

Kadhim, M., Salomaa, S., Wright, E., Hildebrandt, G., Belyakov, O. V., Prise, K. M., & Little, M. P. (2013). Non-targeted effects of ionising radiation-Implications for low dose risk. *Mutation Research - Reviews in Mutation Research*, *752*(2), 84–98. doi:10.1016/j.mrrev.2012.12.001

Kaminski, J. M., Shinohara, E., Summers, J. B., Niermann, K. J., Morimoto, A., & Brousal, J. (2005). The controversial abscopal effect. *Cancer Treatment Reviews*, *31*(3), 159–72. doi:10.1016/j.ctrv.2005.03.004

Kashino, G., Kondoh, T., Nariyama, N., Umetani, K., Ohigashi, T., Shinohara, K., … Ono, K. (2009). Induction of DNA double-strand breaks and cellular migration through bystander effects in cells irradiated with the slit-type microplanar beam of the spring-8 synchrotron.

International Journal of Radiation Oncology, Biology, Physics, *74*(1), 229–36. doi:10.1016/j.ijrobp.2008.09.060

Kass, E. M., & Jasin, M. (2010). Collaboration and competition between DNA double-strand break repair pathways. *FEBS Letters*, *584*(17), 3703–3708. doi:10.1016/j.febslet.2010.07.057

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, *26*(4), 239–57. Retrieved from /pmc/articles/PMC2008650/?report=abstract

Khan, M. A., Hill, R. P., & Van Dyk, J. (1998). Partial volume rat lung irradiation: an evaluation of early DNA damage. *International Journal of Radiation Oncology, Biology, Physics*, *40*(2), 467–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9457837

Khan, M. A., Van Dyk, J., Yeung, I. W. T., & Hill, R. P. (2003). Partial volume rat lung irradiation; Assessment of early DNA damage in different lung regions and effect of radical scavengers. *Radiotherapy and Oncology*, *66*(1), 95–102. doi:10.1016/S0167-8140(02)00325-0

Korystov YuN. (1992). Contributions of the direct and indirect effects of ionizing radiation to reproductive cell death. *Radiation Research*, *129*(2), 228–234.

Koturbash, I., Kutanzi, K., Hendrickson, K., Rodriguez-Juarez, R., Kogosov, D., & Kovalchuk, O. (2008). Radiation-induced bystander effects in vivo are sex specific. *Mutation Research*, *642*(1-2), 28–36. doi:10.1016/j.mrfmmm.2008.04.002

Koval, T. M. (1984). Multiphasic survival response of a radioresistant lepidopteran insect cell line. *Radiation Research*, *98*(3), 642–8.

Krueger, S. a., Collis, S. J., Joiner, M. C., Wilson, G. D., & Marples, B. (2007). Transition in Survival From Low-Dose Hyper-Radiosensitivity to??Increased Radioresistance Is Independent of Activation of ATM SER1981 Activity. *International Journal of Radiation Oncology Biology Physics*, *69*(4), 1262–1271. doi:10.1016/j.ijrobp.2007.08.012

Laissue, J. a, Bartzsch, S., Blattmann, H., Bräuer-Krisch, E., Bravin, A., Dalléry, D., … Slatkin, D. N. (2013). Response of the rat spinal cord to X-ray microbeams. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, *106*(1), 106–11. doi:10.1016/j.radonc.2012.12.007

Laissue, J. A., Blattmann, H., Di Michiel, M., Slatkin, D. N., Lyubimova, N., Guzman, R., … Wagner, H.-P. (2001). The weanling piglet cerebellum: a surrogate for tolerance to MRT (microbeam radiation therapy) in pediatric neuro-oncology. *Penetrating Radiation Systems and Application*, *4508*, 65–73. doi:10.1117/12.450774

Laissue, J. A., Lyubimova, N., Wagner, H.-P., Archer, D. W., Slatkin, D. N., Di Michiel, M., … Blattmann, H. (1999). Microbeam radiation therapy. In H. B. Barber & H. Roehrig (Eds.), *SPIE's International Symposium on Optical Science, Engineering, and Instrumentation* (pp. 38–45). International Society for Optics and Photonics. doi:10.1117/12.368185

Lambin, P., Marples, B., Fertil, B., Malaise, E. P., & Joiner, M. C. (1993). Hypersensitivity of a human tumour cell line to very low radiation doses. *International Journal of Radiation Biology*, *63*(5), 639–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8099110

Lauber, K., Ernst, A., Orth, M., Herrmann, M., & Belka, C. (2012). Dying cell clearance and its impact on the outcome of tumor radiotherapy. *Frontiers in Oncology*, *2*(September), 1–14. doi:10.3389/fonc.2012.00116

Le, M., McNeill, F. E., Seymour, C., Rainbow, A. J., & Mothersill, C. E. (2015). An Observed Effect of Ultraviolet Radiation Emitted from Beta-Irradiated HaCaT Cells upon Non-Beta-Irradiated Bystander Cells. *Radiation Research*, *183*(3), 279–290. doi:10.1667/RR13827.1

Li, H., Ambade, A., & Re, F. (2009). Cutting edge: Necrosis activates the NLRP3 inflammasome. *Journal of Immunology (Baltimore, Md. : 1950)*, *183*(3), 1528–1532. doi:10.4049/jimmunol.0901080

Limoli, C. L., Kaplan, M. I., Giedzinski, E., & Morgan, W. F. (2001). Attenuation of radiation-induced genomic instability by free radical scavengers and cellular proliferation. *Free Radical Biology and Medicine*, *31*(1), 10–19. doi:10.1016/S0891-5849(01)00542-1

Limoli, C. L., Limoli, C. L., Hartmann, A., Hartmann, A., Shephard, L., Shephard, L., … Morgan, W. F. (1998). Apoptosis, Reproductive Failure, and Oxidative Stress in Chinese Hamster Ovary Cells with Compromised Genomic Integrity1. *Life Sciences*, (16).

Lomax, M. E., Folkes, L. K., & O'Neill, P. (2013). Biological consequences of radiation-induced DNA damage: Relevance to radiotherapy. *Clinical Oncology*, *25*(10), 578–585. doi:10.1016/j.clon.2013.06.007

Lorimore, S. a, Coates, P. J., Scobie, G. E., Milne, G., & Wright, E. G. (2001). Inflammatory-type responses after exposure to ionizing radiation in vivo: a mechanism for radiation-induced bystander effects? *Oncogene*, *20*(48), 7085–95. doi:10.1038/sj.onc.1204903

Lyng, F. M., Desplanques, M., Jella, K. K., Garcia, A., & McClean, B. (2012). The importance of serum serotonin levels in the measurement of radiation-induced bystander cell death in HaCaT cells. *International Journal of Radiation Biology*, *88*(10), 770–772. doi:10.3109/09553002.2012.705222

Lyng, F. M., Maguire, P., McClean, B., Seymour, C., & Mothersill, C. (2006). The Involvement of Calcium and MAP Kinase Signaling Pathways in the Production of Radiation-Induced Bystander Effects. *Radiation Research*, *165*(4), 400–409. doi:10.1667/RR3527.1

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2000). Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *British Journal of Cancer*, *83*(9), 1223–30. doi:10.1054/bjoc.2000.1433

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2001). Oxidative stress in cells exposed to low levels of ionizing radiation. *Biochemical Society Transactions*, *29*(2), 350. doi:10.1042/0300-5127:0290350

Lyng, F. M., Seymour, C., & 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*(4), 365–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893237

Maeda, M., Tomita, M., Usami, N., & Kobayashi, K. (2010). Bystander cell death is modified by sites of energy deposition within cells irradiated with a synchrotron X-ray microbeam. *Radiation Research*, *174*(1), 37–45. doi:10.1667/RR2086.1

Maguire, P., Mothersill, C., McClean, B., Seymour, C., & Lyng, F. M. (2007). Modulation of radiation responses by pre-exposure to irradiated cell conditioned medium. *Radiation Research*, *167*(4), 485–92. doi:10.1667/RR0159.1

Mancuso, M., Pasquali, E., Giardullo, P., Leonardi, S., Tanori, M., Di Majo, V., … Saran, A. (2012). The radiation bystander effect and its potential implications for human health. *Current Molecular Medicine*, *12*(5), 613–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22452594

Mancuso, M., Pasquali, E., Leonardi, S., Tanori, M., Rebessi, S., Di Majo, V., … Saran, A. (2008). Oncogenic bystander radiation effects in Patched heterozygous mouse cerebellum. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(34), 12445–12450. doi:10.1073/pnas.0804186105

Marozik, P., Mothersill, C., Seymour, C. B., Mosse, I., & Melnov, S. (2007). Bystander effects induced by serum from survivors of the Chernobyl accident. *Experimental Hematology*, *35*(4 Suppl 1), 55–63. doi:10.1016/j.exphem.2007.01.029

Marples, B., & Joiner, M. C. (1993). The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. *Radiation Research*, *133*(1), 41–51.

Marples, B., Wouters, B. G., Collis, S. J., Chalmers, a J., & Joiner, M. C. (2004). Low-dose hyper-radiosensitivity: a consequence of ineffective cell cycle arrest of radiation-damaged G2-phase cells. *Radiation Research*, *161*(3), 247–55. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14982490

Martin, C., Romero, S., Sánchez-Payá, J., Massutí, B., Arriero, J. M., & Hernández, L. (1999). Bilateral lymphocytic alveolitis: A common reaction after unilateral thoracic irradiation. *European Respiratory Journal*, *13*(4), 727–732. doi:10.1034/j.1399-3003.1999.13d05.x

Martin, L., Marples, B., Coffey, M., Lawler, M., Hollywood, D., & Marignol, L. (2009). Recognition of O6MeG lesions by MGMT and mismatch repair proficiency may be a prerequisite for low-dose radiation hypersensitivity. *Radiation Research*, *172*(4), 405–413. doi:10.1667/RR1717.1

Matsumoto, H., Hayashi, S., Hatashita, M., Ohnishi, K., Shioura, H., Ohtsubo, T., … Kano, E. (2001). Induction of radioresistance by a nitric oxide-mediated bystander effect. *Radiation Research*, *155*(3), 387–396. doi:10.1667/0033-7587(2001)155[0387:IORBAN]2.0.CO;2

Matsumoto, H., Takahashi, A., & Ohnishi, T. (2004). Radiation-induced adaptive responses and bystander effects. *Uchū Seibutsu Kagaku*, *18*(4), 247–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15858392

Mendonca, M. S., Kurohara, W., Antoniono, R., & Redpath, J. L. (1989). Plating efficiency as a function of time postirradiation: evidence for the delayed expression of lethal mutations. *Radiation Research*, *119*(2), 387–393. doi:10.2307/3577629

Mohiuddin, M., Fujita, M., Regine, W. F., Megooni, a S., Ibbott, G. S., & Ahmed, M. M. (1999). High-dose spatially-fractionated radiation (GRID): a new paradigm in the management of advanced cancers. *International Journal of Radiation Oncology, Biology, Physics*, *45*(3), 721–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10524428

Morgan, G. W., & Breit, S. N. (1995). Radiation and the lung: a reevaluation of the mechanisms mediating pulmonary injury. *International Journal of Radiation Oncology, Biology, Physics*, *31*(2), 361–9. doi:10.1016/0360-3016(94)00477-3

Morgan, W. F. (2003). Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. *Radiation Research*, *159*(5), 567–580. doi:10.1667/RRAV19.1

Mothersill, C., Bucking, C., Smith, R., Agnihotri, N., Oneill, a, Kilemade, M., & Seymour, C. (2006). Communication of radiation-induced stress or bystander signals between fish in vivo. *Environmental Science & Technology*, *40*(21), 6859–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17144322

Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., … Seymour, C. (2014). Transmission of signals from rats receiving high doses of microbeam radiation to cage mates: an inter-mammal bystander effect. *Dose-Response : A Publication of International Hormesis Society*, *12*(1), 72–92. doi:10.2203/dose-response.13-011.Mothersill

Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S., & Wright, E. (2005). Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. *Radiation Research*, *163*(4), 391–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15799694

Mothersill, C., Saroya, R., Smith, R. W., Singh, H., & Seymour, C. B. (2010). Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments. *Radiation Research*, *174*(1), 119–23. doi:10.1667/RR2036.1

Mothersill, C., & Seymour, C. (1997). Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology*, *71*(4), 421–7. doi:10.1080/095530097144030

Mothersill, C., & Seymour, C. (2001). Radiation-induced bystander effects: past history and future directions. *Radiation Research*, *155*(6), 759–767. doi:10.1667/0033-7587(2001)155[0759:RIBEPH]2.0.CO;2

Mothersill, C., & Seymour, C. (2002). Bystander and delayed effects after fractionated radiation exposure. *Radiation Research*, *158*(5), 626–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12385640

Mothersill, C., & Seymour, C. (2006). Radiation-induced bystander effects: evidence for an adaptive response to low dose exposures? *Dose-Response : A Publication of International Hormesis Society*, *4*(4), 283–90. doi:10.2203/dose-response.06-111.Mothersill

Mothersill, C., & Seymour, C. (2010). Radiation-induced bystander effects: are they good, bad or both? *Medicine, Conflict, and Survival*, *21*(2), 101–10. doi:10.1080/13623690500073398

MotherSill, C., & Seymour, C. (2012). Changing paradigms in radiobiology. *Mutation Research/Reviews in Mutation Research*, *750*(2), 85–95. doi:10.1016/j.mrrev.2011.12.007

Mothersill, C., & Seymour, C. (2015). RADIATION-INDUCED NON-TARGETED EFFECTS: SOME OPEN QUESTIONS. *Radiation Protection Dosimetry*, 1–6. doi:10.1093/rpd/ncv155

Mothersill, C., & Seymour, C. B. (1998). Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiation Research*, *149*(3), 256–262. doi:10.2307/3579958

Mothersill, C., & Seymour, C. B. (2004). Radiation-induced bystander effects--implications for cancer. *Nature Reviews. Cancer*, *4*(2), 158–164. doi:10.1038/nrc1277

Mothersill, C., Seymour, C. B., & Joiner, M. C. (2002). Relationship between radiation-induced low-dose hypersensitivity and the bystander effect. *Radiation Research*, *157*(5), 526–32. doi:10.1667/0033-7587(2002)157[0526:RBRILD]2.0.CO;2

Mothersill, C., Smith, R. W., Agnihotri, N., & Seymour, C. B. (2007). Characterization of a radiation-induced stress response communicated in vivo between zebrafish. *Environmental Science & Technology*, *41*(9), 3382–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17539553

Mothersill, C., Smith, R. W., Saroya, R., Denbeigh, J., Rowe, B., Banevicius, L., … Seymour, C. B. (2010). Irradiation of rainbow trout at early life stages results in legacy effects in adults. *International Journal of Radiation Biology*, *86*(10), 817–28. doi:10.3109/09553002.2010.486018

Muñoz, L. E., Lauber, K., Schiller, M., Manfredi, A. A., & Herrmann, M. (2010). The role of defective clearance of apoptotic cells in systemic autoimmunity. *Nature Reviews. Rheumatology*, *6*(5), 280–289. doi:10.1038/nrrheum.2010.46

Munro, A. J. (2009). Bystander effects and their implications for clinical radiotherapy. *Journal of Radiological Protection : Official Journal of the Society for Radiological Protection*, *29*(2A), A133–42. doi:10.1088/0952-4746/29/2A/S09

Murphy, J. B., & Morton, J. J. (1915). The Effect Of Roentgen Rays On The Rate Of Growth Of Spontaneous Tumors In Mice. *The Journal of Experimental Medicine*, *22*(6), 800–803. doi:10.1084/jem.22.6.800

Murphy, J. E. J., Nugent, S., Seymour, C., & Mothersill, C. (2005). Mitochondrial DNA point mutations and a novel deletion induced by direct low-LET radiation and by medium from irradiated cells. *Mutation Research*, *585*(1-2), 127–36. doi:10.1016/j.mrgentox.2005.04.011

Nagasawa, H., & Little, J. B. (1992). Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Research*, *52*(22), 6394–6396. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1423287

Narayanan, P. K., Goodwin, E. H., & Lehnert, B. E. (1997). Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Research*, *57*(18), 3963–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9307280

Narayanan, P. K., LaRue, K. E., Goodwin, E. H., & Lehnert, B. E. (1999). Alpha particles induce the production of interleukin-8 by human cells. *Radiation Research*, *152*(1), 57–63. doi:10.2307/3580049

Nuta, O., & Darroudi, F. (2008). The impact of the bystander effect on the low-dose hypersensitivity phenomenon. *Radiation and Environmental Biophysics*, *47*(2), 265–74. doi:10.1007/s00411-007-0145-9

O'Leary, V. B., Ovsepian, S. V., Carrascosa, L. G., Buske, F. A., Radulovic, V., Niyazi, M., … Anastasov, N. (2015). PARTICLE, a Triplex-Forming Long ncRNA, Regulates Locus-Specific Methylation in Response to Low-Dose Irradiation. *Cell Reports*, 474–485. doi:10.1016/j.celrep.2015.03.043

Olivieri, G., Bodycote, J., & Wolff, S. (1984). Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. *Science (New York, N.Y.)*, *223*(4636), 594–597.

Pampfer, S., & Streffer, C. (1989). Increased chromosome aberration levels in cells from mouse fetuses after zygote X-irradiation. *International Journal of Radiation Biology*, *55*(1), 85–92. doi:QMH6QGL9Q26Q1BJ0 [pii]

Pant, G. S. S., & Kamada, N. (1977). Chromosome aberrations in normal leukocytes induced by the plasma of exposed individuals. *Hiroshima Journal of Medical Sciences*, *26*(2-3), 149–54. Retrieved from http://inis.iaea.org/Search/search.aspx?orig_q=RN:9386153

Parsons, W. B., Watkins, C. H., Pease, G. L., & Childs, D. S. (1954). Changes in sternal marrow following roentgen-ray therapy to the spleen in chronic granulocytic leukemia. *Cancer*, *7*(1), 179–189. doi:10.1002/1097-0142(195401)7:1<179::AID-CNCR2820070120>3.0.CO;2-A

Poncy, J. L., Walter, C., Fritsch, P., Masse, R., & Lafuma, J. (1979). Delayed SCE frequency of rat bone marrow cells after radon inhalation. Retrieved from http://www.iaea.org/inis/collection/NCLCollectionStore/_Public/11/535/11535419.pdf

Poon, R. C. C., Agnihotri, N., Seymour, C., & Mothersill, C. (2007). Bystander effects of ionizing radiation can be modulated by signaling amines. *Environmental Research*, *105*(2), 200–11. doi:10.1016/j.envres.2006.12.003

Prise, K. M. (1998). Studies of bystander effects in human fibroblasts using a charged particle microbeam. *International Journal of Radiation Biology*, *74*(6), 793–798. doi:10.1080/095530098141087

Prise, K. M., & O'Sullivan, J. M. (2009). Radiation-induced bystander signalling in cancer therapy. *Nature Reviews. Cancer*, *9*(5), 351–60. doi:10.1038/nrc2603

Prise, K. M., Schettino, G., Folkard, M., & Held, K. D. (2005). New insights on cell death from radiation exposure. *The Lancet Oncology*, *6*(7), 520–8. doi:10.1016/S1470-2045(05)70246-1

Priyadarshika, R. C. U., Crosbie, J. C., Kumar, B., & Rogers, P. a W. (2011). Biodosimetric quantification of short-term synchrotron microbeam versus broad-beam radiation damage to mouse skin using a dermatopathological scoring system. *The British Journal of Radiology*, *84*(1005), 833–42. doi:10.1259/bjr/58503354

Puck, T. T., & Marcus, P. I. (1956). Action of x-rays on mammalian cells. *The Journal of Experimental Medicine*, *103*(5), 653–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10380286

Robertson, J. a. (1978). The CANDU Reactor System: An Appropriate Technology. *Science (New York, N.Y.)*, *199*(4329), 657–664. doi:10.1126/science.199.4329.657

Rodier, F., & Campisi, J. (2011). Four faces of cellular senescence. *Journal of Cell Biology*, *192*(4), 547–556. doi:10.1083/jcb.201009094

Ryan, L. a, Seymour, C. B., Joiner, M. C., & Mothersill, C. E. (2009). Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. *International Journal of Radiation Biology*, *85*(1), 87–95. doi:10.1080/09553000802635062

Rzeszowska-Wolny, J., Przybyszewski, W. M., & Widel, M. (2009). Ionizing radiation-induced bystander effects, potential targets for modulation of radiotherapy. *European Journal of Pharmacology*, *625*(1-3), 156–64. doi:10.1016/j.ejphar.2009.07.028

Saroya, R., Smith, R., Seymour, C., & Mothersill, C. (2009). Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism. *Dose-Response : A Publication of International Hormesis Society*, *8*(3), 317–30. doi:10.2203/dose-response.09-043.Saroya

Sawant, S. G., Zheng, W., Hopkins, K. M., Randers-Pehrson, G., Lieberman, H. B., & Hall, E. J. (2002). The radiation-induced bystander effect for clonogenic survival. *Radiation Research*, *157*(4), 361–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893236

Schettino, G., Folkard, M., Prise, K. M., Vojnovic, B., Held, K. D., & Michael, B. D. (2003). Low-dose studies of bystander cell killing with targeted soft X rays. *Radiation Research*, *160*(5), 505–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14565833

Serduc, R., Berruyer, G., Brochard, T., Renier, M., & Nemoz, C. (2010). In vivo pink-beam imaging and fast alignment procedure for rat brain lesion microbeam radiation therapy. *Journal of Synchrotron Radiation*, *17*(3), 325–31. doi:10.1107/S0909049510006667

Serduc, R., Bouchet, A., Bräuer-Krisch, E., Laissue, J. A., Spiga, J., Sarun, S., … Le Duc, G. (2009). Synchrotron microbeam radiation therapy for rat brain tumor palliation-influence of the microbeam width at constant valley dose. *Physics in Medicine and Biology*, *54*(21), 6711–24. doi:10.1088/0031-9155/54/21/017

Seymour, C. B., Mothersill, C., & Alper, T. (1986). High yields of lethal mutations in somatic mammalian cells that survive ionizing radiation. *International Journal of Radiation Biology and Related Studies in Physics, Chemistry, and Medicine*, *50*(1), 167–179. doi:10.1080/09553008614550541

Seymour, C., & Mothersill, C. (1992). All colonies of CHO-K1 cells surviving γ-irradiation contain non-viable cells. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *267*(1), 19–30. doi:10.1016/0027-5107(92)90107-D

Shao, C., Folkard, M., Michael, B. D., & Prise, K. M. (2004). Targeted cytoplasmic irradiation induces bystander responses. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(37), 13495–500. doi:10.1073/pnas.0404930101

Shao, C., Furusawa, Y., Aoki, M., Matsumoto, H., & Ando, K. (2002). Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells. *International Journal of Radiation Biology*, *78*(9), 837–844. doi:10.1080/09553000210149786

Shao, C., Lyng, F. M., Folkard, M., & Prise, K. M. (2006). Calcium fluxes modulate the radiation-induced bystander responses in targeted glioma and fibroblast cells. *Radiation Research*, *166*(3), 479–487. doi:10.1667/RR3600.1

Shao, C., Stewart, V., Folkard, M., Michael, B. D., & Prise, K. M. (2003). Nitric Oxide-Mediated Signaling in the Bystander Response of Individually Targeted Glioma Cells. *Cancer Research*, *63*(23), 8437–8442.

Short, S. C., Bourne, S., Martindale, C., Woodcock, M., & Jackson, S. P. (2005). DNA damage responses at low radiation doses. *Radiation Research*, *164*(3), 292–302. doi:10.1667/RR3421.1

Short, S. C., Woodcock, M., Marples, B., & Joiner, M. C. (2003). Effects of cell cycle phase on low-dose hyper-radiosensitivity. *International Journal of Radiation Biology*, *79*(2), 99–105. doi:10.1080/0955300021000045646

Silva, M. T. (2010). Secondary necrosis: The natural outcome of the complete apoptotic program. *FEBS Letters*, *584*(22), 4491–4499. doi:10.1016/j.febslet.2010.10.046

Singh, H., Saroya, R., Smith, R., Mantha, R., Guindon, L., Mitchel, R. E. J., … Mothersill, C. (2011). Radiation induced bystander effects in mice given low doses of radiation in vivo. *Dose-Response : A Publication of International Hormesis Society*, *9*(2), 225–42. doi:10.2203/dose-response.09-062.Singh

Skov, K. A. (1994). Molecular, cellular, and genetic basis of radiosensitivity at low doses: a case of inducible repair? *Radiation Research*, *138*(1 Suppl), S1–4.

Skov, K., Marples, B., Matthews, J. B., Joiner, M. C., & Zhou, H. (1994). A preliminary investigation into the extent of increased radioresistance or hyper-radiosensitivity in cells of hamster cell lines known to be deficient in DNA repair. *Radiation Research*, *138*(1 Suppl), S126–S129.

Slatkin, D. N., Spanne, P., Dilmanian, F. a, Gebbers, J. O., & Laissue, J. A. (1995). Subacute neuropathological effects of microplanar beams of x-rays from a synchrotron wiggler. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(19), 8783–8787. doi:10.1073/pnas.92.19.8783

Slatkin, D. N., Spanne, P., Dilmanian, F. A., & Sandborg, M. (1992). Microbeam radiation therapy. *Medical Physics*, *19*(6), 1395–1400. doi:10.1118/1.596771

Smilowitz, H. M., Blattmann, H., Bräuer-Krisch, E., Bravin, a., Di Michiel, M., Gebbers, J. O., … Laissue, J. a. (2006). Synergy of gene-mediated immunoprophylaxis and microbeam radiation therapy for advanced intracerebral rat 9L gliosarcomas. *Journal of Neuro-Oncology*, *78*(2), 135–143. doi:10.1007/s11060-005-9094-9

Smith, R. W., Wang, J., Bucking, C. P., Mothersill, C. E., & Seymour, C. B. (2007). Evidence for a protective response by the gill proteome of rainbow trout exposed to X-ray induced bystander signals. *Proteomics*, *7*(22), 4171–80. doi:10.1002/pmic.200700573

Smith, R. W., Wang, J., Schültke, E., Seymour, C. B., Bräuer-Krisch, E., Laissue, J. A., … Mothersill, C. E. (2013). Proteomic changes in the rat brain induced by homogenous irradiation and by the bystander effect resulting from high energy synchrotron X-ray

microbeams. *International Journal of Radiation Biology*, *89*(2), 118–27. doi:10.3109/09553002.2013.732252

Sokolov, M. V, Smilenov, L. B., Hall, E. J., Panyutin, I. G., Bonner, W. M., & Sedelnikova, O. a. (2005). Ionizing radiation induces DNA double-strand breaks in bystander primary human fibroblasts. *Oncogene*, *24*(49), 7257–65. doi:10.1038/sj.onc.1208886

Souto, J. (1962). Tumour development in the rat induced by blood of irradiated animals. *Nature*, *195*, 1317–1318. doi:10.1038/1951317a0

Strasser, A., O'Connor, L., & Dixit, V. M. (2000). Apoptosis signaling. *Annual Review of Biochemistry*, *69*, 217–245. doi:10.1146/annurev.biochem.69.1.217

Surinov, B. P., Isaeva, V. G., & Dukhova, N. N. (2004). Postirradiation volatile secretions of mice: syngeneic and allogeneic immune and behavioral effects. *Bulletin of Experimental Biology and Medicine*, *138*(4), 384–6. doi:10.1007/s10517-004-0018-z

Surinov, B. P., Isaeva, V. G., & Dukhova, N. N. (2005). Postradiation immunosuppressive and attractive volatile secretions: The "baystander effect" or allelopathy in groups of animals. *Doklady Biological Sciences*, *400*(1-6), 28–30. doi:10.1007/s10630-005-0034-9

Surinov, B. P., Isaeva, V. G., & Karpova, N. A. (1997). Post-radiation communicative induction of blood and immunity disorders. *Patologicheskaia Fiziologiia I Eksperimental'naia Terapiia*, (3), 7–10.

Surinov, B. P., Isaeva, V. G., & Tokarev, O. I. (2001). [Allelopathic activity of volatile secretions in irradiated animals]. *Radiatsionnaia Biologiia, Radioecologiia / Rossiĭskaia Akademiia Nauk*, *41*(6), 645–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11785304

Surinov, B. P., Karpova, N. A., Isaeva, V. G., & Kulish, I. S. (1998). [Communicative behavioral effects and disorders of immunity]. *Zhurnal Vyssheĭ Nervnoĭ Deiatelnosti Imeni I P Pavlova*, *48*(6), 1073–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9929917

Surinov, B. P., Karpova, N. A., Isaeva, V. G., & Kulish, I. S. (2000). Post-stress state and communicative alterations of immunity and blood. *Patologicheskaia Fiziologiia I Eksperimental'naia Terapiia*, (4), 9–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11247137

Tartier, L., Gilchrist, S., Burdak-Rothkamm, S., Folkard, M., & Prise, K. M. (2007). Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells. *Cancer Research*, *67*(12), 5872–9. doi:10.1158/0008-5472.CAN-07-0188

The Canadian Nuclear FAQ - Section D: Safety and Liability. (n.d.). Retrieved July 31, 2015, from http://www.nuclearfaq.ca/cnf_sectionD.htm#x5

Tomita, M., & Maeda, M. (2014). Mechanisms and biological importance of photon-induced bystander responses: do they have an impact on low-dose radiation responses. *J Radiat Res*, 1–15. doi:10.1093/jrr/rru099

Tomita, M., Maeda, M., Maezawa, H., Usami, N., & Kobayashi, K. (2010). Bystander cell killing in normal human fibroblasts is induced by synchrotron X-ray microbeams. *Radiation Research*, *173*(3), 380–5. doi:10.1667/RR1995.1

Trapp, J. V, Warrington, a P., Partridge, M., Philps, a, Leach, M. O., & Webb, S. (2004). 3D measurement of absolute radiation dose in grid therapy. *Journal of Physics: Conference Series*, *3*, 280–283. doi:10.1088/1742-6596/3/1/047

Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., & Kroemer, G. (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. *Nature Reviews. Molecular Cell Biology*, *11*(10), 700–714. doi:10.1038/nrm2970

Weidhaas, J. B., Eisenmann, D. M., Holub, J. M., & Nallur, S. V. (2006). A Caenorhabditis elegans tissue model of radiation-induced reproductive cell death. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(26), 9946–9951. doi:10.1073/pnas.0603791103

Weissenborn, U., & Streffer, C. (1989). Analysis of structural and numerical chromosomal aberrations at the first and second mitosis after X irradiation of two-cell mouse embryos. *Radiation Research*, *117*(2), 214–220.

Willingham, M. C. (1999). Cytochemical methods for the detection of apoptosis. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society*, *47*(9), 1101–1110. doi:10.1177/002215549904700901

Wykes, S. M., Piasentin, E., Joiner, M. C., Wilson, G. D., & Marples, B. (2006). Low-dose hyper-radiosensitivity is not caused by a failure to recognize DNA double-strand breaks. *Radiation Research*, *165*(5), 516–524. doi:10.1667/RR3553.1

Xu, S., Ding, N., Pei, H., Hu, W., Wei, W., Zhang, X., … Wang, J. (2015). MiR-21 is involved in radiation-induced bystander effects. *RNA Biology*, *11*(March 2015), 1161–1170. doi:10.4161/rna.34380

Yang, G., Mei, T., Yuan, H., Zhang, W., Chen, L., Xue, J., … Wang, Y. (2008). Bystander/abscopal effects induced in intact Arabidopsis seeds by low-energy heavy-ion radiation. *Radiation Research*, *170*(3), 372–80. doi:10.1667/RR1324.1

Yang, Y., Crosbie, J. C., Paiva, P., Ibahim, M., Stevenson, A., & Rogers, P. a. W. (2014). In Vitro Study of Genes and Molecular Pathways Differentially Regulated by Synchrotron Microbeam Radiotherapy. *Radiation Research*, *182*(6), 626–639. doi:10.1667/RR13778.1

Yuan, S. S. F., Chang, H. L., & Lee, E. Y. H. P. (2003). Ionizing radiation-induced Rad51 nuclear focus formation is cell cycle-regulated and defective in both ATM-/- and c-Abl-/- cells. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *525*(1-2), 85–92. doi:10.1016/S0027-5107(03)00009-5

Zeng, J., Harris, T. J., Lim, M., Drake, C. G., & Tran, P. T. (2013). Immune modulation and stereotactic radiation: improving local and abscopal responses. *BioMed Research International*, *2013*, 658126. doi:10.1155/2013/658126

Zhong, N., Morris, G. M., Bacarian, T., Rosen, E. M., & Avraham Dilmanian, F. (2003). Response of Rat Skin to High-Dose Unidirectional X-Ray Microbeams: A Histological Study. *Radiation Research*, *160*(2), 133–142. doi:10.1667/3033

Zhou, H., Ivanov, V. N., Gillespie, J., Geard, C. R., Amundson, S. a, Brenner, D. J., … Hei, T. K. (2005). Mechanism of radiation-induced bystander effect: role of the cyclooxygenase-2 signaling pathway. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(41), 14641–6. doi:10.1073/pnas.0505473102

Zhou, H., Ivanov, V. N., Lien, Y.-C., Davidson, M., & Hei, T. K. (2008). Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects. *Cancer Research*, *68*(7), 2233–40. doi:10.1158/0008-5472.CAN-07-5278

Chapter 2

2 INTER-RELATIONSHIP BETWEEN LOW-DOSE HYPER-RADIOSENSITIVITY AND RADIATION-INDUCED BYSTANDER EFFECTS IN THE HUMAN T98G GLIOMA AND THE EPITHELIAL HACAT CELL LINE

Cristian Fernandez-Palomo, Colin Seymour, Carmel Mothersill

This research project was design by the first author, Mothersill and Seymour. The first author performed all experiments and wrote the manuscript. Mothersill and Seymour edited the manuscript before submission to the journal.

The manuscript was submitted to the Journal of Radiation Research on July 22, 2015

2.1 ABSTRACT

The low-dose hyper-radiosensitivity and increased radio-resistance have been investigated for several years in the fields of radiation oncology and biology. The importance lies in both occurring mostly in tumour cell lines. Great progress has been achieved elucidating their signalling pathways; however uncertainties exist when they are studied together with radiation-induced bystander effects. Therefore, the aim of this work was to further investigate this relationship.

We worked with T98G glioma known for showing a strong transition from hyper-radiosensitivity to induced radioresistance and with the HaCaT cells, which do not show low-dose hypersensitivity. Both cell lines were paired using a mix and match protocol, which involved growing un-irradiated cells in culture medium from irradiated cells, and covering all possible combinations between them. The endpoints we analyzed were clonogenic cell survival and live calcium measurements through the cellular membrane.

Our data demonstrated that T98Gs produced bystander signals that decreased the survival of both reporter T98G and HaCaT cells. The bystander effect occurred only when T98Gs were exposed to doses below 1 Gy, and that was corroborated by the induction of calcium fluxes. However, when bystander signals originated from HaCaT cells, the survival fraction increased in reporter T98Gs while it decreased in HaCaTs. Moreover, the corresponding calcium data

showed no calcium fluxes in T98Gs while HaCaTs displayed a biphasic calcium profile.

In conclusion, our research revealed a strong link between low-dose hyper-radiosensitivity and bystander effects. This relationship varies depending on which cell line functions as source of bystander signals. This suggests that the bystander mechanisms are more complex than previously established and caution should be taken when extrapolating bystander results across all cell lines and all doses of radiation.

1.2 INTRODUCTION

The inter-relationship between low-dose hyper-radiosensitivity (HRS), increased radioresistance (IRR), and bystander effects has been studied for several years. Extensive research involving more than 10 different cell lines revealed that populations displaying HRS did not generate bystander effects after exposure to doses of radiation above 0.5Gy. In fact, it was concluded that HRS and bystander effects were mutually exclusive (Carmel Mothersill et al., 2002; Ryan et al., 2009). However new research in the area (Heuskin et al., 2013; Nuta & Darroudi, 2008) has expanded those conclusions. This means we need a re-examination of the interaction between HRS/IRR and bystander effects.

Published literature describes HRS and IRR as two linked mechanisms where the former describes the death of cells due to a greater than expected sensitivity to doses of radiation below 0.5 Gy, while the latter describes how those cells show a more resistant response as the dose increases to about 1 Gy (Joiner et al., 2001; Marples et al., 2004; K. A. Skov, 1994). These responses were first identified in the late eighties (Joiner & Denekamp, 1986) and continued being studied on several normal cell lines (Garcia, Leblanc, Wilkins, & Raaphorst, 2006; Lambin, Fertil, Malaise, & Joiner, 1994; Marples & Joiner, 1993, 1995), malignant cell lines (Garcia et al., 2006; Lambin et al., 1993; S. Short, Mayes, Woodcock, Johns, & Joiner, 1999; Wouters & Skarsgard, 1994), and human

tissue explants (Joiner & Denekamp, 1986; C. Mothersill et al., 1995; Simonsson et al., 2008). There is also clinical evidence of hyper-radiosensitivity of skin after low-dose fractions during radiotherapy (Turesson & Joiner, 1996). Similar biphasic survival responses albeit at much higher doses have also been reported previously in protozoa (Calkins, 1967) and in insect cell lines (Koval, 1984). In order to improve cancer treatments (Schoenherr et al., 2013) and to enable us to exploit HRS/IRR mechanisms to sensitize tumours (Diss et al., 2014), current studies are focusing on elucidating the mechanisms that govern the expression of HRS. Up to now, research shows that the most likely causes seem to be defective DNA repair systems and impaired cell cycle regulation (L. M. Martin, Marples, Lynch, Hollywood, & Marignol, 2014). While the work we present here is looking at bystander signalling as a means of producing HRS.

Authors agree that the increase in cell-kill observed in HRS may have derived from protective mechanism that evolved to prevent cells from developing genomic instability and mutations (L. M. Martin et al., 2014). Detailed mechanistic work has revealed that HRS is not the result of failure to recognise DNA double strand breaks (DSBs) (Wykes et al., 2006). Instead, persistent RAD51 foci at late time points after HRS suggests that DSBs repair pathways are responsible (Yuan et al., 2003). Short et al. have suggested that HRS may occurs due to inefficient or delayed homologous recombination, which is mostly activated in the G2 phase (S C Short et al., 2005). Moreover, mismatch repair deficiency has recently been

correlated with HRS too (Krueger et al., 2007; L. Martin et al., 2009). On the other hand, the involvement of Non-Homologous End Joining (NHEJ) has been linked with IRR (K. Skov et al., 1994), which is the primary DSBs repair mechanism in mammalian cells during G1 and early S phase (Kass & Jasin, 2010). As mentioned earlier, cell cycle regulation is also believed to be involved during HRS. Research in the subject has established that HRS is most intense during G2 (S. C. Short et al., 2003), and several studies also show evasion of the G2/M checkpoint (Collis et al., 2004; Fernet, Mégnin-Chanet, Hall, & Favaudon, 2010; Krueger, Wilson, Piasentin, Joiner, & Marples, 2010; Marples, Wouters, & Joiner, 2003; Marples, 2004; Xue et al., 2009). Conversely, G1 seems less sensitive (S. Short et al., 1999) and S phase and Mitosis are not sensitive at all (Marples et al., 2003).

As previously mentioned, molecular mechanisms involving HRS have also been linked with bystander effects in a great number of cell lines. Bystander effects are radiation-like responses observed in cells that have never been exposed to radiation but that are in direct contact with or have received signals from irradiated cells (Carmel Mothersill & Seymour, 1997; Nagasawa & Little, 1992). Research trying to identify the bystander signals have revealed the involvement of intracellular pathways such as the NF-κB transcription factor, COX2, NOS2 (Iyer & Lehnert, 2000; Kevin M Prise & O'Sullivan, 2009), mitogen-activated protein kinases (MAPKs), mitochondrial disruptions (F.M. Lyng et al.,

2001; J. E. J. Murphy et al., 2005; Zhou et al., 2008), Ca^{++} fluxes (Fiona M Lyng et al., 2002), expression of apoptotic and cell cycle regulatory molecules like p53, $p21^{Waf1}$, p34, and MDM2 (E. Azzam et al., 2004; F. M. Lyng et al., 2006; Carmel Mothersill & Seymour, 2004). Extracellular mediators have also been identified, these involve reactive oxygen and nitrogen species (F M Lyng et al., 2000; Narayanan et al., 1997; Chunlin Shao, Stewart, et al., 2003), transforming growth factor-β1 (Iyer & Lehnert, 2000), interleukin-8 (Narayanan et al., 1999), tumour necrosis factor-α (Iyer & Lehnert, 2000) and serotonin (Fazzari et al., 2012; Saroya et al., 2009). Moreover, the latest research show two new candidates - UV-photon emission (Ahmad et al., 2013; Le, McNeill, Seymour, Rainbow, & Mothersill, 2014) and exosomes (Al-Mayah et al., 2012; K Kumar Jella et al., 2014) both from irradiated cells.

Bystander effects and HRS/IRR are currently well-accepted phenomena. However their inter-relationship has been mostly studied in the dose ranges between 0.5 to 5 Gy. Work developed by Mothersill et. al. was the first study examining their interaction. They showed that out of 13 cell lines exposed, the ones displaying the largest HRS were unable to induce bystander effects (Carmel Mothersill et al., 2002). Later studies developed by Ryan et. al. examined the relationship between HRS/IRR, Bystander effects, and adaptive responses after 0.5, 2 and 5 Gy (Ryan et al., 2009). The authors confirmed that HRS/IRR and bystander effects were mutually exclusive but also showed that adaptive

responses were only seen in cell lines with large HRS. Interestingly, recent studies conducted by Nuta & Darroudi (Nuta & Darroudi, 2008) have shown that HRS might relate to bystander effects after exposing fibroblast to photons at doses below 1 Gy. Moreover, work developed by Heuskin et al. (Heuskin et al., 2013) revealed that HRS and bystander effects were able to coexist after A549 lung carcinoma cells were irradiated with low and high LET charged particles. In fact, their research revealed that bystander effects were the most likely cause of cell-kill during HRS. Moreover, the dose at which HRS transitions to IRR decreased with increasing LET. The conclusion of these researchers makes us wonder to which extent HRS and bystander effects are related, and whether HRS-positive cells are able to induce bystander effect in HRS-negative cell lines.

The goal of the research we present in this manuscript was to look further at the inter-relationship between HRS/IRR and bystander effects. We examined a range of gamma ray doses to examine the effects of doses below and above 1 Gy. We performed a mix and match protocol using two cell lines: the human epithelial HaCaT cell line, which is HRS-negative but exhibits strong bystander effects; and the human T98G glioma, which is HRS-positive and it has been previously described as bystander effect negative. Finally we looked at whether the bystander signalling process was initiated by measuring calcium fluxes through the cellular membrane.

1.3 MATERIALS AND METHODS

2.1.1 Cell lines

The T98G is an immortal and anchorage independent human glioma cell line that arrests in G1 under stationary phase conditions (Stein, 1979). The T98G cells were obtained as a gift from Prof. Brian Marples, (Department of Radiation Oncology, William Beaumont Hospital, USA). The cell line was routinely maintained with Dulbecco's Modified Eagle Medium F12 (DMEM/F12), +L-Glut, +HEPES (Gibco, Oakville, Canada), supplemented with 10% Foetal Bovine Serum (FBS) (Gibco, Oakville Canada).

The HaCaT cell line is a spontaneously immortalized human epithelial cell line that was derived from normal human skin from a patient with melanoma (Boukamp et al., 1999). HaCaTs have been extensively used by our laboratory and others because they exhibit strong radiation induced bystander effects in response to addition of autologous irradiated cell conditioned medium (ICCM) (Carmel Mothersill & Seymour, 1997). HaCaTs were originally derived by Dr. Petra Boukamp in DKFZ, Heidelberg, Germany (Lehman et al., 1993) and given as a kind gift to the Dublin Institute of Technology in Ireland (Dr. Mothersill's Laboratory) in 1996. The cell line was adapted to grown in DMEM/F12, +L-Glut, +HEPES (Gibco, Oakville, Canada); supplemented with 10% FBS (Gibco,

Oakville, Canada), 1µg/ml of Hydrocortisone (Sigma-Aldrich, Oakville, Canada), and 5ml of L-Glutamine (Gibco, Oakville, Canada).

Both cell lines tested negative for mycoplasma before the experiments.

1.3.1 CELL CULTURE TECHNIQUE

Several 75 cm^2 flasks were seeded with T98G and HaCaT cells. Only asynchronous non-confluent flasks were chosen for the experiments. Selected flasks were gently rinsed with 10 ml of calcium and magnesium free DPBS, and detached using a 0.3% (v/v) Trypsin - 0.53 mM EDTA solution (Gibco, Burlington, Canada). Trypsin was neutralized using fresh culture medium, and the cell solution was centrifuged at 125x g-force during 5 minutes. The pellet was re-suspended in 1ml of fresh culture medium, gently pipetted to create a single cell suspension and counted using an automatic cell counter model Z2 (Beckman Coulter, Fullerton, CA). Flasks were seeded according to their purpose: 1) A set of 25 cm^2 flasks was seeded with 500 T98G or HaCaT cells to study their response to radiation using the clonogenic technique developed by Puck and Markus (Puck & Marcus, 1956); 2) A second set of 25 cm^2 flasks was seeded with 500 T98G or HaCaT cells to function as recipients of irradiated cell conditioned media (ICCM) and; 3) A third set of flasks was seeded with 200,000

T98G or HaCaT cells to serve as donors of ICCM to study the induction of bystander effects in the previously described recipient cells. All flasks were placed in a 37° Celsius, 5% CO_2 in air, 95% humidity incubator.

2.1.2 Irradiations

After consultation with Dr. Brian Marples, it was recommended to irradiate the T98G cells 60 to 90 minutes after seeding. HaCaTs however, have been largely reported to induce bystander effects when irradiation occurs 6 hours after seeding. In an attempt to match the irradiation times, HaCaT were irradiated following the T98G protocol. However, the bystander effects were abolished. In order to ensure that T98Gs would show HRS, and HaCaTs would produce bystander effects; we decided to follow the different established procedures for each cell line. T98G were then irradiated 60-90 minutes after seeding based on Dr. Marples recommendation and published protocols (S. Short et al., 1999), while HaCaT were irradiated 6 hours after seeding based on established protocols by Mothersill and Seymour (Carmel Mothersill & Seymour, 1998). Cells were exposed to doses of gamma rays from 0 to 3 Gy using a caesium-137 source. Flasks were placed at 27 cm from the radiation source and irradiated at a dose rate of 0.289 Gy/min. All flasks were placed back in the incubator immediately after irradiation. Donor flasks having 200,000 cells were incubated

for 90 minutes (or 6 hours) prior to medium harvest while reporter flasks containing 500 cells remained undisturbed for 9 days after receiving donor medium to allow for colony formation.

2.1.3 Medium transfer technique and bystander protocol

Since reporter T98Gs and HaCaTs are not irradiated but receive the harvested ICCM from donors, we decided to keep the time between seeding and medium transfer constant across both cell lines based on bystander experiments developed by Mothersill and Seymour (Carmel Mothersill & Seymour, 1997). To accomplish this, reporter flasks were seeded with T98Gs or HaCaTs at clonogenic densities and allowed to attach in an incubator prior medium transfer. Six hours later, the medium from a reporter T98G or HaCaT flask was discarded and replaced by ICCM from a donor flask, which had been previously harvested and filtered using a 0.22 μm filter with HT Turffryn Membrane (Pall Life Sciences). Then, the empty donor flasks were discarded and the reporter flasks were placed back into an incubator and left undisturbed for 9 days to allow for colony formation. The colonies were then stained using a 1:4 (v/v) dilution of Carbol Fuchsin (Ricca Chemical Company) in water, and colonies with more than 50 cells were scored as survivors.

2.1.4 Ratiometric calcium measurements

Our protocol is an adaptation of the one developed by Dr. Fiona Lyng, DIT, Dublin, Ireland and was employed in consultation with her. For this assay 100,000 T98G or HaCaT cells were seeded in Glass Bottom Dishes (MatTek Corporation) containing 2 ml of cell culture media DMEM/F12 (Gibco, Oakville, Canada) supplemented with 10% FBS, and placed in a incubator at 37° Celsius, 5% CO_2 in air for 24 hours. For calcium measurements, the culture media were discarded and the cells were gently washed three times with Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (Cat#: 14025-092, Gibco, Oakville, Canada), supplemented with 25 mM of HEPES (Gibco, Oakville, Canada). The HBSS was discarded and cells were loaded with 200 ul of 8.4 uM of Fura-2/AM (Sigma-Aldrich, Milwaukee, USA), for 1 hour at room temperature in the dark. Loading the Fura-2/AM into the cells at room temperature avoided the compartmentalization of the dye within the cellular organelles, which was a problem we encountered when cells were placed in an incubator at 37° Celsius. Performing the loading at room temperature allowed a better distribution of the fluorescent dye across the cytoplasm. At the end of the loading time, the Fura-2/AM was discarded, the cells were washed three times with HBSS, and 300 ul of the same buffer was added to the dish. Cells were observed with a x40 oil

objective on an Olympus inverted fluorescent microscope (Olympus Canada, Richmond Hill, Canada) and images were captured with a CCD Cool-Snap HQ camera (Photometrics, Tucson, Arizona). Fura 2/AM emits light at 510 nm when is excited at 380 nm and 340 nm. The ratio of emissions between those wavelengths is correlated with the flux of calcium through the cellular membrane. The ratio of emission is presented as a graph where fluorescence is plotted against time. Upon obtaining all fluorescence values, the data from each cell was analyzed and the area under the curve was calculated for each graph/cell. To accomplish this, the distance between the maximum fluorescence point and the baseline was measured for each cell. The values were then averaged according to the conditioned medium tested. Finally the averaged “maximum minus baseline” values were plotted in function of the dose.

2.1.5 Data Analysis

Colony cell survival is presented as mean survival fraction with standard error of the mean (SEM). Experiments were done in triplicate and repeated as 3 independent studies unless otherwise indicated by the n value.

Survival fractions were fitted with the linear-quadratic (LQ) and the induced-repair (IR) equations using the nonlinear least square fit regression. The

calcium data was fitted only to the IR-Model. The algorithm used for the iterations was the Marquardt and Levenberg method (GraphPad Prism 6 software). The LQ model describes the cell survival (S) as function of the dose (D), by:

Equation 2.1 LQ Model

$$\mathrm{S} = \exp(-\alpha \mathrm{D} - \beta \mathrm{D}^2)$$

Where, **α** is the slope of the linear component of the curve, and **β** is the slope of the quadratic component. The IR equation we used was taken from the model described by Joiner (Lambin et al., 1993):

Equation 2.2 IR model

$$S = exp\left(-\alpha r\left(1 + \left(\frac{\alpha s}{\alpha r} - 1\right)e^{-D/D_c}\right)D - \beta D^2\right)$$

Where, **αr** is **α** extrapolated from the conventional LQ dose-response model, thus it represents the slope of the resistant part of the linear component. While **αs** is a new **α** derived from the response at very low doses and it represents the slope of the sensitive part of the linear component.

2.2 RESULTS

2.1.6 Low-dose hyper-radiosensitivity

Figure 1 shows the clonogenic survival of T98G and HaCaT cell lines after direct exposure to gamma rays from a caisium-137 source. The LQ model was fitted to both data sets and shows the different survival responses to the same radiation doses. The IR model was also fitted to both data in order to better describe the effect at doses below 1 Gy, which are underestimated by the LQ model. T98G cells (Fig 2.1A) showed a sharp decrease in survival after exposure to doses below 1Gy. This is a characteristic of hyper-radiosensitive cells, which is better represented by the IR-Model (r^2=0.63). On the contrary, HaCaT cells (Fig 2.1B) showed a clear shoulder region at doses below 1 Gy, which is better represented by the LQ-Model (r^2=0.72).

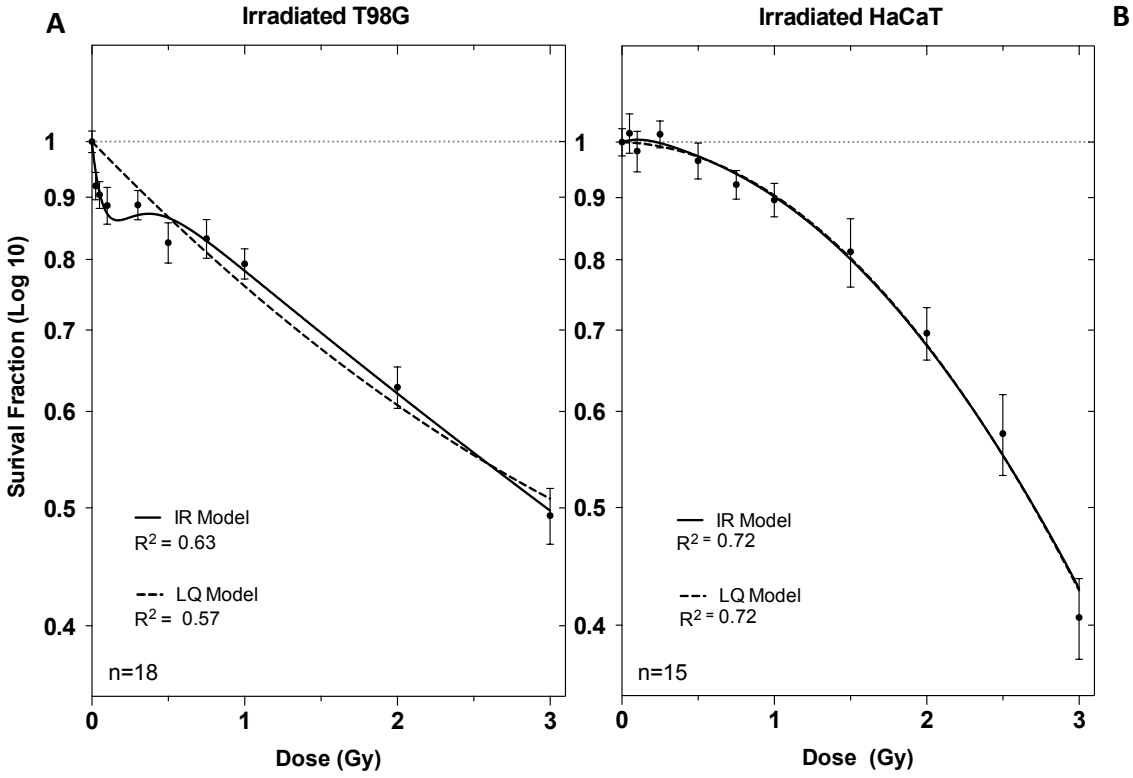

Figure 2.1 Clonogenic Survival of T98G (A) and HaCaT (B) cell lines after irradiation with a caesium-137 gamma source. (LQ) Refers to Linear Quadratic Model. (IR) stands for Induced-repair Model. n = number of flaks (Error bars= SEM)

The values of the best-fit parameters are shown in Table 2.1. The response to radiation (radioresistance) is directly correlated with the α/β values, which for T98Gs and HaCaTs varies widely. For instance, T98G showed an α/β=-12.36, while HaCaTs displayed an α/β=0.11. Dc is another key parameter because it indicates the dose at which cells switch from being sensitive to resistant. In other words, Dc indicates the dose at which cell-kill is higher due to

HRS. For these experiments T98Gs showed the highest HRS cell-kill at ~0.12 Gy, while the value for HaCaT cells becomes irrelevant because they did not show HRS. Nevertheless, it was decided to include these numbers for completeness.

Table 2.1 Best-fit values for the LQ and IR Models on T98G and HaCaT cells irradiated with a Caesium-137 source. 95% confidence limits are shown in parentheses

Parameters	T98G	HaCaT
LQ Model		
α/β	-12.36	0.11
α	0.297 (0.23 to 0.36)	0.01 (-0.053 to 0.072)
β	-0.02 (-0.052 to 0.0034)	0.091 (0.062 to 0.12)
IR Model		
αs/αr	11.15	-6.28
αr	0.25 (0.18 to 0.32)	0.01 (-0.062 to 0.090)
αs	2.78 (1.38 to 4.18)	-0.09 (-1.15 to 0.97)
dc	0.12 (0.062 to 0.18)	0.11 (-0.65 to 0.87)
β	0.02 (-0.088 to 0.13)	-6.36 (-43.20 to 30.49)

2.1.7 Low-dose hypersensitivity and bystander effects.

Figure 2 shows the clonogenic survival of reporter (bystander) cells grown in ICCM. By experimentally fitting the data to both LQ and IR Models, we were

able to show the survival patterns. The IR-Model gave a better fit, thus it was selected as our standard modeling tool for bystander cell survival. The best-fit parameters of the IR-Model for bystander T98G and HaCaT cells are shown in Table 2.2.

Table 2.2 Best fit parameters for the Induced-Repair (IR) Model on bystander cells. 95% confidence limits are shown in parentheses.

Reporter Cell Line	IR Parameters	ICCM from T98G	ICCM from HaCaT
T98G	αs/αr	1060.23	-938.54
	αr	0.00 (-0.002 to 0.003)	0.00 (-0.018 to 0.021)
	αs	0.16 (-2.12 to 2.45)	-1.53 (-4.31 to 1.25)
	dc	0.16 (0.063 to 0.25)	0.16 (-0.15 to 0.46)
	β	-56.77 (-959.4 to 845.8)	-2.40 (-34.74 to 29.93)
HaCaT	αs/αr	358.97	26.26
	αr	0.01 (-0.021 to 0.032)	0.12 (0.013 to 0.22)
	αs	1.92 (0.16 to 3.68)	3.03 (0.52 to 5.55)
	dc	0.16 (0.031 to 0.30)	0.13 (0.034 to 0.23)
	β	-2.39 (-16.21 to 11.43)	0.27 (0.15 to 0.40)

Reporter T98G cells displayed opposite bystander responses depending on whether the ICCM originated from donor T98G or donor HaCaT cells. ICCM from donors T98G decreased the survival of reporter T98Gs after doses below 1 Gy (Fig. 2.2A); achieving the highest cell-kill at ~0.16 Gy (Table 2.2). However,

ICCM from donor HaCaTs increased the survival of reporter T98Gs at doses below 1 Gy (Fig 2.2B); producing the highest survival at ~0.16 Gy.

Reporter HaCaTs however, always showed decrease in survival when exposed to ICCM. But the extent of the cell-kill was also dependent on the donor cell line. ICCM from donor T98Gs decreased the survival of reporter HaCaTs at HRS/IRR doses only (Fig 2.2C); showing the highest cell-kill at ~0.16 Gy. While ICCM from donor HaCaTs decreased the survival of reporter HaCaTs across all doses (Fig 2.2D).

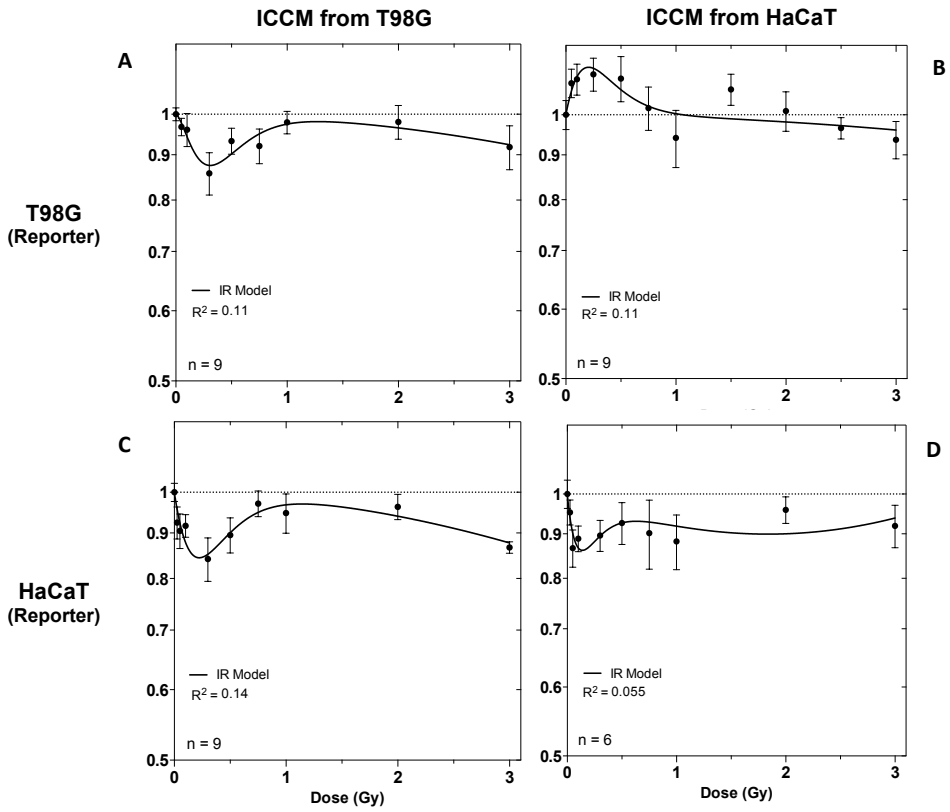

Figure 2.2 Mix-match survival fraction of reporter (bystander) cells exposed to ICCM from T98G or HaCaT. Clonogenic survival of reporter T98G cells grown in ICCM from T98G (A) or HaCaT (B). Clonogenic survival of reporter HaCaT cells grown in ICCM from T98G (C) or HaCaT (D). The solid line represents the best fit for the IR Model for each plot. (IR) stands for Induced-repair Model. n = number of flaks (Error bars=SEM)

2.1.8 Low-dose hypersensitivity and calcium fluxes in bystander cells.

Figure 2.3 shows the induction of calcium flux through the cellular membrane in bystander T98G and HaCaTs. The data were plotted as Total Peak Area for each dose and fitted to the IR-Model.

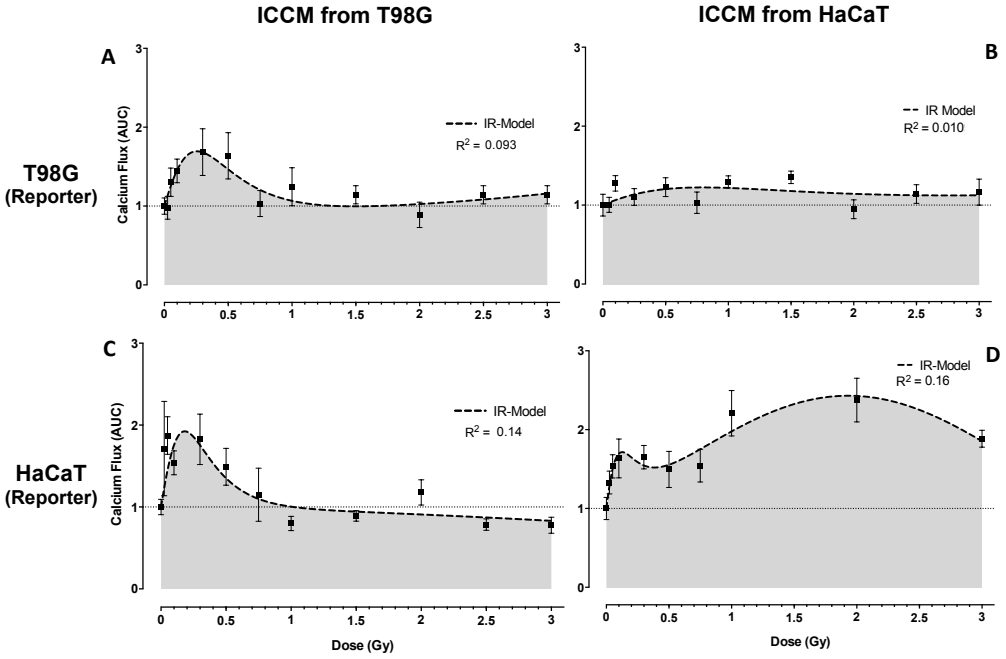

Figure 2.3 Calcium Flux on reporter (bystander) cells induced by ICCM from T98G or HaCaT. Fura 2/AM was used to perform ratiometric calcium measurements on T98G and HaCaT cells using ICCM as a triggering agent. The data show the influx of calcium ions through the cellular membrane for both bystander T98G (A & B) and bystander HaCat cells (C & D). Data plotted corresponds to the value of the Peak Y axis minus the baseline "1" for each dose. Data was fitted to the Induced Repair (IR) Model.

The calcium flux profile of reporter T98G cells varied depending on the origin of the ICCM. When bystander T98G cells were exposed to ICCM from donor T98G (Fig 2.3A), there was an intense increase in calcium flux in the HRS region, which reached maximum intensity at ~0.34 Gy (Table 2.3). This rapidly decreased to background levels as the doses approached 1 Gy**.** However, when ICCM originated from the HaCaT cell line, the calcium fluxes in T98G cells were very low (Fig 3B). On the other hand, when ICCM from irradiated T98Gs was applied to reporter HaCaTs (Fig 3C), there was fast increase in calcium ions that reach a maximum concentration at ~0.17 Gy (Table 2.2)**,** and then rapidly returned to background levels as the dose neared 1 Gy. This response resembles the one observed in T98G reporter cells (Fig 2.3A). Finally, when HaCaTs were exposed to ICCM originated from HaCaTs (Fig 2.3D), there was rapid and constant increase in calcium fluxes that were sustained at all doses. This calcium profile however, seemed to have two peaks. The first occurred in the HRS region and the second appeared in the high dose region. While the first peak was estimated at ~0.1 Gy**,** the IR-Model cannot provide a value for the second peak. After visual examination of the data we estimated it at ~2 Gy.

Table 2.3 Best-fit parameters for the calcium data according to the IR Model. 95% confidence limits are shown in parentheses.

Reporter Cell Line	IR Parameters	ICCM from T98G	ICCM from HaCaT
T98G	αs/αr	-130.87	51.81
	αr	0.04 (-0.24 to 0.32)	-0.01 (-0.22 to 0.19)
	αs	-5.47 (-8.37 to -2.57)	-0.70 (-2.19 to 0.80)
	dc	0.34 (-0.21 to 0.89)	0.60 (-1.83 to 1.00)
	β	0.73 (-1.92 to 3.38)	-0.39 (-13.14 to 12.35)
HaCaT	αs/αr	-472.48	13.86
	αr	0.02 (-0.32 to 0.36)	-0.92 (-1.12 to -0.72)
	αs	-9.67 (-14.39 to -4.95)	-12.74 (-21.18 to -4.31)
	dc	0.17 (-0.22 to 0.55)	0.10 (0.032 to 0.18)
	β	-0.67 (-18.12 to 16.77)	0.26 (0.22 to 0.30)

1.4 DISCUSSION

Hyper-radiosensitivity and increased radio-resistance are two phenomena that occur at low doses of radiation in a number of cancer cell lines. Understanding the mechanisms behind these two events may provide an opportunity to emulate and increase the cell-killing effect to improve radiotherapy treatments. While some laboratories have focused on elucidating the signalling pathways, others have focused in understanding the inter-relationship between HRS/IRR, adaptive responses and bystander effects. For instance, Ryan et al. (2009) established that cell lines that exhibited adaptive responses were also HRS/IRR-positive but lacked the ability to produce bystander effects. Although their work provided great insight about the mechanism of action of these three

phenomena, bystander effects were not studied at doses below 0.5 Gy, which is were HRS dominates the dose response. We postulated that bystander effects might occur in the HRS region of the survival curve only and might be lost as the dose increased to levels where IRR mechanisms predominated. To examine this hypothesis we sought a direct link between bystander effects and cell lines showing HRS at low doses. We performed a mix and match bystander protocol focusing on the doses that produce HRS responses in the T98G cell line.

The data show that T98G cells exposed to HRS doses produced and responded to bystander signals. The results demonstrate that bystander effects can occur at doses below 1 Gy in a cell line with large HRS/IRR transition (T98G). While strong bystander effects are displayed across all doses in a cell line that is not hyper-radiosensitive (HaCaT). The data therefore fully support our hypothesis and clarify previously published work by Ryan et al. (Ryan et al., 2009) and earlier work by Mothersill et al (Carmel Mothersill et al., 2002) were it was suggested that bystander effects and HRS/IRR were mutually exclusive.

In the current experiments we studied 6 doses below 1 Gy (0.025, 0.05, 0.1, 0.25, 0.5 and 0.75 Gy) and 4 above it (1.5, 2, 2.5 and 3 Gy). This allowed us to replicate the HRS/IRR response (Fig 2.1A) and to study whether any of those doses, if not all, would induce bystander effects. We showed that bystander effects in T98G cells reached maximum strength at approximately 0.16 Gy; then

they gradually weakened as IRR took place, until they finally disappeared at around 1 Gy (Fig 2.2A). Our results are in accordance with work developed by Heuskin et. al. (Heuskin et al., 2013) where they exposed the A549 lung carcinoma to protons and alpha particles and found that bystander effects and HRS can coexist in that particular cell line.

In order to confirm further our bystander clonogenic assay results, we decided to perform live calcium measurements in the reporter cells. Calcium fluxes through the cellular membrane have been extensively linked with bystander effects (F. M. Lyng et al., 2006; Fiona M Lyng et al., 2002; Carmel Mothersill et al., 2005b; Chunlin Shao et al., 2006). During our experiments we were able to create a profile of calcium changes for the reporter cells. The calcium flux profile of T98Gs showed an inverse relation with the bystander cell death, which is evident at the HRS/IRR dose levels (Fig 2.3A). These data are supported by previous work from our laboratory (Fazzari et al., 2012; Fernandez-Palomo et al., 2015; Saroya et al., 2009; Singh et al., 2011) and colleagues (F. M. Lyng et al., 2006; Fiona M Lyng et al., 2002; Carmel Mothersill et al., 2005b; Chunlin Shao et al., 2006), which demonstrate that transient calcium fluxes are related with bystander effects. Moreover, work developed by Shao et al., revealed that bystander responses in T98G cells were eliminated when cells were treated with the calcium channel blocker calcicludine (Chunlin Shao et al., 2006), which further supports our findings.

T98G cells were also able to induce bystander effects on reporter HaCaTs. This was shown using a mix and match protocol, which aims to isolate the signal component and the response component of bystander effects. The clonogenic survival reported that medium from T98G cells is able induce bystander effects on reporter HaCaTs. The HaCaT cell line has been extensively studied due to its ability to produce and respond in a stable way to bystander signals (Fernandez-Palomo et al., 2015; K Kumar Jella et al., 2014; C Mothersill, Smith, Hinton, Aizawa, & Seymour, 2009; Carmel Mothersill & Seymour, 1997, 1998; Ryan et al., 2009). Published work shows that HaCaT cells have responded to bystander signals originated from human (K Kumar Jella et al., 2014; Le et al., 2014; Carmel Mothersill & Seymour, 1998; Ryan et al., 2009), fish (C Mothersill et al., 2009) and rat (Fernandez-Palomo et al., 2015) cell lines. Thus, discovering that HaCaT cells also respond to signals from T98G cells confirm them as universal reporter if a signal has been produced.

Bystander effects in HaCaT reporter cells occurred only when the medium had originated from T98G cells exposed to HRS doses and not doses in the IRR region (Fig 2.2C). Reporter HaCaT cells exposed to medium from irradiated HaCaT cells showed a decrease in survival across all doses (Fig 2.2D). This “partial” bystander response by HaCaT cells suggests that irradiated T98Gs can only produce bystander signals at HRS doses. This hypothesis is supported by our calcium results where medium from T98G cells exposed to HRS doses were

capable of triggering transient calcium fluxes in HaCaT cells; but medium from T98G cells exposed to doses in the IRR region of the dose response curve did not trigger calcium fluxes in the HaCaT reporter cells (Fig 2.3C). Therefore, our results further confirm that T98Gs produce bystander signals only when exposed to HRS doses and that HaCaT cells are able respond to them.

Reporter T98G cells showed an increase in survival when they were grown in medium from donor HaCaT cells (Fig 2.2B), while calcium fluxes were absent (Fig 2.3B). Increases in cell proliferation due to bystander effects although rare, have been described in the literature. Work by our group reported recently that Synchrotron-irradiated Fisher rats harbouring F98 tumours induced increase in survival in the reporter HaCaT cells compared to tumour-free rats (Fernandez-Palomo et al., 2015). The increase in survival was attributed to the presence of the F98 tumour, which seemed to counteract the cell-kill effects of bystander signals. Also, work developed by Iyer and Lehnert (Iyer & Lehnert, 2000) showed increase in survival when human lung fibroblast were treated with supernatants from alpha irradiated cells. Moreover, work developed by Shao et al. (Chunlin Shao, Aoki, & Furusawa, 2003) shows that the plating efficiency of bystander Neoplastic Human Salivary Gland (NSG) cells was increased when co-cultured with irradiated NGSs. Our current calcium data suggest that HaCaTs have the ability to induced increase in survival on the reporter T98G by means of independent calcium fluxes. These results together with the published literature

further suggest that bystander effects involve complex mechanisms where the response of one cell type is not necessary the same as that of others.

Reporter HaCaTs receiving medium from donor HaCaTs showed clear bystander effects (Fig 2.2D), which were accompanied by a broad biphasic calcium flux profile (Fig 2.3D). The clonogenic survival results are in accordance with vast published literature by Mothersill, Lyng, Ryan and others, where reporter HaCaT cells showed a marked decrease in survival as a consequence of bystander effects (K Kumar Jella et al., 2014; Fiona M. Lyng et al., 2012; Carmel Mothersill et al., 2002; Ryan et al., 2009). The present study focussed on the bystander calcium profile across a wide range of doses from 0.05 to 3 Gy. The results show a biphasic calcium flux profile in the HaCaT cells. One related to the HRS dose levels and another related to higher doses. We identify the bystander calcium profile as a biphasic curve, where the first phase (from 0.05 Gy to 0.5 Gy) resembles the calcium response observed when T98Gs were donors (Fig 2.3A & 2.3C), while the second phase is much more intense. A possible explanation is that two different mechanisms govern the response of cells to radiation. One predominates in the low dose region and the other at high doses. It would be interesting to focus future studies on whether other HRS/IRR-negative cells can produce similar biphasic calcium responses.

Bystander cell killing due to ICCM from donor T98G cells exposed to doses in the HRS region decreased the clonogenic survival of the reporter cells to nearly 88% for T98G (Fig 2A) and to 85% for HaCaTs (Fig 2C). Those values were extremely close to the 87% of survival observed in the direct irradiated T98G (Fig 1A). This strongly suggests that the cell death produced in the HRS region may be entirely attributable to bystander effects. Similar results were first reported by Mothersill et al. (Carmel Mothersill et al., 2002) after observing that the cell killing in the human colon adenocarcinoma SW48 cell line was linked to the bystander effect at low doses of X-rays (0.01 to 1 Gy). Published literature regarding human keratinocytes also shows that very low doses of low-LET photons (0.01-0.05 Gy) induced clonogenic cell death that was linked to bystander effects only (Colin B. Seymour & Mothersill, 2000). Those results are supported further by data from Nuta and Darroui (Nuta & Darroudi, 2008), who suggested that HRS might be causally related to bystander signals. Therefore, we propose that bystander effects may play a significant role in cell killing at HRS doses.

In conclusion, we have shown that T98G cells exposed to doses of ionising radiation associated with HRS response produce bystander signals detectable in HaCaT or T98G reporter cells. The signals are not produced by

T98G cells exposed to higher doses where IRR response predominates. Mix and match protocols showed that the bystander responses vary depending on which cell line was donor or reporter. Taken together with previous work by this group and others, the data suggest that bystander mechanisms need to be studied carefully and that responses in particular cell lines or at specific doses cannot not be generalized. Our results do however suggest that the major cause of cell killing in the HRS region is due to bystander signalling mechanisms.

The results may be important in radiotherapy where increasing use of imaging techniques exposes both tumour cells and associated normal tissues to doses of radiation in the range where HRS occurs.

2.5 CONFLICT OF INTERESTS

The authors declare to have no conflict of interests

2.6 ACKNOWLEDGMENTS

We would like to acknowledge the assistance of Prof. Brian Marples during the design of the experimental protocol. We acknowledge support from the Natural Science and Engineering Research Council (NSERC), the Canada

Research Chairs Programme and the Chronic Fatigue and Immune Deficiency Syndrome (CFIDS) Foundation. We would also like to acknowledge the National Commission for Scientific and Technological Research of Chile, which is funding Cristian Fernandez-Palomo's doctoral studies.

2.7 REFERENCES

Ahmad, S. B., McNeill, F. E., Byun, S. H., Prestwich, W. V, Mothersill, C., Seymour, C., … Fernandez, C. (2013). Ultra-Violet Light Emission from HPV-G Cells Irradiated with Low Let Radiation From (90)Y; Consequences for Radiation Induced Bystander Effects. Dose-Response : A Publication of International Hormesis Society, 11, 498–516. doi:10.2203/dose-response.12-048.Ahmad

Al-Mayah, A. H. J., Irons, S. L., Pink, R. C., Carter, D. R. F., & Kadhim, M. A. (2012). Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. Radiation Research, 177(5), 539–45. doi:10.1667/RR2868.1

Arbetter, K. R., Prakash, U. B., Tazelaar, H. D., & Douglas, W. W. (1999). Radiation-induced pneumonitis in the "nonirradiated" lung. Mayo Clinic Proceedings. Mayo Clinic, 74(1), 27–36. doi:10.4065/74.1.27

Audette-Stuart, M., & Yankovich, T. (2011). Bystander effects in bullfrog tadpoles. Radioprotection, 46(6), S497–S497. doi:http://dx.doi.org/10.1051/radiopro/20116770

Autsavapromporn, N., Suzuki, M., Funayama, T., Usami, N., Plante, I., Yokota, Y., … Murakami, T. (2013). Gap junction communication and the propagation of bystander effects induced by microbeam irradiation in human fibroblast cultures: the impact of radiation quality. Radiation Research, 180(4), 367–75. doi:10.1667/RR3111.1

Azzam, E., de Toledo, S., & Little, J. (2004). Stress Signaling from Irradiated to Non-Irradiated Cells. Current Cancer Drug Targets, 4(1), 53–64. doi:10.2174/1568009043481641

Azzam, E. I., de Toledo, S. M., Gooding, T., & 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(5), 497–504. doi:10.2307/3579865

Azzam, E. I., de Toledo, S. M., & Little, J. B. (2001). Direct evidence for the participation of gap junction-mediated intercellular communication in the transmission of damage signals from alpha - particle irradiated to nonirradiated cells. Proceedings of the National Academy of Sciences of the United States of America, 98(2), 473–8. doi:10.1073/pnas.011417098

Azzam, E. I., de Toledo, S. M., & Little, J. B. (2003). Expression of CONNEXIN43 Is Highly Sensitive to Ionizing Radiation and Other Environmental Stresses. Cancer Res., 63(21), 7128–7135.

Azzam, E. I., de Toledo, S. M., & Little, J. B. (2003). Oxidative metabolism, gap junctions and the ionizing radiation-induced bystander effect. Oncogene, 22(45), 7050–7. doi:10.1038/sj.onc.1206961

Azzam, E. I., de Toledo, S. M., Raaphorst, G. P., & Mitchel, R. E. (1996). Low-dose ionizing radiation decreases the frequency of neoplastic transformation to a level below the spontaneous rate in C3H 10T1/2 cells. Radiation Research, 146(4), 369–373. doi:10.2307/3579298

Azzam, E. I., De Toledo, S. M., Spitz, D. R., & Little, J. B. (2002). Oxidative metabolism modulates signal transduction and micronucleus formation in bystander cells from ??-particle-irradiated normal human fibroblast cultures. Cancer Research, 62(19), 5436–5442.

Baskar, R., Balajee, A. S., Geard, C. R., & Hande, M. P. (2008). Isoform-specific activation of protein kinase c in irradiated human fibroblasts and their bystander cells. The International Journal of Biochemistry & Cell Biology, 40(1), 125–34. doi:10.1016/j.biocel.2007.07.002

Blattmann, H., Gebbers, J.-O., Bräuer-Krisch, E., Bravin, A., Le Duc, G., Burkard, W., … Laissue, J. a. (2005). Applications of synchrotron X-rays to radiotherapy. Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment, 548(1-2), 17–22. doi:10.1016/j.nima.2005.03.060

Blyth, B. J., & Sykes, P. J. (2011). Radiation-induced bystander effects: what are they, and how relevant are they to human radiation exposures? Radiation Research, 176(2), 139–57. doi:10.1667/RR2548.1

Bouchet, A., Bidart, M., Miladi, I., Le Clec'h, C., Serduc, R., Coutton, C., … Le Duc, G. (2014). Characterization of the 9L gliosarcoma implanted in the Fischer rat: an orthotopic model for a grade IV brain tumor. Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine, 35(7), 6221–33. doi:10.1007/s13277-014-1783-6

Bouchet, A., Sakakini, N., El Atifi, M., Le Clec'h, C., Brauer, E., Moisan, A., … Pelletier, L. (2013). Early gene expression analysis in 9L orthotopic tumor-bearing rats identifies immune modulation in molecular response to synchrotron microbeam radiation therapy. PloS One, 8(12), e81874. doi:10.1371/journal.pone.0081874

Boukamp, P., Popp, S., Bleuel, K., Tomakidi, E., Bürkle, A., & Fusenig, N. E. (1999). Tumorigenic conversion of immortal human skin keratinocytes (HaCaT) by elevated temperature. Oncogene, 18(41), 5638–45. doi:10.1038/sj.onc.1202934

Bräuer-Krisch, E., Serduc, R., Siegbahn, E. a, Le Duc, G., Prezado, Y., Bravin, A., … Laissue, J. A. (2010). Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. Mutation Research, 704(1-3), 160–6. doi:10.1016/j.mrrev.2009.12.003

Brooks, A., Retherford, J. C., & McClellan, R. (1974). Effect of239 PuO2 Particle Number and Size on the Frequency and Distribution of Chromosome Aberrations in the Liver of the Chinese Hamster. Radiation Research, 59(3), 693–709.

Brown, J. M., & Wilson, G. (2003). Apoptosis genes and resistance to cancer therapy: what does the experimental and clinical data tell us? Cancer Biology & Therapy, 2(5), 477–490.

Burdak-Rothkamm, S., & Prise, K. M. (2009). New molecular targets in radiotherapy: DNA damage signalling and repair in targeted and non-targeted cells. European Journal of Pharmacology, 625(1-3), 151–5. doi:10.1016/j.ejphar.2009.09.068

Calkins, J. (1967). An unusual form of response in x-irradiated protozoa and a hypothesis as to its origin. International Journal of Radiation Biology and Related Studies in Physics, Chemistry, and Medicine, 12(3), 297–301.

Camphausen, K., Moses, M. a., Ménard, C., Sproull, M., Beecken, W. D., Folkman, J., & O'Reilly, M. S. (2003). Radiation abscopal antitumor effect is mediated through p53. Cancer Research, 63(8), 1990–1993. doi:10.1016/S0360-3016(02)03449-1

Chang, W. P., & Little, J. B. (1992). Persistently elevated frequency of spontaneous mutations in progeny of CHO clones surviving X-irradiation: Association with delayed reproductive death phenotype. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 270(2), 191–199. doi:10.1016/0027-5107(92)90130-T

Clutton, S. M., Townsend, K. M., Walker, C., Ansell, J. D., & Wright, E. G. (1996). Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. Carcinogenesis, 17(8), 1633–1639. doi:10.1093/carcin/17.8.1633

Collis, S. J., Schwaninger, J. M., Ntambi, A. J., Keller, T. W., Nelson, W. G., Dillehay, L. E., & DeWeese, T. L. (2004). Evasion of early cellular response mechanisms following low level radiation-induced DNA damage. Journal of Biological Chemistry, 279(48), 49624–49632. doi:10.1074/jbc.M409600200

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., … Yuan, J. (2005). Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nature Chemical Biology, 1(2), 112–119. doi:10.1038/nchembio711

Demoise, C. F., & Conrad, R. A. (1972). Effects of age and radiation exposure on chromosomes in a Marshall Island population. Journal of Gerontology, 27(2), 197–201. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5060721

Deshpande, a, Goodwin, E. H., Bailey, S. M., Marrone, B. L., & Lehnert, B. E. (1996). Alpha-particle-induced sister chromatid exchange in normal human lung fibroblasts: evidence for an extranuclear target. Radiation Research, 145(3), 260–267. doi:10.2307/3578980

Dewey, W. C., Ling, C. C., & Meyn, R. E. (1995). Radiation-induced apoptosis: Relevance to radiotherapy. International Journal of Radiation Oncology Biology Physics, 33(4), 781–796. doi:10.1016/0360-3016(95)00214-8

Dilmanian, F. a, Qu, Y., Liu, S., Cool, C. D., Gilbert, J., Hainfeld, J. F., … McDonald, J. W. (2005). X-ray microbeams: Tumor therapy and central nervous system research. Nuclear Instruments & Methods in Physics Research. Section A, Accelerators, Spectrometers, Detectors and Associated Equipment, 548(1-2), 30–37. doi:10.1016/j.nima.2005.03.062

Dilmanian, F. A., Button, T. M., Le Duc, G., Zhong, N., Peña, L. A., Smith, J. A. L., … Ren, B. (2002). Response of rat intracranial 9L gliosarcoma to microbeam radiation therapy. Neuro-Oncology, 4(1), 26. doi:10.1016/j.ejrad.2008.04.055

Dilmanian, F. A., Morris, G. M., Le Duc, G., Huang, X., Ren, B., Bacarian, T., … Shivaprasad, H. L. (2001). Response of avian embryonic brain to spatially segmented x-ray microbeams. Cellular and Molecular Biology (Noisy-Le-Grand, France), 47(3), 485–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11441956

Dilmanian, F. A., Morris, G. M., Zhong, N., Bacarian, T., Hainfeld, J. F., Kalef-Ezra, J., … Rosen, E. M. (2003). Murine EMT-6 carcinoma: high therapeutic efficacy of microbeam radiation therapy. Radiation Research, 159(5), 632–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12710874

Dilmanian, F. A., Qu, Y., Feinendegen, L. E., Peña, L. A., Bacarian, T., Henn, F. A., … McDonald, J. W. (2007). Tissue-sparing effect of x-ray microplanar beams particularly in the CNS: is a bystander effect involved? Experimental Hematology, 35(4 Suppl 1), 69–77. doi:10.1016/j.exphem.2007.01.014

Diss, E., Nalabothula, N., Nguyen, D., Chang, E., Kwok, Y., & Carrier, F. (2014). Vorinostat(SAHA) Promotes Hyper-Radiosensitivity in Wild Type p53 Human Glioblastoma Cells. Journal of Clinical Oncology and Research, 2(1), 1–16. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4219415&tool=pmcentrez&rendertype=abstract

Elkind, M. M., & Whitmore, G. F. (1967). The radiobiology of cultured mammalian cells. Gordon and Breach. Retrieved from https://books.google.com/books?id=UQe2AAAAIAAJ&pgis=1

Elmore, S. (2007). Apoptosis: a review of programmed cell death. Toxicologic Pathology, 35(4), 495–516. doi:10.1080/01926230701320337

Emerit, I., Levy, a, Cernjavski, L., Arutyunyan, R., Oganesyan, N., Pogosian, a, … Quastel, M. (1994). Transferable clastogenic activity in plasma from persons exposed as salvage personnel of the Chernobyl reactor. Journal of Cancer Research and Clinical Oncology, 120(9), 558–61. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8045922

Faguet, G. B., Reichard, S. M., & Welter, D. a. (1984). Radiation-induced clastogenic plasma factors. Cancer Genetics and Cytogenetics, 12(1), 73–83. doi:10.1016/0165-4608(84)90010-4

Fazzari, J., Mersov, A., Smith, R., Seymour, C., & Mothersill, C. (2012). Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect. International Journal of Radiation Biology, 88(10), 786–90. doi:10.3109/09553002.2012.714519

Fei, P., & El-Deiry, W. S. (2003). P53 and radiation responses. Oncogene, 22(37), 5774–5783. doi:10.1038/sj.onc.1206677

Fernandez-Palomo, C., Bräuer-Krisch, E., Laissue, J., Vukmirovic, D., Blattmann, H., Seymour, C., … Mothersill, C. (2015). Use of synchrotron medical microbeam irradiation to investigate radiation-induced bystander and abscopal effects in vivo. Physica Medica : PM : An International Journal Devoted to the Applications of Physics to Medicine and Biology : Official Journal of the Italian Association of Biomedical Physics (AIFB), 1–12. doi:10.1016/j.ejmp.2015.03.004

Fernandez-Palomo, C., Schültke, E., Smith, R., Bräuer-Krisch, E., Laissue, J., Schroll, C., … Mothersill, C. (2013). Bystander effects in tumor-free and tumor-bearing rat brains following irradiation by synchrotron X-rays. International Journal of Radiation Biology, 89(6), 445–53. doi:10.3109/09553002.2013.766770

Fernet, M., Mégnin-Chanet, F., Hall, J., & Favaudon, V. (2010). Control of the G2/M checkpoints after exposure to low doses of ionising radiation: Implications for hyper-radiosensitivity. DNA Repair, 9(1), 48–57. doi:10.1016/j.dnarep.2009.10.006

Freeman, S. M., Abboud, C. N., Whartenby, K. A., Packman, C. H., Koeplin, D. S., Moolten, F. L., & Abraham, G. N. (1993). The “bystander effect”: Tumor regression when a fraction of the tumor mass is genetically modified. Cancer Research, 53(21), 5274–5283.

Gamulin, M., Garaj-Vrhovac, V., & Kopjar, N. (2007). Evaluation of DNA damage in radiotherapy-treated cancer patients using the alkaline comet assay. Collegium Antropologicum, 31(3), 837–845.

Garcia, L. M., Leblanc, J., Wilkins, D., & Raaphorst, G. P. (2006). Fitting the linear-quadratic model to detailed data sets for different dose ranges. Physics in Medicine and Biology, 51(11), 2813–2823. doi:10.1088/0031-9155/51/11/009

Goh K-O. (1975). Total-body irradiation and human chromosomes. V. Additional evidence of a transferable substance in the plasma of irradiated persons to induce chromosomal breakages. Journal of Medicine, 6(1), 51–60.

Goh, K., & 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(1), 171–181.

Gow, M. D., Seymour, C. B., Byun, S.-H., & Mothersill, C. E. (2008). Effect of dose rate on the radiation-induced bystander response. Physics in Medicine and Biology, 53(1), 119–32. doi:10.1088/0031-9155/53/1/008

Guan, X., Bonney, W. J., & Ruch, R. J. (1995). Changes in gap junction permeability, gap junction number, and connexin43 expression in lindane-treated rat liver epithelial cells. Toxicology and Applied Pharmacology, 130(1), 79–86. doi:10.1006/taap.1995.1011

Hall, E. J. (2003). THE BYSTANDER EFFECT. Health Physics, 85(1), 31. Retrieved from http://journals.lww.com/health-physics/Abstract/2003/07000/The_Bystander_Effect.8.aspx

Hall, E. J., & Giaccia, A. J. (2006). Radiobiology for the Radiologist. Lippincott Williams & Wilkins. Retrieved from http://books.google.ca/books/about/Radiobiology_for_the_Radiologist.html?id=6HhjwRyqBzgC&pgis=1

Hamada, N., Matsumoto, H., Hara, T., & Kobayashi, Y. (2007). Intercellular and Intracellular Signaling Pathways Mediating Ionizing Radiation-Induced Bystander Effects. Journal of Radiation Research, 48(2), 87–95. doi:10.1269/jrr.06084

Hei, T. K., Zhou, H., Ivanov, V. N., Hong, M., Lieberman, H. B., Brenner, D. J., … Geard, C. R. (2008). Mechanism of radiation-induced bystander effects: a unifying model. The Journal of Pharmacy and Pharmacology, 60(8), 943–50. doi:10.1211/jpp.60.8.0001

Heuskin, A.-C., Wéra, A.-C., Riquier, H., Michiels, C., & Lucas, S. (2013). Low-Dose Hypersensitivity and Bystander Effect are Not Mutually Exclusive in A549 Lung Carcinoma Cells after Irradiation with Charged Particles. Radiation Research, 180(5), 491–8. doi:10.1667/RR13358.1

Hickman, a W., Jaramillo, R. J., Lechner, J. F., & Johnson, N. F. (1994). Alpha-particle-induced p53 protein expression in a rat lung epithelial cell strain. Cancer Research, 54(22), 5797–5800.

Hollowell, J. G. J., & Littlefield, L. G. (1967). CHROMOSOME ABERRATIONS INDUCED BY PLASMA FROM IRRADIATED PATIENTS: A BRIEF REPORT. J. S. C. Med. Ass., 63: 437 442(Dec. 19, 1967). Retrieved from http://www.osti.gov/scitech/biblio/4798922

Hollowell, J. G., & Littlefield, L. G. (1968). Chromosome damage induced by plasma of x-rayed patients: an indirect effect of x-ray. Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine (New York, N.Y.), 129(1), 240–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/5686521

Hu, B., Shen, B., Su, Y., Geard, C. R., & Balajee, A. S. (2009). Protein kinase C epsilon is involved in ionizing radiation induced bystander response in human cells. The International Journal of Biochemistry & Cell Biology, 41(12), 2413–21. doi:10.1016/j.biocel.2009.06.012

Iyer, R., & Lehnert, B. E. (2000). Factors underlying the cell growth-related bystander responses to ?? particles. Cancer Research, 60(5), 1290–1298. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10728689

Jella, K. K., Garcia, A., McClean, B., Byrne, H. J., & Lyng, F. M. (2013). Cell death pathways in directly irradiated cells and cells exposed to medium from irradiated cells. International Journal of Radiation Biology, 89(3), 182–90. doi:10.3109/09553002.2013.734942

Jella, K. K., Rani, S., O'Driscoll, L., McClean, B., Byrne, H. J., & Lyng, F. M. (2014). Exosomes are involved in mediating radiation induced bystander signaling in human keratinocyte cells. Radiation Research, 181(2), 138–45. doi:10.1667/RR13337.1

Joiner, M. C., & Denekamp, J. (1986). The effect of small radiation doses on mouse skin. The British Journal of Cancer. Supplement, 7, 63–6. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2149747&tool=pmcentrez&rendertype=abstract

Joiner, M. C., Marples, B., Lambin, P., Short, S. C., & Turesson, I. (2001). Low-dose hypersensitivity: current status and possible mechanisms. International Journal of Radiation Oncology, Biology, Physics, 49(2), 379–89. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11173131

Kadhim, M. A., & Hill, M. A. (2015). NON-TARGETED EFFECTS OF RADIATION EXPOSURE: RECENT ADVANCES AND IMPLICATIONS. Radiation Protection Dosimetry, 1–7. doi:10.1093/rpd/ncv167

Kadhim, M. A., Macdonald, D. A., Goodhead, D. T., Lorimore, S. A., Marsden, S. J., & Wright, E. G. (1992). Transmission of chromosomal instability after plutonium alpha-particle irradiation. Nature, 355(6362), 738–740. doi:10.1038/355738a0

Kadhim, M., Salomaa, S., Wright, E., Hildebrandt, G., Belyakov, O. V., Prise, K. M., & Little, M. P. (2013). Non-targeted effects of ionising radiation-Implications for low dose risk. Mutation Research - Reviews in Mutation Research, 752(2), 84–98. doi:10.1016/j.mrrev.2012.12.001

Kaminski, J. M., Shinohara, E., Summers, J. B., Niermann, K. J., Morimoto, A., & Brousal, J. (2005). The controversial abscopal effect. Cancer Treatment Reviews, 31(3), 159–72. doi:10.1016/j.ctrv.2005.03.004

Kashino, G., Kondoh, T., Nariyama, N., Umetani, K., Ohigashi, T., Shinohara, K., … Ono, K. (2009). Induction of DNA double-strand breaks and cellular migration through bystander effects in cells irradiated with the slit-type microplanar beam of the spring-8 synchrotron. International Journal of Radiation Oncology, Biology, Physics, 74(1), 229–36. doi:10.1016/j.ijrobp.2008.09.060

Kass, E. M., & Jasin, M. (2010). Collaboration and competition between DNA double-strand break repair pathways. FEBS Letters, 584(17), 3703–3708. doi:10.1016/j.febslet.2010.07.057

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer, 26(4), 239–57. Retrieved from /pmc/articles/PMC2008650/?report=abstract

Khan, M. A., Hill, R. P., & Van Dyk, J. (1998). Partial volume rat lung irradiation: an evaluation of early DNA damage. International Journal of Radiation Oncology, Biology, Physics, 40(2), 467–76. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9457837

Khan, M. A., Van Dyk, J., Yeung, I. W. T., & Hill, R. P. (2003). Partial volume rat lung irradiation; Assessment of early DNA damage in different lung regions and effect of radical scavengers. Radiotherapy and Oncology, 66(1), 95–102. doi:10.1016/S0167-8140(02)00325-0

Korystov YuN. (1992). Contributions of the direct and indirect effects of ionizing radiation to reproductive cell death. Radiation Research, 129(2), 228–234.

Koturbash, I., Kutanzi, K., Hendrickson, K., Rodriguez-Juarez, R., Kogosov, D., & Kovalchuk, O. (2008). Radiation-induced bystander effects in vivo are sex specific. Mutation Research, 642(1-2), 28–36. doi:10.1016/j.mrfmmm.2008.04.002

Koval, T. M. (1984). Multiphasic survival response of a radioresistant lepidopteran insect cell line. Radiation Research, 98(3), 642–8.

Krueger, S. a., Collis, S. J., Joiner, M. C., Wilson, G. D., & Marples, B. (2007). Transition in Survival From Low-Dose Hyper-Radiosensitivity to??Increased Radioresistance Is Independent of Activation of ATM SER1981 Activity. International Journal of Radiation Oncology Biology Physics, 69(4), 1262–1271. doi:10.1016/j.ijrobp.2007.08.012

Krueger, S. a., Wilson, G. D., Piasentin, E., Joiner, M. C., & Marples, B. (2010). The effects of G2-phase enrichment and checkpoint abrogation on low-dose hyper-radiosensitivity. International Journal of Radiation Oncology Biology Physics, 77(5), 1509–1517. doi:10.1016/j.ijrobp.2010.01.028

Laissue, J. a, Bartzsch, S., Blattmann, H., Bräuer-Krisch, E., Bravin, A., Dalléry, D., … Slatkin, D. N. (2013). Response of the rat spinal cord to X-ray microbeams. Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology, 106(1), 106–11. doi:10.1016/j.radonc.2012.12.007

Laissue, J. A., Blattmann, H., Di Michiel, M., Slatkin, D. N., Lyubimova, N., Guzman, R., … Wagner, H.-P. (2001). The weanling piglet cerebellum: a surrogate for tolerance to MRT (microbeam radiation therapy) in pediatric neuro-oncology. Penetrating Radiation Systems and Application, 4508, 65–73. doi:10.1117/12.450774

Laissue, J. A., Lyubimova, N., Wagner, H.-P., Archer, D. W., Slatkin, D. N., Di Michiel, M., … Blattmann, H. (1999). Microbeam radiation therapy. In H. B. Barber & H. Roehrig (Eds.), SPIE's International Symposium on Optical Science, Engineering, and Instrumentation (pp. 38–45). International Society for Optics and Photonics. doi:10.1117/12.368185

Lambin, P., Fertil, B., Malaise, E. P., & Joiner, M. C. (1994). Multiphasic survival curves for cells of human tumor cell lines: induced repair or hypersensitive subpopulation? Radiation Research, 138(1 Suppl), S32–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8146321

Lambin, P., Marples, B., Fertil, B., Malaise, E. P., & Joiner, M. C. (1993). Hypersensitivity of a human tumour cell line to very low radiation doses. International Journal of Radiation Biology, 63(5), 639–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8099110

Lauber, K., Ernst, A., Orth, M., Herrmann, M., & Belka, C. (2012). Dying cell clearance and its impact on the outcome of tumor radiotherapy. Frontiers in Oncology, 2(September), 1–14. doi:10.3389/fonc.2012.00116

Le, M., McNeill, F. E., Seymour, C., Rainbow, A. J., & Mothersill, C. E. (2014). An observed effect of ultraviolet radiation emitted from β-irradiated HaCaT cells upon non-β-irradiated bystander cells. Radiation Research, (In Print).

Le, M., McNeill, F. E., Seymour, C., Rainbow, A. J., & Mothersill, C. E. (2015). An Observed Effect of Ultraviolet Radiation Emitted from Beta-Irradiated HaCaT Cells upon Non-Beta-Irradiated Bystander Cells. Radiation Research, 183(3), 279–290. doi:10.1667/RR13827.1

Lehman, T. A., Modali, R., Boukamp, P., Stanek, J., Bennett, W. P., Welsh, J. A., … Rogan, E. M. (1993). p53 mutations in human immortalized epithelial cell lines. Carcinogenesis, 14(5), 833–839.

Li, H., Ambade, A., & Re, F. (2009). Cutting edge: Necrosis activates the NLRP3 inflammasome. Journal of Immunology (Baltimore, Md. : 1950), 183(3), 1528–1532. doi:10.4049/jimmunol.0901080

Limoli, C. L., Kaplan, M. I., Giedzinski, E., & Morgan, W. F. (2001). Attenuation of radiation-induced genomic instability by free radical scavengers and cellular proliferation. Free Radical Biology and Medicine, 31(1), 10–19. doi:10.1016/S0891-5849(01)00542-1

Limoli, C. L., Limoli, C. L., Hartmann, A., Hartmann, A., Shephard, L., Shephard, L., … Morgan, W. F. (1998). Apoptosis, Reproductive Failure, and Oxidative Stress in Chinese Hamster Ovary Cells with Compromised Genomic Integrity1. Life Sciences, (16).

Lomax, M. E., Folkes, L. K., & O'Neill, P. (2013). Biological consequences of radiation-induced DNA damage: Relevance to radiotherapy. Clinical Oncology, 25(10), 578–585. doi:10.1016/j.clon.2013.06.007

Lorimore, S. a, Coates, P. J., Scobie, G. E., Milne, G., & Wright, E. G. (2001). Inflammatory-type responses after exposure to ionizing radiation in vivo: a mechanism for radiation-induced bystander effects? Oncogene, 20(48), 7085–95. doi:10.1038/sj.onc.1204903

Lyng, F. M., Desplanques, M., Jella, K. K., Garcia, A., & McClean, B. (2012). The importance of serum serotonin levels in the measurement of radiation-induced bystander cell death in HaCaT cells. International Journal of Radiation Biology, 88(10), 770–772. doi:10.3109/09553002.2012.705222

Lyng, F. M., Maguire, P., McClean, B., Seymour, C., & Mothersill, C. (2006). The Involvement of Calcium and MAP Kinase Signaling Pathways in the Production of Radiation-Induced Bystander Effects. Radiation Research, 165(4), 400–409. doi:10.1667/RR3527.1

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2000). Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. British Journal of Cancer, 83(9), 1223–30. doi:10.1054/bjoc.2000.1433

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2001). Oxidative stress in cells exposed to low levels of ionizing radiation. Biochemical Society Transactions, 29(2), 350. doi:10.1042/0300-5127:0290350

Lyng, F. M., Seymour, C., & 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(4), 365–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893237

Maeda, M., Tomita, M., Usami, N., & Kobayashi, K. (2010). Bystander cell death is modified by sites of energy deposition within cells irradiated with a synchrotron X-ray microbeam. Radiation Research, 174(1), 37–45. doi:10.1667/RR2086.1

Maguire, P., Mothersill, C., McClean, B., Seymour, C., & Lyng, F. M. (2007). Modulation of radiation responses by pre-exposure to irradiated cell conditioned medium. Radiation Research, 167(4), 485–92. doi:10.1667/RR0159.1

Mancuso, M., Pasquali, E., Giardullo, P., Leonardi, S., Tanori, M., Di Majo, V., … Saran, A. (2012). The radiation bystander effect and its potential implications for human health. Current Molecular Medicine, 12(5), 613–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22452594

Mancuso, M., Pasquali, E., Leonardi, S., Tanori, M., Rebessi, S., Di Majo, V., … Saran, A. (2008). Oncogenic bystander radiation effects in Patched heterozygous mouse cerebellum. Proceedings of the National Academy of Sciences of the United States of America, 105(34), 12445–12450. doi:10.1073/pnas.0804186105

Marozik, P., Mothersill, C., Seymour, C. B., Mosse, I., & Melnov, S. (2007). Bystander effects induced by serum from survivors of the Chernobyl accident. Experimental Hematology, 35(4 Suppl 1), 55–63. doi:10.1016/j.exphem.2007.01.029

Marples, B. (2004). Is low-dose hyper-radiosensitivity a measure of G2-phase cell radiosensitivity? Cancer and Metastasis Reviews. doi:10.1023/B:CANC.0000031761.61361.2a

Marples, B., & Joiner, M. C. (1993). The response of Chinese hamster V79 cells to low radiation doses: evidence of enhanced sensitivity of the whole cell population. Radiation Research, 133(1), 41–51.

Marples, B., & Joiner, M. C. (1995). The elimination of low-dose hypersensitivity in Chinese hamster V79-379A cells by pretreatment with X rays or hydrogen peroxide. Radiation Research, 141(2), 160–169. doi:10.2307/3579043

Marples, B., Wouters, B. G., Collis, S. J., Chalmers, a J., & Joiner, M. C. (2004). Low-dose hyper-radiosensitivity: a consequence of ineffective cell cycle arrest of radiation-damaged G2-phase cells. Radiation Research, 161(3), 247–55. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14982490

Marples, B., Wouters, B. G., & 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(1), 38–45. doi:10.1667/RR3013

Martin, C., Romero, S., Sánchez-Payá, J., Massutí, B., Arriero, J. M., & Hernández, L. (1999). Bilateral lymphocytic alveolitis: A common reaction after unilateral thoracic irradiation. European Respiratory Journal, 13(4), 727–732. doi:10.1034/j.1399-3003.1999.13d05.x

Martin, L. M., Marples, B., Lynch, T. H., Hollywood, D., & Marignol, L. (2014). Exposure to low dose ionising radiation: Molecular and clinical consequences. Cancer Letters, 349(1), 98–106. doi:10.1016/j.canlet.2013.12.015

Martin, L., Marples, B., Coffey, M., Lawler, M., Hollywood, D., & Marignol, L. (2009). Recognition of O6MeG lesions by MGMT and mismatch repair proficiency may be a prerequisite for low-dose radiation hypersensitivity. Radiation Research, 172(4), 405–413. doi:10.1667/RR1717.1

Matsumoto, H., Hayashi, S., Hatashita, M., Ohnishi, K., Shioura, H., Ohtsubo, T., … Kano, E. (2001). Induction of radioresistance by a nitric oxide-mediated bystander effect. Radiation Research, 155(3), 387–396. doi:10.1667/0033-7587(2001)155[0387:IORBAN]2.0.CO;2

Matsumoto, H., Takahashi, A., & Ohnishi, T. (2004). Radiation-induced adaptive responses and bystander effects. Uchū Seibutsu Kagaku, 18(4), 247–54. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15858392

Mendonca, M. S., Kurohara, W., Antoniono, R., & Redpath, J. L. (1989). Plating efficiency as a function of time postirradiation: evidence for the delayed expression of lethal mutations. Radiation Research, 119(2), 387–393. doi:10.2307/3577629

Mohiuddin, M., Fujita, M., Regine, W. F., Megooni, a S., Ibbott, G. S., & Ahmed, M. M. (1999). High-dose spatially-fractionated radiation (GRID): a new paradigm in the management of advanced cancers. International Journal of Radiation Oncology, Biology, Physics, 45(3), 721–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10524428

Morgan, G. W., & Breit, S. N. (1995). Radiation and the lung: a reevaluation of the mechanisms mediating pulmonary injury. International Journal of Radiation Oncology, Biology, Physics, 31(2), 361–9. doi:10.1016/0360-3016(94)00477-3

Morgan, W. F. (2003). Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects in vitro. Radiation Research, 159(5), 567–580. doi:10.1667/RRAV19.1

Mothersill, C., Bucking, C., Smith, R., Agnihotri, N., Oneill, a, Kilemade, M., & Seymour, C. (2006). Communication of radiation-induced stress or bystander signals between fish in vivo. Environmental Science & Technology, 40(21), 6859–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17144322

Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., … Seymour, C. (2014). Transmission of signals from rats receiving high doses of microbeam radiation to cage mates: an inter-mammal bystander effect. Dose-Response : A Publication of International Hormesis Society, 12(1), 72–92. doi:10.2203/dose-response.13-011.Mothersill

Mothersill, C., Harney, J., Lyng, F., Cottell, D., Parsons, K., Murphy, D. M., & Seymour, C. B. (1995). Primary Explants of Human Uroepithelium Show an Unusual Response to Low-Dose Irradiation with Cobalt-60 Gamma Rays. Radiation Research, 142(2), 181. doi:10.2307/3579027

Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S., & Wright, E. (2005a). Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. Radiation Research, 163(4), 391–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15799694

Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S., & Wright, E. (2005b). Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. Radiation Research, 163(4), 391–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15799694

Mothersill, C., Saroya, R., Smith, R. W., Singh, H., & Seymour, C. B. (2010). Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments. Radiation Research, 174(1), 119–23. doi:10.1667/RR2036.1

Mothersill, C., & Seymour, C. (1997). Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. International Journal of Radiation Biology, 71(4), 421–7. doi:10.1080/095530097144030

Mothersill, C., & Seymour, C. (2001). Radiation-induced bystander effects: past history and future directions. Radiation Research, 155(6), 759–767. doi:10.1667/0033-7587(2001)155[0759:RIBEPH]2.0.CO;2

Mothersill, C., & Seymour, C. (2002). Bystander and delayed effects after fractionated radiation exposure. Radiation Research, 158(5), 626–33. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12385640

Mothersill, C., & Seymour, C. (2006). Radiation-induced bystander effects: evidence for an adaptive response to low dose exposures? Dose-Response : A Publication of International Hormesis Society, 4(4), 283–90. doi:10.2203/dose-response.06-111.Mothersill

Mothersill, C., & Seymour, C. (2010). Radiation-induced bystander effects: are they good, bad or both? Medicine, Conflict, and Survival, 21(2), 101–10. doi:10.1080/13623690500073398

MotherSill, C., & Seymour, C. (2012). Changing paradigms in radiobiology. Mutation Research/Reviews in Mutation Research, 750(2), 85–95. doi:10.1016/j.mrrev.2011.12.007

Mothersill, C., & Seymour, C. (2015). RADIATION-INDUCED NON-TARGETED EFFECTS: SOME OPEN QUESTIONS. Radiation Protection Dosimetry, 1–6. doi:10.1093/rpd/ncv155

Mothersill, C., & Seymour, C. B. (1998). Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. Radiation Research, 149(3), 256–262. doi:10.2307/3579958

Mothersill, C., & Seymour, C. B. (2004). Radiation-induced bystander effects--implications for cancer. Nature Reviews. Cancer, 4(2), 158–164. doi:10.1038/nrc1277

Mothersill, C., Seymour, C. B., & Joiner, M. C. (2002). Relationship between radiation-induced low-dose hypersensitivity and the bystander effect. Radiation Research, 157(5), 526–32. doi:10.1667/0033-7587(2002)157[0526:RBRILD]2.0.CO;2

Mothersill, C., Smith, R. W., Agnihotri, N., & Seymour, C. B. (2007). Characterization of a radiation-induced stress response communicated in vivo between zebrafish. Environmental Science & Technology, 41(9), 3382–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17539553

Mothersill, C., Smith, R. W., Hinton, T. G., Aizawa, K., & Seymour, C. B. (2009). Communication of radiation-induced signals in vivo between DNA repair deficient and proficient medaka (Oryzias latipes). Environmental Science & Technology, 43(9), 3335–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19534155

Mothersill, C., Smith, R. W., Saroya, R., Denbeigh, J., Rowe, B., Banevicius, L., … Seymour, C. B. (2010). Irradiation of rainbow trout at early life stages results in legacy effects in adults. International Journal of Radiation Biology, 86(10), 817–28. doi:10.3109/09553002.2010.486018

Muñoz, L. E., Lauber, K., Schiller, M., Manfredi, A. A., & Herrmann, M. (2010). The role of defective clearance of apoptotic cells in systemic autoimmunity. Nature Reviews. Rheumatology, 6(5), 280–289. doi:10.1038/nrrheum.2010.46

Munro, A. J. (2009). Bystander effects and their implications for clinical radiotherapy. Journal of Radiological Protection : Official Journal of the Society for Radiological Protection, 29(2A), A133–42. doi:10.1088/0952-4746/29/2A/S09

Murphy, J. B., & Morton, J. J. (1915). The Effect Of Roentgen Rays On The Rate Of Growth Of Spontaneous Tumors In Mice. The Journal of Experimental Medicine, 22(6), 800–803. doi:10.1084/jem.22.6.800

Murphy, J. E. J., Nugent, S., Seymour, C., & Mothersill, C. (2005). Mitochondrial DNA point mutations and a novel deletion induced by direct low-LET radiation and by medium from irradiated cells. Mutation Research, 585(1-2), 127–36. doi:10.1016/j.mrgentox.2005.04.011

Nagasawa, H., & Little, J. B. (1992). Induction of sister chromatid exchanges by extremely low doses of alpha-particles. Cancer Research, 52(22), 6394–6396. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1423287

Narayanan, P. K., Goodwin, E. H., & Lehnert, B. E. (1997). Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. Cancer Research, 57(18), 3963–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9307280

Narayanan, P. K., LaRue, K. E., Goodwin, E. H., & Lehnert, B. E. (1999). Alpha particles induce the production of interleukin-8 by human cells. Radiation Research, 152(1), 57–63. doi:10.2307/3580049

Nuta, O., & Darroudi, F. (2008). The impact of the bystander effect on the low-dose hypersensitivity phenomenon. Radiation and Environmental Biophysics, 47(2), 265–74. doi:10.1007/s00411-007-0145-9

O'Leary, V. B., Ovsepian, S. V., Carrascosa, L. G., Buske, F. A., Radulovic, V., Niyazi, M., … Anastasov, N. (2015). PARTICLE, a Triplex-Forming Long ncRNA, Regulates Locus-Specific Methylation in Response to Low-Dose Irradiation. Cell Reports, 474–485. doi:10.1016/j.celrep.2015.03.043

Olivieri, G., Bodycote, J., & Wolff, S. (1984). Adaptive response of human lymphocytes to low concentrations of radioactive thymidine. Science (New York, N.Y.), 223(4636), 594–597.

Pampfer, S., & Streffer, C. (1989). Increased chromosome aberration levels in cells from mouse fetuses after zygote X-irradiation. International Journal of Radiation Biology, 55(1), 85–92. doi:QMH6QGL9Q26Q1BJ0 [pii]

Pant, G. S. S., & Kamada, N. (1977). Chromosome aberrations in normal leukocytes induced by the plasma of exposed individuals. Hiroshima Journal of Medical Sciences, 26(2-3), 149–54. Retrieved from http://inis.iaea.org/Search/search.aspx?orig_q=RN:9386153

Parsons, W. B., Watkins, C. H., Pease, G. L., & Childs, D. S. (1954). Changes in sternal marrow following roentgen-ray therapy to the spleen in chronic granulocytic leukemia. Cancer, 7(1), 179–189. doi:10.1002/1097-0142(195401)7:1<179::AID-CNCR2820070120>3.0.CO;2-A

Poncy, J. L., Walter, C., Fritsch, P., Masse, R., & Lafuma, J. (1979). Delayed SCE frequency of rat bone marrow cells after radon inhalation. Retrieved from http://www.iaea.org/inis/collection/NCLCollectionStore/_Public/11/535/11535419.pdf

Poon, R. C. C., Agnihotri, N., Seymour, C., & Mothersill, C. (2007). Bystander effects of ionizing radiation can be modulated by signaling amines. Environmental Research, 105(2), 200–11. doi:10.1016/j.envres.2006.12.003

Prise, K. M. (1998). Studies of bystander effects in human fibroblasts using a charged particle microbeam. International Journal of Radiation Biology, 74(6), 793–798. doi:10.1080/095530098141087

Prise, K. M., & O'Sullivan, J. M. (2009). Radiation-induced bystander signalling in cancer therapy. Nature Reviews. Cancer, 9(5), 351–60. doi:10.1038/nrc2603

Prise, K. M., Schettino, G., Folkard, M., & Held, K. D. (2005). New insights on cell death from radiation exposure. The Lancet Oncology, 6(7), 520–8. doi:10.1016/S1470-2045(05)70246-1

Priyadarshika, R. C. U., Crosbie, J. C., Kumar, B., & Rogers, P. a W. (2011). Biodosimetric quantification of short-term synchrotron microbeam versus broad-beam radiation damage to mouse skin using a dermatopathological scoring system. The British Journal of Radiology, 84(1005), 833–42. doi:10.1259/bjr/58503354

Puck, T. T., & Marcus, P. I. (1956). Action of x-rays on mammalian cells. The Journal of Experimental Medicine, 103(5), 653–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10380286

Robertson, J. a. (1978). The CANDU Reactor System: An Appropriate Technology. Science (New York, N.Y.), 199(4329), 657–664. doi:10.1126/science.199.4329.657

Rodier, F., & Campisi, J. (2011). Four faces of cellular senescence. Journal of Cell Biology, 192(4), 547–556. doi:10.1083/jcb.201009094

Ryan, L. a, Seymour, C. B., Joiner, M. C., & Mothersill, C. E. (2009). Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. International Journal of Radiation Biology, 85(1), 87–95. doi:10.1080/09553000802635062

Rzeszowska-Wolny, J., Przybyszewski, W. M., & Widel, M. (2009). Ionizing radiation-induced bystander effects, potential targets for modulation of radiotherapy. European Journal of Pharmacology, 625(1-3), 156–64. doi:10.1016/j.ejphar.2009.07.028

Saroya, R., Smith, R., Seymour, C., & Mothersill, C. (2009). Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism. Dose-Response : A Publication of International Hormesis Society, 8(3), 317–30. doi:10.2203/dose-response.09-043.Saroya

Sawant, S. G., Zheng, W., Hopkins, K. M., Randers-Pehrson, G., Lieberman, H. B., & Hall, E. J. (2002). The radiation-induced bystander effect for clonogenic survival. Radiation Research, 157(4), 361–4. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893236

Schettino, G., Folkard, M., Prise, K. M., Vojnovic, B., Held, K. D., & Michael, B. D. (2003). Low-dose studies of bystander cell killing with targeted soft X rays. Radiation Research, 160(5), 505–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14565833

Schoenherr, D., Krueger, S. A., Martin, L., Marignol, L., Wilson, G. D., & Marples, B. (2013). Determining if low dose hyper-radiosensitivity (HRS) can be exploited to provide a therapeutic advantage: a cell line study in four glioblastoma multiforme (GBM) cell lines. International Journal of Radiation Biology, 89(12), 1009–16. doi:10.3109/09553002.2013.825061

Serduc, R., Berruyer, G., Brochard, T., Renier, M., & Nemoz, C. (2010). In vivo pink-beam imaging and fast alignment procedure for rat brain lesion microbeam radiation therapy. Journal of Synchrotron Radiation, 17(3), 325–31. doi:10.1107/S0909049510006667

Serduc, R., Bouchet, A., Bräuer-Krisch, E., Laissue, J. A., Spiga, J., Sarun, S., … Le Duc, G. (2009). Synchrotron microbeam radiation therapy for rat brain tumor palliation-influence of the microbeam width at constant valley dose. Physics in Medicine and Biology, 54(21), 6711–24. doi:10.1088/0031-9155/54/21/017

Seymour, C. B., & Mothersill, C. (2000). Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. Radiation Research, 153(5 Pt 1), 508–11. doi:10.1667/0033-7587(2000)153

Seymour, C. B., Mothersill, C., & Alper, T. (1986). High yields of lethal mutations in somatic mammalian cells that survive ionizing radiation. International Journal of Radiation Biology and Related Studies in Physics, Chemistry, and Medicine, 50(1), 167–179. doi:10.1080/09553008614550541

Seymour, C., & Mothersill, C. (1992). All colonies of CHO-K1 cells surviving γ-irradiation contain non-viable cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 267(1), 19–30. doi:10.1016/0027-5107(92)90107-D

Shao, C., Aoki, M., & Furusawa, Y. (2003). Bystander effect on cell growth stimulation in neoplastic HSGc cells induced by heavy-ion irradiation. Radiation and Environmental Biophysics, 42(3), 183–187. doi:10.1007/s00411-003-0202-y

Shao, C., Folkard, M., Michael, B. D., & Prise, K. M. (2004). Targeted cytoplasmic irradiation induces bystander responses. Proceedings of the National Academy of Sciences of the United States of America, 101(37), 13495–500. doi:10.1073/pnas.0404930101

Shao, C., Furusawa, Y., Aoki, M., Matsumoto, H., & Ando, K. (2002). Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells. International Journal of Radiation Biology, 78(9), 837–844. doi:10.1080/09553000210149786

Shao, C., Lyng, F. M., Folkard, M., & Prise, K. M. (2006). Calcium fluxes modulate the radiation-induced bystander responses in targeted glioma and fibroblast cells. Radiation Research, 166(3), 479–487. doi:10.1667/RR3600.1

Shao, C., Stewart, V., Folkard, M., Michael, B. D., & Prise, K. M. (2003). Nitric Oxide-Mediated Signaling in the Bystander Response of Individually Targeted Glioma Cells. Cancer Research, 63(23), 8437–8442.

Short, S. C., Bourne, S., Martindale, C., Woodcock, M., & Jackson, S. P. (2005). DNA damage responses at low radiation doses. Radiation Research, 164(3), 292–302. doi:10.1667/RR3421.1

Short, S. C., Woodcock, M., Marples, B., & Joiner, M. C. (2003). Effects of cell cycle phase on low-dose hyper-radiosensitivity. International Journal of Radiation Biology, 79(2), 99–105. doi:10.1080/0955300021000045646

Short, S., Mayes, C., Woodcock, M., Johns, H., & Joiner, M. C. (1999). Low dose hypersensitivity in the T98G human glioblastoma cell line. International Journal of Radiation Biology, 75(7), 847–55. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10489896

Silva, M. T. (2010). Secondary necrosis: The natural outcome of the complete apoptotic program. FEBS Letters, 584(22), 4491–4499. doi:10.1016/j.febslet.2010.10.046

Simonsson, M., Qvarnström, F., Nyman, J., Johansson, K. A., Garmo, H., & Turesson, I. (2008). Low-dose hypersensitive γH2AX response and infrequent apoptosis in epidermis from radiotherapy patients. Radiotherapy and Oncology, 88(3), 388–397. doi:10.1016/j.radonc.2008.04.017

Singh, H., Saroya, R., Smith, R., Mantha, R., Guindon, L., Mitchel, R. E. J., … Mothersill, C. (2011). Radiation induced bystander effects in mice given low doses of radiation in vivo. Dose-Response : A Publication of International Hormesis Society, 9(2), 225–42. doi:10.2203/dose-response.09-062.Singh

Skov, K. A. (1994). Molecular, cellular, and genetic basis of radiosensitivity at low doses: a case of inducible repair? Radiation Research, 138(1 Suppl), S1–4.

Skov, K., Marples, B., Matthews, J. B., Joiner, M. C., & Zhou, H. (1994). A preliminary investigation into the extent of increased radioresistance or hyper-radiosensitivity in cells of hamster cell lines known to be deficient in DNA repair. Radiation Research, 138(1 Suppl), S126–S129.

Slatkin, D. N., Spanne, P., Dilmanian, F. a, Gebbers, J. O., & Laissue, J. A. (1995). Subacute neuropathological effects of microplanar beams of x-rays from a synchrotron wiggler. Proceedings of the National Academy of Sciences of the United States of America, 92(19), 8783–8787. doi:10.1073/pnas.92.19.8783

Slatkin, D. N., Spanne, P., Dilmanian, F. A., & Sandborg, M. (1992). Microbeam radiation therapy. Medical Physics, 19(6), 1395–1400. doi:10.1118/1.596771

Smilowitz, H. M., Blattmann, H., Bräuer-Krisch, E., Bravin, a., Di Michiel, M., Gebbers, J. O., … Laissue, J. a. (2006). Synergy of gene-mediated immunoprophylaxis and microbeam radiation therapy for advanced intracerebral rat 9L gliosarcomas. Journal of Neuro-Oncology, 78(2), 135–143. doi:10.1007/s11060-005-9094-9

Smith, R. W., Wang, J., Bucking, C. P., Mothersill, C. E., & Seymour, C. B. (2007). Evidence for a protective response by the gill proteome of rainbow trout exposed to X-ray induced bystander signals. Proteomics, 7(22), 4171–80. doi:10.1002/pmic.200700573

Smith, R. W., Wang, J., Schültke, E., Seymour, C. B., Bräuer-Krisch, E., Laissue, J. A., … Mothersill, C. E. (2013). Proteomic changes in the rat brain induced by homogenous irradiation and by the bystander effect resulting from high energy synchrotron X-ray microbeams. International Journal of Radiation Biology, 89(2), 118–27. doi:10.3109/09553002.2013.732252

Sokolov, M. V, Smilenov, L. B., Hall, E. J., Panyutin, I. G., Bonner, W. M., & Sedelnikova, O. a. (2005). Ionizing radiation induces DNA double-strand breaks in bystander primary human fibroblasts. Oncogene, 24(49), 7257–65. doi:10.1038/sj.onc.1208886

Souto, J. (1962). Tumour development in the rat induced by blood of irradiated animals. Nature, 195, 1317–1318. doi:10.1038/1951317a0

Stein, G. H. (1979). T98G: an anchorage-independent human tumor cell line that exhibits stationary phase G1 arrest in vitro. Journal of Cellular Physiology, 99(1), 43–54. doi:10.1002/jcp.1040990107

Strasser, A., O'Connor, L., & Dixit, V. M. (2000). Apoptosis signaling. Annual Review of Biochemistry, 69, 217–245. doi:10.1146/annurev.biochem.69.1.217

Surinov, B. P., Isaeva, V. G., & Dukhova, N. N. (2004). Postirradiation volatile secretions of mice: syngeneic and allogeneic immune and behavioral effects. Bulletin of Experimental Biology and Medicine, 138(4), 384–6. doi:10.1007/s10517-004-0018-z

Surinov, B. P., Isaeva, V. G., & Dukhova, N. N. (2005). Postradiation immunosuppressive and attractive volatile secretions: The "baystander effect" or allelopathy in groups of animals. Doklady Biological Sciences, 400(1-6), 28–30. doi:10.1007/s10630-005-0034-9

Surinov, B. P., Isaeva, V. G., & Karpova, N. A. (1997). Post-radiation communicative induction of blood and immunity disorders. Patologicheskaia Fiziologiia I Eksperimental'naia Terapiia, (3), 7–10.

Surinov, B. P., Isaeva, V. G., & Tokarev, O. I. (2001). [Allelopathic activity of volatile secretions in irradiated animals]. Radiatsionnaia Biologiia, Radioecologiia / Rossiĭskaia Akademiia Nauk, 41(6), 645–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11785304

Surinov, B. P., Karpova, N. A., Isaeva, V. G., & Kulish, I. S. (1998). [Communicative behavioral effects and disorders of immunity]. Zhurnal Vysshei Nervnoĭ Deiatelnosti Imeni I P Pavlova, 48(6), 1073–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9929917

Surinov, B. P., Karpova, N. A., Isaeva, V. G., & Kulish, I. S. (2000). Post-stress state and communicative alterations of immunity and blood. Patologicheskaia Fiziologiia I Eksperimental'naia Terapiia, (4), 9–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11247137

Tartier, L., Gilchrist, S., Burdak-Rothkamm, S., Folkard, M., & Prise, K. M. (2007). Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells. Cancer Research, 67(12), 5872–9. doi:10.1158/0008-5472.CAN-07-0188

The Canadian Nuclear FAQ - Section D: Safety and Liability. (n.d.). Retrieved July 31, 2015, from http://www.nuclearfaq.ca/cnf_sectionD.htm#x5

Tomita, M., & Maeda, M. (2014). Mechanisms and biological importance of photon-induced bystander responses: do they have an impact on low-dose radiation responses. J Radiat Res, 1–15. doi:10.1093/jrr/rru099

Tomita, M., Maeda, M., Maezawa, H., Usami, N., & Kobayashi, K. (2010). Bystander cell killing in normal human fibroblasts is induced by synchrotron X-ray microbeams. Radiation Research, 173(3), 380–5. doi:10.1667/RR1995.1

Trapp, J. V, Warrington, a P., Partridge, M., Philps, a, Leach, M. O., & Webb, S. (2004). 3D measurement of absolute radiation dose in grid therapy. Journal of Physics: Conference Series, 3, 280–283. doi:10.1088/1742-6596/3/1/047

Turesson, I., & Joiner, M. C. (1996). Clinical evidence of hypersensitivity to low doses in radiotherapy. Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology, 40(1), 1–3.

Vandenabeele, P., Galluzzi, L., Vanden Berghe, T., & Kroemer, G. (2010). Molecular mechanisms of necroptosis: an ordered cellular explosion. Nature Reviews. Molecular Cell Biology, 11(10), 700–714. doi:10.1038/nrm2970

Weidhaas, J. B., Eisenmann, D. M., Holub, J. M., & Nallur, S. V. (2006). A Caenorhabditis elegans tissue model of radiation-induced reproductive cell death. Proceedings of the National Academy of Sciences of the United States of America, 103(26), 9946–9951. doi:10.1073/pnas.0603791103

Weissenborn, U., & Streffer, C. (1989). Analysis of structural and numerical chromosomal aberrations at the first and second mitosis after X irradiation of two-cell mouse embryos. Radiation Research, 117(2), 214–220.

Willingham, M. C. (1999). Cytochemical methods for the detection of apoptosis. The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society, 47(9), 1101–1110. doi:10.1177/002215549904700901

Wouters, B. G., & Skarsgard, L. D. (1994). The response of a human tumor cell line to low radiation doses: evidence of enhanced sensitivity. Radiation Research, 138(1 Suppl), S76–80.

Wykes, S. M., Piasentin, E., Joiner, M. C., Wilson, G. D., & Marples, B. (2006). Low-dose hyper-radiosensitivity is not caused by a failure to recognize DNA double-strand breaks. Radiation Research, 165(5), 516–524. doi:10.1667/RR3553.1

Xu, S., Ding, N., Pei, H., Hu, W., Wei, W., Zhang, X., … Wang, J. (2015). MiR-21 is involved in radiation-induced bystander effects. RNA Biology, 11(March 2015), 1161–1170. doi:10.4161/rna.34380

Xue, L., Yu, D., Furusawa, Y., Cao, J., Okayasu, R., & Fan, S. (2009). ATM-Dependent Hyper-Radiosensitivity in Mammalian Cells Irradiated by Heavy Ions. International Journal of Radiation Oncology Biology Physics, 75(1), 235–243. doi:10.1016/j.ijrobp.2009.04.088

Yang, G., Mei, T., Yuan, H., Zhang, W., Chen, L., Xue, J., … Wang, Y. (2008). Bystander/abscopal effects induced in intact Arabidopsis seeds by low-energy heavy-ion radiation. Radiation Research, 170(3), 372–80. doi:10.1667/RR1324.1

Yang, Y., Crosbie, J. C., Paiva, P., Ibahim, M., Stevenson, A., & Rogers, P. a. W. (2014). In Vitro Study of Genes and Molecular Pathways Differentially Regulated by Synchrotron Microbeam Radiotherapy. Radiation Research, 182(6), 626–639. doi:10.1667/RR13778.1

Yuan, S. S. F., Chang, H. L., & Lee, E. Y. H. P. (2003). Ionizing radiation-induced Rad51 nuclear focus formation is cell cycle-regulated and defective in both ATM-/- and c-Abl-/- cells. Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis, 525(1-2), 85–92. doi:10.1016/S0027-5107(03)00009-5

Zeng, J., Harris, T. J., Lim, M., Drake, C. G., & Tran, P. T. (2013). Immune modulation and stereotactic radiation: improving local and abscopal responses. BioMed Research International, 2013, 658126. doi:10.1155/2013/658126

Zhong, N., Morris, G. M., Bacarian, T., Rosen, E. M., & Avraham Dilmanian, F. (2003). Response of Rat Skin to High-Dose Unidirectional X-Ray Microbeams: A Histological Study. Radiation Research, 160(2), 133–142. doi:10.1667/3033

Zhou, H., Ivanov, V. N., Gillespie, J., Geard, C. R., Amundson, S. a, Brenner, D. J., … Hei, T. K. (2005). Mechanism of radiation-induced bystander effect: role of the cyclooxygenase-2 signaling pathway. Proceedings of the National Academy of Sciences of the United States of America, 102(41), 14641–6. doi:10.1073/pnas.0505473102

Zhou, H., Ivanov, V. N., Lien, Y.-C., Davidson, M., & Hei, T. K. (2008). Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects. Cancer Research, 68(7), 2233–40. doi:10.1158/0008-5472.CAN-07-5278

Chapter 3

3 OPPOSITE RADIATION-INDUCED BYSTANDER EFFECTS IN THE C6 AND F98 RAT GLIOMAS: A COMPARISON BETWEEN SYNCHROTRON X-RAYS AND GAMMA IRRADIATION.

Cristian Fernandez-Palomo, Elisabeth Schültke, Elke Bräuer-Krisch, Colin Seymour, Carmel Mothersill

---

The first author and Mothersill designed this research project. The co-authors Schültke and Bräuer-Krisch participated during the Synchrotron irradiation process. The first author completed the cell preparation, gamma-irradiation, medium harvest & transfer, data collection and analysis. The first author wrote the manuscript, and Mothersill edited it.

The submission of the manuscript will be attempted to the International Journal of Synchrotron Radiation

3.1 ABSTRACT

Experimental radiotherapy treatments using Synchrotron radiation and involving animals harbouring the C6 and the F98 tumour have shown contradictory results. While tissue explants from rats harbouring the C6 glioma induced stronger bystander effects than the tumour-free animals, explants from rats harbouring the F98 tumour produced weaker bystander effects than their tumour-free irradiated controls. We wondered whether in-vitro radiation exposure would produce similar results in the two tumour cell lines. The work we present here investigates the response of the C6 and the F98 rat gliomas to low doses of gamma and Synchrotron radiation and their ability to induce bystander effects.

We examined the survival of both cell lines to doses between 0 to 3 Gy of gamma photons, with a focus on the low-dose hyper-radiosensitive region (<1 Gy). We also measured the cell capacity to induce bystander effects by performing clonogenic bioassays and calcium measurements using the conditioned medium from irradiated donors as the source of bystander signals. For the bystander experiments, both cell lines were exposed to Synchrotron or gamma radiation. Synchrotron radiation was given as an array of parallel rectangular microbeams with intermediate gaps, while gamma-photons were delivered as a broad beam.

Our results show that both cell lines behaved as hyper-radiosensitive at low doses and showed increased radioresistance as the dose increased. However, while the bystander signals from F98 glioma induced a decrease in survival in their reporters, bystander signals from C6 glioma produced an increase in survival in their reporters. We suspect that the differing p53 status of the cell lines may underlie this difference.

In conclusion, although both the C6 and the F98 rat gliomas produced bystander effects at low doses, the F98 cells promoted survival and the C6s induced cell death. These results suggest two different mechanisms for the low-dose effects in these rat glioma cell lines, which concords with our previous findings in animal models.

Keywords: F98, C6, Synchrotron radiation, Gamma-rays, radiation-induced bystander effects, hyper-radiosensitivity.

3.2 INTRODUCTION

Synchrotron microbeam radiation is currently used in the development of innovative radiotherapy treatments for brain cancer. The high flux of Synchrotron–generated X-rays is spatially fractionated by the insertion of a multislit collimator that generates a microbeam array of very large doses with sharp edges. Currently, there are two modalities being investigated by our team at McMaster University and the European Synchrotron Radiation Facility (ESRF). The first is Microbeam Radiation Therapy (MRT), which is aimed at treating primary brain tumours. MRT is composed by quasi-parallel rectangular beamlets of 25 to 50 μm width with intermediate gaps of 200 to 400 μm. The second technique is called Pencilbeam (PB) Therapy, which is aimed at treating brain metastasis. The PB array is formed by pencil-like square beams of 50 by 50 μm spaced by gaps of 200 to 400 μm. Both treatments are still in the pre-clinical stage, but MRT, which has been under study for a longer time, is already showing great advantages compared to broad beam irradiation.

An accumulation of evidence suggests that single fractions of MRT produce a higher therapeutic index than broad beam irradiation. This has been shown in animal models harbouring the aggressive 9L gliosarcoma (Bouchet et al., 2013; Dilmanian et al., 2002; Laissue et al., 1998), F98 tumours (Biston et al., 2004; Schültke et al., 2008), the murine mammary carcinoma transplanted

subcutaneously (Crosbie et al., 2010) and the aggressive and radioresistant murine squamous carcinoma (Miura et al., 2006). The efficacy of MRT has been attributed to the effects of the high valley doses that are reinforced by the peak doses (Bräuer-Krisch et al., 2010). Peak doses are those deposited in the tissue by each beamlet while the Valley doses expose the tissue between the microbeam paths and are formed by the scattered photons generated from the peak doses. The combination of peak and valley doses seem to be sufficient to avoid damaging effects in the normal tissue while tumours show high susceptibility due to their tortuous microvasculature (Bräuer-Krisch et al., 2010; Slatkin, Spanne, Dilmanian, & Sandborg, 1992). In situ studies by our group looking at DNA damage have revealed that the delivered microbeam array correlated very well with the distribution of the dose across the brain tissue (Fernandez-Palomo, Mothersill, et al., 2015). The results also suggested that tissue exposed to peak doses of 350 Gy may have released bystander signals that increased the DNA damage towards the valley areas.

Radiation-induced bystander effects are radiation-like responses observed in unirradiated cells or tissues after they receive signals from irradiated targets (Carmel Mothersill & Seymour, 1997; Nagasawa & Little, 1992). Experiments have shown that the signals are transported from neighbouring cells through Gap Junction Intercellular communication (E. I. Azzam, de Toledo, Gooding, & Little, 1998) or by exposing the unirradiated cells to conditioned media that originated

from irradiated cells (Carmel Mothersill & Seymour, 1997, 1998). Scientists investigating the bystander phenomenon have found the involvement of several mediators and intracellular pathways. Some of there are: reactive oxygen and nitrogen species (F M Lyng, Seymour, & Mothersill, 2000; Narayanan, Goodwin, & Lehnert, 1997; Shao, Stewart, Folkard, Michael, & Prise, 2003), tumour necrosis factor-α (Iyer & Lehnert, 2000), transforming growth factor-β1 (Iyer & Lehnert, 2000), serotonin (Fazzari, Mersov, Smith, Seymour, & Mothersill, 2012; Saroya, Smith, Seymour, & Mothersill, 2009), mitochondrial disruptions (F.M. Lyng, Seymour, & Mothersill, 2001; Murphy, Nugent, Seymour, & Mothersill, 2005; Zhou, Ivanov, Lien, Davidson, & Hei, 2008), Ca^{2+} fluxes (Fiona M Lyng, Seymour, & Mothersill, 2002), mitogen-activated protein kinases (F. M. Lyng, Maguire, McClean, Seymour, & Mothersill, 2006), p53, $p21^{Waf1}$, p34 and MDM2 (E. Azzam, de Toledo, & Little, 2004; F. M. Lyng et al., 2006; Carmel Mothersill & Seymour, 2004). Recently, two candidates for bystander triggering factors have been identified: UV-photon emission from irradiated cells (Ahmad et al., 2013; Le, McNeill, Seymour, Rainbow, & Mothersill, 2014) and long-non-coding RNA (O'Leary et al., 2015), which are also found is exosomes (Al-Mayah, Irons, Pink, Carter, & Kadhim, 2012; Jella et al., 2014).

Bystander effects are relevant to Synchrotron studies because the tissue exposed to valley doses is susceptible to receiving signals from the tissue exposed to the peak doses. Dilmanian et al. suggested that bystander signals

could be involved in the enhanced tissue restoration observed after exposing aortic endothelial cells to MRT. Research by our group looked at whether MRT-irradiated tissue would produce bystander effects on the reporter HaCat cell line (Carmel Mothersill et al., 2014). The results revealed that abscopal and bystander effects were induced. Moreover, the same investigation showed that bystander effects could be communicated from irradiated to unirradiated animals. We also looked at whether MRT would modulate the bystander effects differently than broad beam (Fernandez-Palomo et al., 2013). Although bystander effect occurred, no significant differences were found between the two modalities when MRT was delivered as an array of 25 μm with gaps of 200 μm. However, the same research revealed that the Wistar rats that were harbouring the C6 glioma in their brains induced amplified bystander effects across all doses and radiation modality.

Given the previous findings, our group further investigated the occurrence of bystander effects between animals but using the F98 glioma as tumour model and the Fisher rats as new host (Fernandez-Palomo, Bräuer-Krisch, et al., 2015). In these experiments, it was revealed that bystander effects were also communicated from irradiated to unirradiated animals. However, the presence of the F98 tumour blocked/weakened the bystander effects compared to the tumour-free control rats. These experiments together with the previous finding made us wonder what type of respond the C6 and F98 glioma cell lines would

have after in vitro exposures to ionising radiation. In an attempt to answer that question, we developed the study presented in this paper. The approach we used was to irradiate both cell lines with low doses, which are relevant for bystander effects and hyper-radiosensitive cells, and we performed medium transfers to evaluate clonogenic survival as means of bystander response.

3.3 MATERIALS AND METHODS

3.3.1 Cell Lines

The tumour cell lines selected for these experiments were the C6 and F98 rat gliomas. These lines have been used by our group and others in multiple animals studies involving Synchrotron radiation because they share a broad range of characteristics with the glioblastoma multiforme (Barth & Kaur, 2009; Desmarais et al., 2012, 2015; Farrell, Stewart, & Del Maestro, 1987; Gil, Sarun, Biete, Prezado, & Sabés, 2011). Both cell lines proliferate rapidly once injected into the brain and for solid tumours (Barth & Kaur, 2009). However, F98s have a mutant p53 status (Schlegel et al., 1999; Senatus et al., 2006) while C6 are reported to be wild-type p53 (Asai et al., 1994; Strigari et al., 2014; Yang et al., 2014). Both cell lines originated from different sources. The F98s were developed in Fisher rats after exposing foetuses to N-ethyl-N-nitrosourea (Koestner, Swenberg, & Wechsler, 1971) while the C6s were produced in random-bred

Wistar-Furth rats by exposure to the same chemical compound (Benda, Lightbody, Sato, Levine, & Sweet, 1968). Another difference between the cell lines is their immunogenicity. The F98 glioma is reported to be weakly immunogenic (Tzeng, Barth, Orosz, & James, 1991), while C6s show high immunogenicity (Barth, 1998). These difference made us wonder how the cells would behave if we studied them in vitro after radiation.

Non-tumorigenic HaCaTs cells were also used during our experiments. HaCaTs are human epithelial cells, which are regularly used by our group at McMaster University because of their reliable response to bystander signals (Carmel Mothersill & Seymour, 1997). The cell line became spontaneously immortal in culture but derived originally from the normal skin surrounding a melanoma (Boukamp et al., 1999). Reports indicate that HaCaTs display three p53 point mutations; one in codon 179 of exon 5 of one allele, and two mutations in codons 281 & 282 of exon 8 on the other allele (Lehman et al., 1993). Despite these mutations, HaCaTs remain functional with respect to apoptosis (Henseleit et al., 1997) and behave like wild-type p53 regarding bystander effects (Ryan, Seymour, Joiner, & Mothersill, 2009).

For these experiments, both tumour cell lines were obtained from ATCC, while the HaCaTs were originally derived by Dr. Petra Boukamp in DKFZ, Hidelberg, Germany (Lehman et al., 1993) and given as an in-kind gift to the

Dublin Institute of Technology in Ireland (Dr. Mothersill's Laboratory) in 1996. All cell lines were individually maintained in 75 cm^2 flasks containing 25 ml of Dulbecco's Modified Eagle Medium F12-formula (Gibco, Canada) +HEPEs, +15mM L-glutamine, supplemented with 10% of foetal bovine serum (Gibco, Canada) and 1% penicillin/streptomycin (Gibco, Canada).

3.3.2 Cell culture technique:

Flasks housing 90% confluent cells were selected for the experiments and a solution 1:1 (v/v) 0.05% Trypsin in Dulbecco's Phosphate-Buffered Solution was used to detach the cells. The cell suspension was then centrifuged at 125g for 5 minutes. The supernatant was discarded, and the cells were re-suspended in 1 ml of fresh medium. Cells were counted using an automatic cell counter (Beckman Coulter, Fullerton, CA). F98 and C6 gliomas were seeded in three different groups according to their purpose. Group 1 was seeded at a density of 250,000 cells in 25 cm^2 flasks containing 5 ml of the previously described culture medium. This group was irradiated and served as the donor of irradiated cell conditioned medium (ICCM), which provides the radiation-induced bystander signals. The response of cells from groups 2 and 3 was studied using the clonogenic technique developed by Puck and Markus (Puck & Marcus, 1956). Group 2 was formed by sets of 25 cm^2 flasks seeded with clonogenic densities 500 of C6 or F98 cells, which functioned as recipients of the ICCM harvested

from the donors. Group 3 was also formed by sets of flasks seeded as in Group 2, but these were irradiated following the same doses as their donor counterparts. HaCat cells, however, were only seeded as reporters (Group 2). All flasks were placed in a 37° Celsius, 5% CO_2 in air, 95% humidity incubator.

3.3.3 Irradiation

All flasks were irradiated six hours after seeding according to well-established bystander protocols published by Mothersill and Seymour (Carmel Mothersill & Seymour, 1998). The C6 and F98 cells irradiated with gamma rays were exposed to 12 doses from 0 to 3 Gy from a caesium-137 source at McMaster University. Flasks were placed 27 cm from the radiation source to achieve a dose rate of 0.289 Gy/min and the time of exposure was controlled to deliver the appropriate doses. All flasks were kept in an incubator after irradiation. Donor flasks were incubated for 90 minutes prior to medium harvest while reporter flasks remained undisturbed for about 9-11 days to allow for colony formation.

Irradiation with Synchrotron-generated X-rays was performed in the biomedical beamline of the ESRF in Grenoble, France. Only donor cells were irradiated, and their medium harvested and transported to McMaster University. For the irradiation C6 and F98 donor cells were exposed to the same doses as

above. The only dose that could not be delivered, because it was too low for the beamline to deliver it, was 0.05 Gy. The lowest dose given was 0.1 Gy, and a new dose of 0.35 Gy was added to replace the one that we were not able to deliver. Synchrotron radiation was given as an array of parallel rectangular microbeams of 50 μm width with an intermediate gap of 400 μm. The flasks were single-irradiated by standing vertically with their cell-surface area facing the incident beam. Since the biomedical beamline does not have a biosafety cabinet, discarding and replacing the culture medium before and after irradiation was not allowed for biosafety reasons. Therefore, the culture medium remained at the bottom of the standing flask. However, medium and sham irradiated controls were included. After irradiation, all cells were transported back to the biosafety level-2 laboratory and placed in the incubator until medium harvest.

3.3.4 Medium Transfer technique and bystander protocol

All medium transfers were performed six hours after reporter cells were seeded according to the bystander protocols developed by Mothersill and Seymour (Carmel Mothersill & Seymour, 1998).

The medium from the gamma-irradiated donors was harvested 1.5 hours after irradiation in a biosafety cabinet. The medium was directly poured into a 5 ml syringe, the plunger of which had been previously removed and then replaced. The medium was then filtered using a 0.22 μm filter with HT Turffryn Membrane

(Pall Life Sciences) and transferred into the reporter flasks containing 500 un-irradiated C6 or F98 cells. It is important to highlight that we did not perform a mix and match protocol. Instead, the media from donor C6 cells were transferred onto reporter C6s while the same technique was applied for F98 cells. The empty donors were discarded, and the reporter flasks were placed back into an incubator and left undisturbed for nine to eleven days in order to allow for colony formation.

The medium from the Synchrotron-irradiated donors had to be harvested three to four hours after irradiation. The delayed harvesting time compared to the gamma-irradiated groups was a consequence of an unanticipated technical limitation of the synchrotron technique, which only allowed us to irradiate single flasks instead of a set of them. This situation tripled the irradiation session, hence delaying the harvesting of the medium. Nevertheless, the collection process followed similar steps as the protocol mentioned above. Briefly, the medium was collected from the donors, filtered, transferred into to 5ml tubes, and stored in a +4°C fridge until transported to McMaster University. Upon arrival, the ICCM was transferred onto reporter F98 or C6 cells seeded six hours in advance. Flasks were then placed in the incubator and allowed to form colonies over nine to eleven days.

All flasks that received ICCM from synchrotron-irradiated F98 cells showed no living cells a few days after the medium transfer. Interestingly, there was no sign of bacterial contamination, and the local plating efficiency and medium transfer controls were normal. For this reason, we decided to re-harvest, re-filter, and transfer the media into flasks containing 300 cells of our well-established HaCaT reporter cell line. Although approximately 3 ml of medium were transferred, the cells grew well this time. Thus, the colonies were stained after ten days using a solution of 1:4 (v/v) of Carbol Fuchsin (Ricca Chemical Company) in water. Survivors were scored when colonies showed fifty or more cells.

3.3.5 Ratiometric Calcium measurements

The protocol used for these experiments has been used previously by our group (Fernandez-Palomo, Bräuer-Krisch, et al., 2015; C. Mothersill et al., 2014) but is an adaptation of the one developed by Dr. Fiona Lyng, DIT, Dublin, Ireland. Briefly, 100,000 F98 or C6 cells were seeded in glass bottom dishes (MatTek Corporation) containing 2 ml of the culture medium previously described. Dishes were placed in an incubator at 37° Celsius, 5% CO2 in air for twenty-four hours. Cells were then gently washed twice with Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (Cat#: 14025-092, Gibco, Oakville, Canada) supplemented with 25 mM of HEPES (Gibco, Oakville, Canada). HBSS was discarded and the cells were loaded with 200 µl of 4.6 µM of Fura-2/AM (Sigma-

Aldrich, Milwaukee, USA) for forty-five minutes at room temperature in the dark. In our experience, incubation at room temperature allows for better distribution of the dye in the cytoplasm while significantly reducing compartmentalization within the cellular organelles. The cells were washed three more times, then 300 µl of buffer were added to the dish and the cells were left undisturbed in the dark for fifteen minutes to allow for further Fura2/AM cleavage. The cells were then observed with an x40 oil objective on an Olympus inverted fluorescent microscope (Olympus Canada, Richmond Hill, Canada). Ten cells were selected and after sixty seconds of acquisition 100 µl of ICCM was added and the calcium fluxes were tracked over time. Fura2-free and fura2-calcium bound emit light at 510 nm when they are excited at 380 nm and 340 nm, respectively. The ratio of emissions between those wavelengths is correlated with the flux of calcium through the cellular membrane. Once the calcium values are obtained, the ratio of each cell is analyzed and the area under the curve is calculated. The value obtained for the maximum peak is selected and then plotted as a function of the dose.

3.3.6 Data Analysis

The survival data was obtained after the experiments were done in triplicate and repeated three times. However, the irradiation involving Synchrotron x-rays was done only once, but it included 9 flasks per treatment group. The

values of the figures are presented as mean survival fraction with the standard error of the mean (SEM). The calcium data were obtained after selecting ten cells per dish and the measurements were repeated three times. The data were analyzed and outliers – cells showing no calcium fluxes – were removed before proceeding to calculate the area under the curve. All figures were plotted using the GraphPad Prism software “Version 6.0f” for Mac. Calcium data was fitted to the IR-Model explained below.

Clonogenic survival data was fitted using the linear-quadratic (LQ) and the induced-repair (IR) equations. The algorithm used for the iterations was the Marquardt and Levenberg method under the nonlinear least-square fit regression of the GraphPad Prism software “Version 6.0f” for Mac.

The LQ model describes the cell survival (S) as a function of the dose (D), where (α) is the slope of the linear component of the curve and (β) is the slope of the quadratic component:

Equation 3.1LQ Model

$$S = \exp(-\alpha D - \beta D^2)$$

We selected the IR equation described by Lambin et al. (Lambin, Marples, Fertil, Malaise, & Joiner, 1993). (**αr)** is (**α)** extrapolated from the LQ model and

therefore represents the slope of the resistant part of the linear component. (**αs)** is a new (**α)** derived from the response at very low doses and represents the slope of the sensitive part of the linear component.

Equation 3.2 IR Model

$$S = exp\left(-\alpha r\left(1 + \left(\frac{\alpha s}{\alpha r} - 1\right)e^{-D/D_c}\right)D - \beta D^2\right)$$

3.4 RESULTS

3.4.1 Response of the directly irradiated C6 and F98 gliomas

Figure 3.1 shows the clonogenic survival of the gamma-irradiated C6 and F98 glioma cell lines. The LQ and IR equations were fitted to the data of both cell lines. The C6 glioma (Fig 1A) shows a hyper-radiosensitive (HRS) response at very low doses that is well demonstrated by the fit of the IR-Model (r^2 = 0.84) while the LQ-Model displays slightly lower fit (r^2 = 0.82). The F98 cells (Fig 1B) also displayed HRS at low doses. The data revealed that the IR model likewise exhibited a better fit (r^2 = 0.76) than the LQ-Model (r^2 = 0.75). Upon visual comparison of both survival fractions, the C6 glioma displays a wider shoulder than the F98 cells.

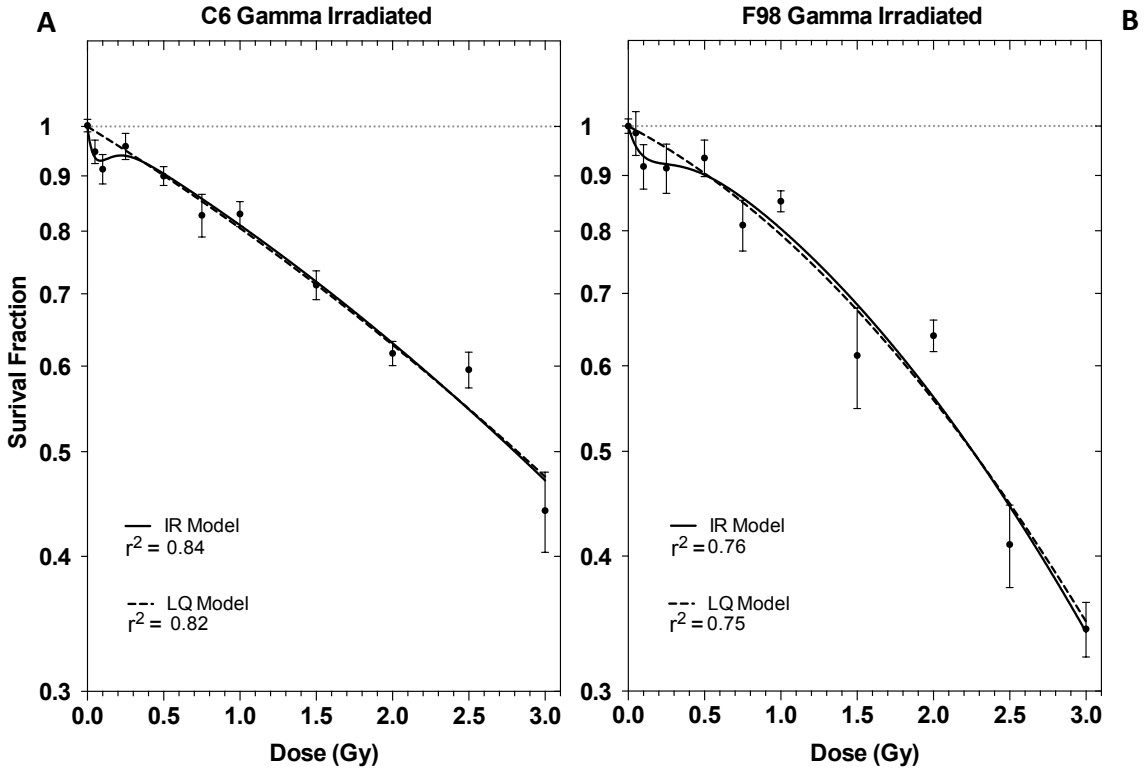

Figure 3.1 Clonogenic survivals of C6 and F98 glioma after gamma irradiation. The C6 and F98 cell lines were exposed to gamma photos from a caesium-137 source. A) Shows the response of the C6 gliomas while B) shows the response of the F98 glioma. The IR-Model and LQ-Model were fitted to the survival data. Error bars show SEM.

Table 3.1 shows the best-fit values for the IR and LQ models. The α/β ratio is a numerical value that indicates the response to radiation. What is clear is the different α/β ratios between both cell lines. The C6 glioma exhibited a high α/β ratio = 12.45, which is related to a radioresistance response and correlates with the wide shoulder previously described. The F98 glioma, however, has a much lower α/β ratio of 2.84, which suggests a more sensitive response to radiation.

The αs/αr factor measures the magnitude of the difference between the sensitive and the resistant α components derived from the LQ Model. In other words, it indicates the strength of the transition from HRS to increased radioresistance (IRR). Our models indicate that the C6 glioma displays a more evident HRS/IRR transition (αs/αr = 13.73) than the F98 glioma (αs/αr = 7.94). Our best-fit values also indicate that the dose at which HRS shifts to IRR is lower in the C6 cells (Dc = 0.066 Gy) than in the F98 cells (Dc = 0.127 Gy). The slopes for the sensitive (αs), resistant (αr) and quadratic (β) components are presented for completeness.

Table 3.1 Best-fit values of the LQ and IR Models for the C6 and F98 cells irradiated with gamma photons from a caesium-137 source. 95% confidence limits are shown in parentheses.

Parameters	C6	F98
LQ Model		
α/β	**12.45**	**2.84**
α	0.20 (0.15 to 0.26)	0.17 (0.082 to 0.26)
β	0.02 (-0.008 to 0.041)	0.06 (0.017 to 0.10)
IR Model		
αs/αr	**13.73**	**7.94**
αr	0.19 (0.14 to 0.24)	0.15 (0.044 to 0.25)
αs	2.60 (-1.26 to 6.46)	1.17 (-0.97 to 3.32)
Dc	0.07 (-0.014 to 0.15)	0.13 (-0.08 to 0.33)
β	-0.11 (-0.27 to 0.049)	-0.48 (-1.14 to 0.18)

3.4.2 Response of bystander C6 and F98 glioma

Figure 3.2 shows the clonogenic survival of the reporter C6 and F98 gliomas, which were grown in the ICCM from donor F98 or C6 cells exposed to gamma photons from a caesium-137 source. The figure also shows bystander C6 and bystander HaCaT cells grown in ICCM from Synchrotron-irradiated C6 or F98 gliomas. The bystander C6 cells that were grown in the ICCM from donor C6 that were exposed to gamma photons (Fig 3.2A) display an increase in survival at the same doses that induced HRS in the directly irradiated cells (Fig 3.1A). The best-fit parameters in Table 2 indicate both a maximum survival effect achieved at 0.13 Gy and a strong shift between the influence of HRS and IRR ($\alpha s/\alpha r$ = 61.21).

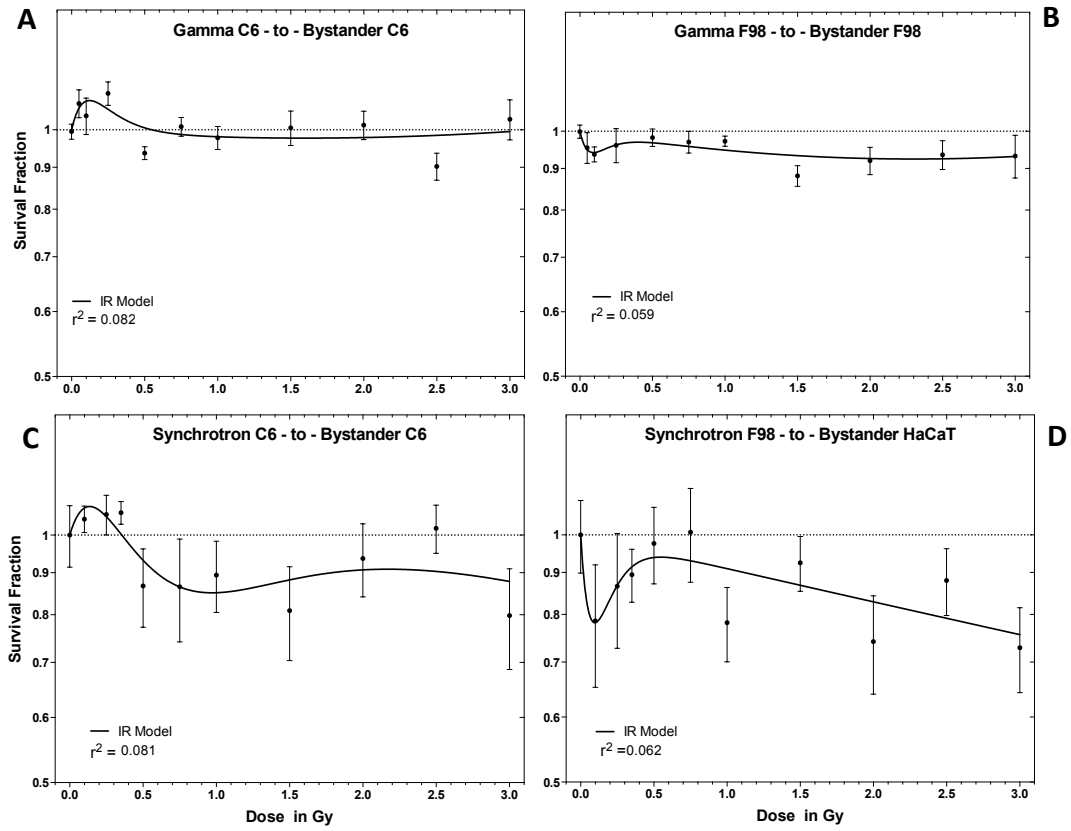

Figure 3.2 Clonogenic survival of bystander C6, F98 and HaCaT cells grown in ICCM from Gamma-irradiated or Synchrotron-irradiated C6 or F98 glioma.
The ICCM originated from donor flasks containing 250,000 F98 or C6 cells, which were irradiated with either Gamma photons from a caesium-137 source at McMaster University or Synchrotron photons at the ESRF. A) Shows the survival of bystander C6 cells grown in ICCM from donor C6s exposed to Gamma photons. B) Shows bystander F98 cells grown in ICCM from donor F98s exposed to Gamma photons. C) Shows bystander C6 cells grown in ICCM from donor C6 exposed to Synchrotron X-raysa and D) shows bystander HaCaT cells grown in ICCM from F98 donors irradiated with Synchrotron X-rays. The IR-Model was used to fit the data. Error bars show SEM.

Table 3.2 Best-fit values of the IR Model for the C6 and F98 grown in the ICCM from donor cells. 95% confidence limits are shown in parentheses.

IR Parameters	Gamma C6 to bystander C6	Gamma F98 to bystander F98	Synchrotron C6 to bystander C6	Synchrotron F98 to bystander HaCaT
αs/αr	**-61.21**	**26.72**	**253.08**	**73.68**
αr	0.03 (-0.04 to 0.10)	0.07 (0.01 to 0.13)	-0.01 (-0.01 to 0.003)	0.10 (-0.09 to 0.28)
αs	-1.80 (-3.82 to 0.22)	1.82 (-1.33 to 4.97)	-1.40 (-3.7 to 0.88)	7.00 (-7.3 to 21)
Dc	0.13 (0.01 to 0.26)	0.09 (-0.04 to 0.21)	0.36 (0.12 to 0.59)	0.09 (-0.01 to 0.19)
β	0.32 (0.04 to 0.59)	0.22 (0.05 to 0.38)	2.81 (1.3 to 4.3)	0.01 (-0.78 to 0.79)

The bystander F98 glioma, however, exhibited a decrease in clonogenic survival after being grown in the ICCM from donor F98 cells that were irradiated at HRS doses (Fig 1B). The clonogenic survival partially recovered at 0.5 Gy but remained below control levels as the dose increased. The best-fit parameters (Table 3.2) estimate that the maximum cell-kill induced by HRS in F98 glioma is achieved at ~0.09 Gy while the shift from HRS to IRR is almost half the value of that for the C6 glioma (αs/αr = 26.72).

The bystander C6 glioma grown in ICCM from Synchrotron-irradiated C6 cells (Fig 3.2C) displayed a marked increased in survival at low doses with respect to their controls – a response that was similar to their counterparts (Fig 3.2A). The maximum HRS influence on the C6 gliomas was estimated at Valley doses of 0.36 Gy (Table 3.2). The best-fit parameters indicate that the transition between HRS and IRR doses or the influence of them is very high under these

conditions (αs/αr = 253.08). It was also observed that ICCM from C6 cells exposed to Synchrotron valley doses of around and above 0.5 Gy induced a constant decrease in survival until at least 3 Gy.

Unexpectedly, bystander F98 gliomas exposed to ICCM from Synchrotron-irradiated F98 cells did not survive. Visual examination under the inverted microscope indicated that cells underwent up to two divisions before they showed signs of apoptosis. In an attempt to salvage the experiment, the ICCM was re-harvested, re-filtered, and transferred onto HaCaT cells seeded in clonogenic densities six hours in advance. The bystander HaCaT cells grew well and showed a decrease in survival with respect to their controls (Fig 3.2D). The bystander response in HaCaTs was similar but more intense than the one observed on reporter F98 grown in ICCM from gamma-irradiated F98 (Fig 3.2B). The best-fit parameters estimate that the maximum bystander decrease in survival induced by HRS doses is achieved at ~0.1 Gy, while the transition between HRS/IRR denotes a αs/αr factor of 73.68.

Figure 3.3 shows calcium flux through the cellular membrane of living bystander C6 and F98 gliomas. The agent used to trigger the flux was the ICCM from the respective donors exposed to gamma photons. The bystander C6 cells displayed a weakened calcium flux when the ICCM originated from donor C6 exposed to HRS gamma doses. The best-fit parameters from Table 3 indicate that the dose at which the calcium flux was lower in the bystander C6 glioma corresponds to 0.07 Gy. However, the F98 glioma exhibited an increase in

calcium flux when the F98 donor cells producing the ICCM were exposed to HRS gamma doses. The best-fit values estimate that the peak influx of calcium is achieved at 0.2 Gy.

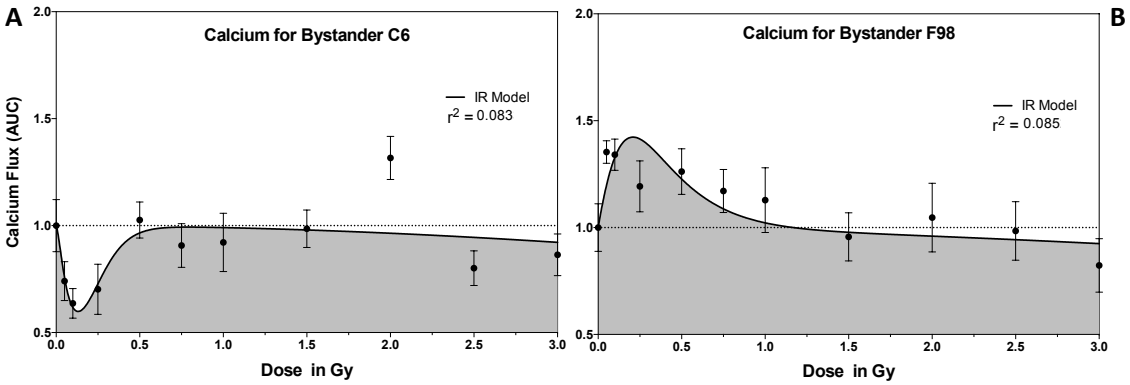

Figure 3.3 Calcium flux of bystander C6 and F98 glioma induced by ICCM from C6 and F98 respectivelly.
ICCM from 250,000 C6 or F98 donor cells was used to trigger calcium fluxes through the cellular membrane of reporter C6 or F98 cells. The caclium fluxes are displayed as a function of area under the curve. A) Shows the influx of calcium of the bystander C6 cells while B) shows the influx of calcium of the F98 glioma. Error bars show SEM.

Table 3.3 Best-fit values of the IR Model for the calcium data from C6 and F98. 95% confidence limits are shown in parentheses.

IR Parameters	C6 exposed to ICCM from Gamma C6	F98 exposed to ICCM from Gamma F98
αs/αr	**20380**	**-446**
αr	0.0001 (-0.002 to 0.002)	0.0102 (-0.26 to 0.28)
αs	2.31 (-36.14 to 40.75)	-4.56 (-7.83 to -1.29)
Dc	0.07 (0.009 to 0.128)	0.19 (-0.52 to 0.90)
β	-79.50 (-1697 to 1538)	-0.51 (-24.27 to 23.25)

3.5 DISCUSSION

Several experiments performed by our group show that synchrotron radiation induces abscopal and bystander effects in mice and rats harbouring the C6 and the F98 gliomas. Published data show that the presence of these cell lines in the brains of the animals seems to induce opposite bystander effects in the reporter cells. The aim of work we present here is to deepen those findings by investigating how the F98 and C6 gliomas respond after in vitro irradiation of Synchrotron X-rays or gamma photons.

Our data indicate that the C6 glioma is more radioresistant than the F98 cells to gamma irradiation in vitro. This is suggested by the different α/β ratios, which are 12.45 for the C6 cells and 2.84 for the F98 cells. The α/β ratio has

been largely associated with acute or late tissue response to radiation. A larger α/β ratio indicates a predominance of the linear component of the survival curve and thus a lower killing effect by the dose (M. V Williams, Denekamp, & Fowler, 1985), which is a consequence of the cellular ability to better deal with radiation damage. To our knowledge, this is the first in vitro observation that these two widely-used rat glioma cell lines have different radioresponses. The most likely explanation of the dissimilar radiosensitivity is the different p53 statuses of both cell lines. Published literature shows that the C6 glioma has wild-type p53 status (Asai et al., 1994; Strigari et al., 2014; Yang et al., 2014) while the F98 glioma is described has having a point mutation localized at the amino acid 54 (Gly-to-Ala) (Schlegel et al., 1999; Senatus et al., 2006). Our findings are in accordance with data published by Williams et al. (J. R. Williams et al., 2008) where they compared the radiosensitivity of twenty-seven human tumour cell lines after exposure to caesium-137. Their results indicated that the wild-type p53 cells became more radioresistant while the mutant-p53 cells became more radiosensitive. Data from Berggvist et al. (Bergqvist et al., 2003) also supports our findings by showing that mutations in the exon 7 of the p53 gene were associated with increased radiosensitivity in human lung cancer cells. Okaichi et al. (Okaichi et al., 2008) performed a detailed analysis of the mutated p53 codons and their involvement in radiation sensitivity. Those experiments revealed that cells with mutations in codons 123, 195, 238 and 242 were radiosensitive. However, they also established that mutations in codons 175, 244, 245, 273 and

282 were present in radioresistant cells, suggesting that the location of the point mutation in the p53 gene is an indication of cellular radioresponse rather than hypersensitivity. Also, the influence of two or more point mutations was not studied and remains to be elucidated. Nevertheless, Okaichi et al.'s research is compatible with our findings and allows for further investigations to clarify the involvement of the mutated p53 gene in the radiosensitivity of the F98 glioma.

Focusing on the effects of low doses of gamma irradiation, both glioma cell lines exhibited hyper-radiosensitivity and increased radioresistance. These are two inter-related phenomena where the first represents the excessive sensitivity to very low doses of ionising radiation (below 0.5 Gy) and the second describes the radioresistant behaviour of the same cell line as the dose increases from 0.5 to near 1 Gy. An accumulation of research suggests that the most likely causes of HRS are defective DNA double-strand breaks (DSBs) repair pathways (Yuan, Chang, & Lee, 2003), rather than failure to recognize DSBs (Wykes, Piasentin, Joiner, Wilson, & Marples, 2006). Defective cell cycle regulation has also been suggested (Martin, Marples, Lynch, Hollywood, & Marignol, 2014). It has been reported that evasion of the G2/M checkpoint (Collis et al., 2004; Fernet, Mégnin-Chanet, Hall, & Favaudon, 2010; Krueger, Wilson, Piasentin, Joiner, & Marples, 2010; Marples, Wouters, & Joiner, 2003; Marples, 2004; Xue et al., 2009) and HRS are more intense during G2 (Short, Woodcock, Marples, & Joiner, 2003). Although, the occurrence of HRS was considered in our hypothesis, it is

interesting to note that the HRS/IRR transition happened at a slightly lower dose than commonly expected. Published data show that HRS switches to IRR at doses between 0.16 to 0.2 Gy in the T98G glioma cell line (Schoenherr et al., 2013; Short et al., 2003), at 0.19 Gy in the U87MG cells (Schoenherr et al., 2013), and at 0.20 Gy in the U138 (Schoenherr et al., 2013) cell line. The best-fit values we present here estimate that the F98 glioma switches at 0.13 Gy while the C6 does so at 0.06 Gy. Accordingly, this paper also appears to be the first study reporting HRS in these two rat glioma cell lines.

Donor F98 cells exposed to gamma photons induced bystander effects on the reporter F98, which was corroborated by the influx of calcium. The incidence of bystander effects on cells that display large HRS is a recent discovery. It was previously thought that bystander effects and HRS were mutually exclusive (Carmel Mothersill, Seymour, & Joiner, 2002; Ryan et al., 2009). However, work developed in BJ human fibroblasts by Nuta & Darroudi (Nuta & Darroudi, 2008) suggested that those cells would release radiation-induced bystander factors after gamma and X-ray exposure that may lead to cell inactivation and thus HRS. Published data by Heuskin et al. (Heuskin, Wéra, Riquier, Michiels, & Lucas, 2013) revealed that the HRS-positive A549 lung carcinoma was successful at inducing bystander effects after exposure to alpha particles. In these experiments, the researchers observed an enhanced cell-kill after HRS doses, which was abolished by using Lindane, an inhibitor of the GJIC, demonstrating

that bystander signals were responsible for the enhanced cell-kill observed. The work we present here further supports those findings, and it provides new means of confirming the occurrence of bystander effects by measuring the flux of calcium ions through the cellular membrane. We observed a direct relationship between the cell-kill generated at HRS doses in the F98 cell lines and the influx of calcium ions on the reported F98 cells, which was triggered by ICCM from F98 glioma cells exposed to HRS doses. The link between bystander effects and calcium fluxes is supported by a vast literature from several scientific teams (F. M. Lyng et al., 2006; Fiona M Lyng et al., 2002; Carmel Mothersill et al., 2005; Shao, Lyng, Folkard, & Prise, 2006) and from our laboratory (Fazzari et al., 2012; Fernandez-Palomo, Bräuer-Krisch, et al., 2015; Saroya et al., 2009; Singh et al., 2011). Therefore, transient calcium fluxes provide further evidence to support the hypothesis that bystander signals may indeed participate in the HRS response.

Our work also reported that donor C6 cells exposed to gamma rays induced an increase in survival on the reporter C6 glioma, which was correlated with a decrease in calcium fluxes. The stimulation of survival occurred only at HRS doses and then decreased to control levels as IRR and higher doses took place. Similarly, work developed by Shao et al. (Shao, Aoki, & Furusawa, 2003) involving a cancer cell line revealed that the survival of the Neoplastic Human Salivary Gland (NSG) increased when it was co-cultured with irradiated NGSs. Iyer and Lehnert (Iyer & Lehnert, 2000) have also reported cell proliferation due

to bystander effects. In their experiments, they observed increased survival in human lung fibroblast that had been exposed to supernatants from alpha-irradiated cells. Although the reasons explaining the radiation-induced increase in survival remains to be elucidated, the work we present here provides the measurement of calcium fluxes as a new element in the signalling process. Interestingly, the influx of calcium ions on the reporter C6 was reduced by the ICCM from donor C6 cells (Fig 3.3A). Moreover, since the flux of calcium is lower than in the control group, it is probable that the signals contained in the ICCM had blocked or prevented the normal flux of calcium. More research is needed to fully support that hypothesis.

Synchrotron-irradiated donor cells induced similar survival patterns on their reporter cells than their gamma-irradiated counterparts. Moreover, the bystander effects caused by synchrotron-irradiated donor cells were much more intense on their reporter, especially at higher doses, compared with the results from the gamma exposures. For instance, C6 donors exposed to HRS gamma doses only induced cell proliferation (Fig 3.2A). However Synchrotron radiation induced both cell survival at HRS doses and then cell-kill when the origin of the ICCM shifted from HRS-irradiated to IRR-irradiated C6 cells (Fig 3.2C). Similarly, F98 cells exposed to gamma photons induced a decrease in survival across all doses but with a focus on the HRS region (Fig 3.2B). In comparison, Synchrotron radiation induced larger cell-kill on their reporter cells in both the HRS dose levels

and at higher doses. Whether the amplified effect is entirely due to Synchrotron radiation or a consequence of using reporter HaCaT cells instead of F98, we do not know. The most likely explanation of the enhanced effect observed after Synchrotron radiation may lie in the microbeam configuration. Previous studies by our group looked at the different induction of bystander effects in vivo after exposure to Synchrotron microbeam and broad beam (Fernandez-Palomo et al., 2013). Those results showed no significant differences between the two modalities. However, the microbeam array in those experiments was different from the one used in the present study and that may be an important difference. In the same in vivo experiments we used microbeams of 25 μm width and intermediate gaps of 200 μm while the in vitro work we present here was done with microbeams of 50 μm width and a center to center distance of 400 μm. The latter configuration involves lower valley doses and also a much larger volume of tissue exposed to them, which could explain the amplification of the bystander effects. Further research should study the role of different peak-to-valley dose ratios during bystander effects.

A clear finding of our experiments was that the C6 and F98 donor cells induced opposite bystander effects at HRS doses either after exposure to gamma or Synchrotron X-rays. The reason behind these different responses is currently unknown. However, we were expecting this type of response given previously published and unpublished in vivo work by our group. Investigations involving

tumour-free and C6-bearing Wistar rats treated with MRT revealed that the induction of bystander effects was more intense by the tumour bearing animals than by the tumour-free rats (Fernandez-Palomo et al., 2013). Conversely, when the F98 glioma was inoculated in the brain of Fisher rats, the bystander effects were weakened/blocked after Synchrotron Radiation compared to the normal control rats (Fernandez-Palomo, Bräuer-Krisch, et al., 2015). Moreover, athymic nude mice harbouring the F98 glioma also induced a weakened bystander effect compared with tumour-free animals after Synchrotron radiation. While those in vivo responses could be associated with the different immunogenicity of the tumours, it cannot explain our results in vitro. A more likely explanation could be attributed again to the different p53 statuses of these cell lines. Published work has previously demonstrated that a different p53 status affects the response of cells to bystander signals. For instance, Mothersill et al. looked at the different induction of bystander effects in the human colon tumour cell line 116 comparing the wild-type p53 and the null-p53 HCT116 types (Carmel Mothersill et al., 2011). Those experiments revealed that both cell lines produced bystander signals, but only the wild-type responded to them. Similarly, the different p53 statuses of the C6 and F98 glioma could be behind the opposite bystander effects observed at HRS doses.

In conclusion, our in vitro studies have revealed that the C6 glioma is more radioresistant than the F98 cells. The two cell lines induced opposite bystander

effects after exposure to HRS doses using gamma and Synchrotron X-rays, which were corroborated with opposite fluxes of calcium ions through the cellular membrane after HRS doses. The data presented here together with published work suggest that a larger volume of tissue exposed to Synchrotron valley doses relative to the volume exposed to peak doses may be more efficient at inducing bystander effects.

3.6 REFERENCES

Ahmad, S. B., McNeill, F. E., Byun, S. H., Prestwich, W. V, Mothersill, C., Seymour, C., … Fernandez, C. (2013). Ultra-Violet Light Emission from HPV-G Cells Irradiated with Low Let Radiation From (90)Y; Consequences for Radiation Induced Bystander Effects. *Dose-Response : A Publication of International Hormesis Society*, *11*, 498–516. doi:10.2203/dose-response.12-048.Ahmad

Al-Mayah, A. H. J., Irons, S. L., Pink, R. C., Carter, D. R. F., & Kadhim, M. A. (2012). Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. *Radiation Research*, *177*(5), 539–45. doi:10.1667/RR2868.1

Asai, A., Miyagi, Y., Sugiyama, A., Gamanuma, M., Hong, S. H., Takamoto, S., … Kuchino, Y. (1994). Negative effects of wild-type p53 and s-Myc on cellular growth and tumorigenicity of glioma cells. Implication of the tumor suppressor genes for gene therapy. *Journal of Neuro-Oncology*, *19*(3), 259–68. doi:10.1007/BF01053280

Azzam, E., de Toledo, S., & Little, J. (2004). Stress Signaling from Irradiated to Non-Irradiated Cells. *Current Cancer Drug Targets*, *4*(1), 53–64. doi:10.2174/1568009043481641

Azzam, E. I., de Toledo, S. M., Gooding, T., & 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*(5), 497–504. doi:10.2307/3579865

Barth, R. F. (1998). Rat brain tumor models in experimental neuro-oncology: the 9L, C6, T9, F98, RG2 (D74), RT-2 and CNS-1 gliomas. *Journal of Neuro-Oncology*, *36*(1), 91–102. doi:10.1007/s11060-009-9875-7

Barth, R. F., & Kaur, B. (2009). Rat brain tumor models in experimental neuro-oncology: the C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. *Journal of Neuro-Oncology*, *94*(3), 299–312. doi:10.1007/s11060-009-9875-7

Benda, P., Lightbody, J., Sato, G., Levine, L., & Sweet, W. (1968). Differentiated rat glial cell strain in tissue culture. *Science (New York, N.Y.)*, *161*(3839), 370–1. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4873531

Bergqvist, M., Brattström, D., Gullbo, J., Hesselius, P., Brodin, O., & Wagenius, G. (2003). p53 status and its in vitro relationship to radiosensitivity and chemosensitivity in lung cancer.

Anticancer Research, *23*(2B), 1207–12. Retrieved from http://europepmc.org/abstract/med/12820372

Biston, M.-C., Joubert, A., Adam, J.-F., Elleaume, H., Bohic, S., Charvet, A.-M., … Balosso, J. (2004). Cure of Fisher rats bearing radioresistant F98 glioma treated with cis-platinum and irradiated with monochromatic synchrotron X-rays. *Cancer Research*, *64*(7), 2317–23. doi:10.1158/0008-5472.CAN-03-3600

Bouchet, A., Lemasson, B., Christen, T., Potez, M., Rome, C., Coquery, N., … Serduc, R. (2013). Synchrotron microbeam radiation therapy induces hypoxia in intracerebral gliosarcoma but not in the normal brain. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, *108*(1), 143–8. doi:10.1016/j.radonc.2013.05.013

Boukamp, P., Popp, S., Bleuel, K., Tomakidi, E., Bürkle, A., & Fusenig, N. E. (1999). Tumorigenic conversion of immortal human skin keratinocytes (HaCaT) by elevated temperature. *Oncogene*, *18*(41), 5638–45. doi:10.1038/sj.onc.1202934

Bräuer-Krisch, E., Serduc, R., Siegbahn, E. a, Le Duc, G., Prezado, Y., Bravin, A., … Laissue, J. A. (2010). Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. *Mutation Research*, *704*(1-3), 160–6. doi:10.1016/j.mrrev.2009.12.003

Collis, S. J., Schwaninger, J. M., Ntambi, A. J., Keller, T. W., Nelson, W. G., Dillehay, L. E., & DeWeese, T. L. (2004). Evasion of early cellular response mechanisms following low level radiation-induced DNA damage. *Journal of Biological Chemistry*, *279*(48), 49624–49632. doi:10.1074/jbc.M409600200

Crosbie, J. C., Anderson, R. L., Rothkamm, K., Restall, C. M., Cann, L., Ruwanpura, S., … Rogers, P. a W. (2010). Tumor cell response to synchrotron microbeam radiation therapy differs markedly from cells in normal tissues. *International Journal of Radiation Oncology, Biology, Physics*, *77*(3), 886–94. doi:10.1016/j.ijrobp.2010.01.035

Desmarais, G., Charest, G., Fortin, D., Bujold, R., Mathieu, D., & Paquette, B. (2015). Cyclooxygenase-2 inhibitor prevents radiation-enhanced infiltration of F98 glioma cells in brain of Fischer rat. *International Journal of Radiation Biology*, 1–10. doi:10.3109/09553002.2015.1043756

Desmarais, G., Fortin, D., Bujold, R., Wagner, R., Mathieu, D., & Paquette, B. (2012). Infiltration of glioma cells in brain parenchyma stimulated by radiation in the F98/Fischer rat model. *International Journal of Radiation Biology*, *88*(8), 565–74. doi:10.3109/09553002.2012.692495

Dilmanian, F. A., Button, T. M., Le Duc, G., Zhong, N., Peña, L. A., Smith, J. A. L., … Ren, B. (2002). Response of rat intracranial 9L gliosarcoma to microbeam radiation therapy. *Neuro-Oncology*, *4*(1), 26. doi:10.1016/j.ejrad.2008.04.055

Farrell, C. L., Stewart, P. a, & Del Maestro, R. F. (1987). A new glioma model in rat: the C6 spheroid implantation technique permeability and vascular characterization. *Journal of Neuro-Oncology*, *4*(4), 403–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3572471

Fazzari, J., Mersov, A., Smith, R., Seymour, C., & Mothersill, C. (2012). Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect. *International Journal of Radiation Biology*, *88*(10), 786–90. doi:10.3109/09553002.2012.714519

Fernandez-Palomo, C., Bräuer-Krisch, E., Laissue, J., Vukmirovic, D., Blattmann, H., Seymour, C., … Mothersill, C. (2015). Use of synchrotron medical microbeam irradiation to investigate

radiation-induced bystander and abscopal effects in vivo. *Physica Medica : PM : An International Journal Devoted to the Applications of Physics to Medicine and Biology : Official Journal of the Italian Association of Biomedical Physics (AIFB)*, 1–12. doi:10.1016/j.ejmp.2015.03.004

Fernandez-Palomo, C., Mothersill, C., Bräuer-Krisch, E., Laissue, J., Seymour, C., & Schültke, E. (2015). γ-H2AX as a Marker for Dose Deposition in the Brain of Wistar Rats after Synchrotron Microbeam Radiation. *PLOS ONE*, *10*(3), e0119924. doi:10.1371/journal.pone.0119924

Fernandez-Palomo, C., Schültke, E., Smith, R., Bräuer-Krisch, E., Laissue, J., Schroll, C., … Mothersill, C. (2013). Bystander effects in tumor-free and tumor-bearing rat brains following irradiation by synchrotron X-rays. *International Journal of Radiation Biology*, *89*(6), 445–53. doi:10.3109/09553002.2013.766770

Fernet, M., Mégnin-Chanet, F., Hall, J., & Favaudon, V. (2010). Control of the G2/M checkpoints after exposure to low doses of ionising radiation: Implications for hyper-radiosensitivity. *DNA Repair*, *9*(1), 48–57. doi:10.1016/j.dnarep.2009.10.006

Gil, S., Sarun, S., Biete, A., Prezado, Y., & Sabés, M. (2011). Survival analysis of F98 glioma rat cells following minibeam or broad-beam synchrotron radiation therapy. *Radiation Oncology (London, England)*, *6*(1), 37. doi:10.1186/1748-717X-6-37

Henseleit, U., Zhang, J., Wanner, R., Haase, I., Kolde, G., & Rosenbach, T. (1997). Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *The Journal of Investigative Dermatology*. doi:10.1111/1523-1747.ep12340708

Heuskin, A.-C., Wéra, A.-C., Riquier, H., Michiels, C., & Lucas, S. (2013). Low-Dose Hypersensitivity and Bystander Effect are Not Mutually Exclusive in A549 Lung Carcinoma Cells after Irradiation with Charged Particles. *Radiation Research*, *180*(5), 491–8. doi:10.1667/RR13358.1

Iyer, R., & Lehnert, B. E. (2000). Factors underlying the cell growth-related bystander responses to ?? particles. *Cancer Research*, *60*(5), 1290–1298. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10728689

Jella, K. K., Rani, S., O'Driscoll, L., McClean, B., Byrne, H. J., & Lyng, F. M. (2014). Exosomes are involved in mediating radiation induced bystander signaling in human keratinocyte cells. *Radiation Research*, *181*(2), 138–45. doi:10.1667/RR13337.1

Koestner, a, Swenberg, J. a, & Wechsler, W. (1971). Transplacental production with ethylnitrosourea of neoplasms of the nervous system in Sprague-Dawley rats. *The American Journal of Pathology*, *63*(1), 37–56. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2047464&tool=pmcentrez&rendertype=abstract

Krueger, S. a., Wilson, G. D., Piasentin, E., Joiner, M. C., & Marples, B. (2010). The effects of G2-phase enrichment and checkpoint abrogation on low-dose hyper-radiosensitivity. *International Journal of Radiation Oncology Biology Physics*, *77*(5), 1509–1517. doi:10.1016/j.ijrobp.2010.01.028

Laissue, J. A., Geiser, G., Spanne, P. O., Dilmanian, F. a, Gebbers, J. O., Geiser, M., … Slatkin, D. N. (1998). Neuropathology of ablation of rat gliosarcomas and contiguous brain tissues using a microplanar beam of synchrotron-wiggler-generated X rays. *International Journal of Cancer. Journal International Du Cancer*, *78*(5), 654–60. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med4&NEWS=N&AN=9808538

Lambin, P., Marples, B., Fertil, B., Malaise, E. P., & Joiner, M. C. (1993). Hypersensitivity of a human tumour cell line to very low radiation doses. *International Journal of Radiation Biology*, *63*(5), 639–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8099110

Le, M., McNeill, F. E., Seymour, C., Rainbow, A. J., & Mothersill, C. E. (2014). An observed effect of ultraviolet radiation emitted from β-irradiated HaCaT cells upon non-β-irradiated bystander cells. *Radiation Research*, (In Print).

Lehman, T. A., Modali, R., Boukamp, P., Stanek, J., Bennett, W. P., Welsh, J. A., … Rogan, E. M. (1993). p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis*, *14*(5), 833–839.

Lyng, F. M., Maguire, P., McClean, B., Seymour, C., & Mothersill, C. (2006). The Involvement of Calcium and MAP Kinase Signaling Pathways in the Production of Radiation-Induced Bystander Effects. *Radiation Research*, *165*(4), 400–409. doi:10.1667/RR3527.1

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2000). Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *British Journal of Cancer*, *83*(9), 1223–30. doi:10.1054/bjoc.2000.1433

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2001). Oxidative stress in cells exposed to low levels of ionizing radiation. *Biochemical Society Transactions*, *29*(2), 350. doi:10.1042/0300-5127:0290350

Lyng, F. M., Seymour, C., & 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*(4), 365–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893237

Marples, B. (2004). Is low-dose hyper-radiosensitivity a measure of G2-phase cell radiosensitivity? *Cancer and Metastasis Reviews*. doi:10.1023/B:CANC.0000031761.61361.2a

Marples, B., Wouters, B. G., & 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*(1), 38–45. doi:10.1667/RR3013

Martin, L. M., Marples, B., Lynch, T. H., Hollywood, D., & Marignol, L. (2014). Exposure to low dose ionising radiation: Molecular and clinical consequences. *Cancer Letters*, *349*(1), 98–106. doi:10.1016/j.canlet.2013.12.015

Miura, M., Blattmann, H., Bräuer-Krisch, E., Bravin, A., Hanson, A. L., Nawrocky, M. M., … Laissue, J. A. (2006). Radiosurgical palliation of aggressive murine SCCVII squamous cell carcinomas using synchrotron-generated X-ray microbeams. *The British Journal of Radiology*, *79*(937), 71–5. doi:10.1259/bjr/50464795

Mothersill, C., Austin, D., Fernandez-Palomo, C., Seymour, C., Auchinachie, N., & Austin, B. (2014). Rescue of fish exposed to a lethal dose of pathogen, by signals from sublethally exposed survivors. *FEMS Microbiology Letters*, *362*(October 2014), fnu058–fnu058. doi:10.1093/femsle/fnu058

Mothersill, C., Bristow, R. G., Harding, S. M., Smith, R. W., Mersov, A., & Seymour, C. B. (2011). A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells. *International Journal of Radiation Biology*, *87*(11), 1120–5. doi:10.3109/09553002.2011.610866

Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., … Seymour, C. (2014). Transmission of signals from rats receiving high doses of microbeam

radiation to cage mates: an inter-mammal bystander effect. *Dose-Response : A Publication of International Hormesis Society*, *12*(1), 72–92. doi:10.2203/dose-response.13-011.Mothersill

Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S., & Wright, E. (2005). Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. *Radiation Research*, *163*(4), 391–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15799694

Mothersill, C., & Seymour, C. (1997). Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology*, *71*(4), 421–7. doi:10.1080/095530097144030

Mothersill, C., & Seymour, C. B. (1998). Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiation Research*, *149*(3), 256–262. doi:10.2307/3579958

Mothersill, C., & Seymour, C. B. (2004). Radiation-induced bystander effects--implications for cancer. *Nature Reviews. Cancer*, *4*(2), 158–164. doi:10.1038/nrc1277

Mothersill, C., Seymour, C. B., & Joiner, M. C. (2002). Relationship between radiation-induced low-dose hypersensitivity and the bystander effect. *Radiation Research*, *157*(5), 526–32. doi:10.1667/0033-7587(2002)157[0526:RBRILD]2.0.CO;2

Murphy, J. E. J., Nugent, S., Seymour, C., & Mothersill, C. (2005). Mitochondrial DNA point mutations and a novel deletion induced by direct low-LET radiation and by medium from irradiated cells. *Mutation Research*, *585*(1-2), 127–36. doi:10.1016/j.mrgentox.2005.04.011

Nagasawa, H., & Little, J. B. (1992). Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Research*, *52*(22), 6394–6396. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1423287

Narayanan, P. K., Goodwin, E. H., & Lehnert, B. E. (1997). Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Research*, *57*(18), 3963–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9307280

Nuta, O., & Darroudi, F. (2008). The impact of the bystander effect on the low-dose hypersensitivity phenomenon. *Radiation and Environmental Biophysics*, *47*(2), 265–74. doi:10.1007/s00411-007-0145-9

O'Leary, V. B., Ovsepian, S. V., Carrascosa, L. G., Buske, F. A., Radulovic, V., Niyazi, M., … Anastasov, N. (2015). PARTICLE, a Triplex-Forming Long ncRNA, Regulates Locus-Specific Methylation in Response to Low-Dose Irradiation. *Cell Reports*, 474–485. doi:10.1016/j.celrep.2015.03.043

Okaichi, K., Ide-Kanematsu, M., Izumi, N., Morita, N., Okumura, Y., & Ihara, M. (2008). Variations in sensitivity to ionizing radiation in relation to p53 mutation point. *Anticancer Research*, *28*(5 A), 2687–2690.

Puck, T. T., & Marcus, P. I. (1956). Action of x-rays on mammalian cells. *The Journal of Experimental Medicine*, *103*(5), 653–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10380286

Ryan, L. a, Seymour, C. B., Joiner, M. C., & Mothersill, C. E. (2009). Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. *International Journal of Radiation Biology*, *85*(1), 87–95. doi:10.1080/09553000802635062

Saroya, R., Smith, R., Seymour, C., & Mothersill, C. (2009). Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism. *Dose-Response : A Publication of International Hormesis Society*, *8*(3), 317–30. doi:10.2203/dose-response.09-043.Saroya

Schlegel, J., Piontek, G., Kersting, M., Schuermann, M., Kappler, R., Scherthan, H., … Mennel, H. (1999). The p16/Cdkn2a/Ink4a gene is frequently deleted in nitrosourea-induced rat glial tumors. *Pathobiology : Journal of Immunopathology, Molecular and Cellular Biology*, *67*(4), 202–6. doi:28073

Schoenherr, D., Krueger, S. A., Martin, L., Marignol, L., Wilson, G. D., & Marples, B. (2013). Determining if low dose hyper-radiosensitivity (HRS) can be exploited to provide a therapeutic advantage: a cell line study in four glioblastoma multiforme (GBM) cell lines. *International Journal of Radiation Biology*, *89*(12), 1009–16. doi:10.3109/09553002.2013.825061

Schültke, E., Juurlink, B. H. J., Ataelmannan, K., Laissue, J., Blattmann, H., Bräuer-Krisch, E., … Fourney, D. (2008). Memory and survival after microbeam radiation therapy. *European Journal of Radiology*, *68*(3 SUPPL.), 142–146. doi:10.1016/j.ejrad.2008.04.051

Senatus, P. B., Li, Y., Mandigo, C., Nichols, G., Moise, G., Mao, Y., … Fine, R. L. (2006). Restoration of p53 function for selective Fas-mediated apoptosis in human and rat glioma cells in vitro and in vivo by a p53 COOH-terminal peptide. *Molecular Cancer Therapeutics*, *5*(1), 20–8. doi:10.1158/1535-7163.MCT-05-0181

Shao, C., Aoki, M., & Furusawa, Y. (2003). Bystander effect on cell growth stimulation in neoplastic HSGc cells induced by heavy-ion irradiation. *Radiation and Environmental Biophysics*, *42*(3), 183–187. doi:10.1007/s00411-003-0202-y

Shao, C., Lyng, F. M., Folkard, M., & Prise, K. M. (2006). Calcium fluxes modulate the radiation-induced bystander responses in targeted glioma and fibroblast cells. *Radiation Research*, *166*(3), 479–487. doi:10.1667/RR3600.1

Shao, C., Stewart, V., Folkard, M., Michael, B. D., & Prise, K. M. (2003). Nitric Oxide-Mediated Signaling in the Bystander Response of Individually Targeted Glioma Cells. *Cancer Research*, *63*(23), 8437–8442.

Short, S. C., Woodcock, M., Marples, B., & Joiner, M. C. (2003). Effects of cell cycle phase on low-dose hyper-radiosensitivity. *International Journal of Radiation Biology*, *79*(2), 99–105. doi:10.1080/0955300021000045646

Singh, H., Saroya, R., Smith, R., Mantha, R., Guindon, L., Mitchel, R. E. J., … Mothersill, C. (2011). Radiation induced bystander effects in mice given low doses of radiation in vivo. *Dose-Response : A Publication of International Hormesis Society*, *9*(2), 225–42. doi:10.2203/dose-response.09-062.Singh

Slatkin, D. N., Spanne, P., Dilmanian, F. A., & Sandborg, M. (1992). Microbeam radiation therapy. *Medical Physics*, *19*(6), 1395–1400. doi:10.1118/1.596771

Strigari, L., Mancuso, M., Ubertini, V., Soriani, A., Giardullo, P., Benassi, M., … Bossi, G. (2014). Abscopal effect of radiation therapy: Interplay between radiation dose and p53 status. *International Journal of Radiation Biology*, *90*(3), 248–55. doi:10.3109/09553002.2014.874608

Tzeng, J. J., Barth, R. F., Orosz, C. G., & James, S. M. (1991). Phenotype and functional activity of tumor-infiltrating lymphocytes isolated from immunogenic and nonimmunogenic rat brain tumors. *Cancer Research*, *51*(9), 2373–2378.

Williams, J. R., Zhang, Y., Zhou, H., Gridley, D. S., Koch, C. J., Slater, J. M., & Little, J. B. (2008). Overview of Radiosensitivity of Human Tumor Cells to Low-Dose-Rate Irradiation. *International Journal of Radiation Oncology Biology Physics*, *72*(3), 909–917. doi:10.1016/j.ijrobp.2008.06.1928

Williams, M. V, Denekamp, J., & Fowler, J. F. (1985). A review of alpha/beta ratios for experimental tumors: implications for clinical studies of altered fractionation. *International Journal of Radiation Oncology, Biology, Physics*, *11*(1), 87–96. doi:10.1016/0360-3016(85)90366-9

Wykes, S. M., Piasentin, E., Joiner, M. C., Wilson, G. D., & Marples, B. (2006). Low-dose hyper-radiosensitivity is not caused by a failure to recognize DNA double-strand breaks. *Radiation Research*, *165*(5), 516–524. doi:10.1667/RR3553.1

Xue, L., Yu, D., Furusawa, Y., Cao, J., Okayasu, R., & Fan, S. (2009). ATM-Dependent Hyper-Radiosensitivity in Mammalian Cells Irradiated by Heavy Ions. *International Journal of Radiation Oncology Biology Physics*, *75*(1), 235–243. doi:10.1016/j.ijrobp.2009.04.088

Yang, S.-H., Wang, S.-M., Syu, J.-P., Chen, Y., Wang, S.-D., Peng, Y.-S., … Kung, H.-N. (2014). Andrographolide induces apoptosis of C6 glioma cells via the ERK-p53-caspase 7-PARP pathway. *BioMed Research International*, *2014*, 312847. doi:10.1155/2014/312847

Yuan, S. S. F., Chang, H. L., & Lee, E. Y. H. P. (2003). Ionizing radiation-induced Rad51 nuclear focus formation is cell cycle-regulated and defective in both ATM-/- and c-Abl-/- cells. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, *525*(1-2), 85–92. doi:10.1016/S0027-5107(03)00009-5

Zhou, H., Ivanov, V. N., Lien, Y.-C., Davidson, M., & Hei, T. K. (2008). Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects. *Cancer Research*, *68*(7), 2233–40. doi:10.1158/0008-5472.CAN-07-5278

Chapter 4

4 USE OF SYNCHROTRON MEDICAL MICROBEAM IRRADIATION TO INVESTIGATE RADIATION-INDUCED BYSTANDER AND ABSCOPAL EFFECTS IN VIVO.

Cristian Fernandez-Palomo, Elke Bräuer-Krisch, Jean Laissue, Dusan Vukmirovic, Hans Blattmann, Colin Seymour, Elisabeth Schültke, Carmel Mothersill

---

This research project was design by Mothersill and the first author. The irradiations were performed by the first author, Schültke, Bräuer-Krisch and Seymour. Tissue harvest, sampling, clonogenic assays and calcium measurements were performed by Blattmann, Laissue, Mothersill, Vukmirovic and the first author. Data analysis was performed by the first author. The manuscript was written by the first author and Mothersill; edited by Schültke, Laissue and Bräuerk-Krisch.

The manuscript is published in Physica Medica.

Published September, 2015. Volume 31; issue 6; pages 584-595,

doi: 10.1016/j.ejmp.2015.03.004

4.1 ABSTRACT

The question of whether bystander and abscopal effects are the same is unclear. Our experimental system enables us to address this question by allowing irradiated organisms to partner with unexposed individuals. Organs from both animals and appropriate sham and scatter dose controls are tested for expression of several endpoints such as calcium flux, role of 5HT, reporter assay cell death and proteomic profile. The results show that membrane related functions of calcium and 5HT are critical for true bystander effect expression. Our original inter-animal experiments used fish species whole body irradiated with low doses of X-rays, which prevented us from addressing the abscopal effect question. Data which are much more relevant in radiotherapy are now available for rats which received high dose local irradiation to the implanted right brain glioma. The data were generated using quasi-parallel microbeams at the biomedical beamline at the European Synchrotron Radiation Facility in Grenoble France. This means we can directly compare abscopal and “true” bystander effects in a rodent tumour model. Analysis of right brain hemisphere, left brain and urinary bladder in the directly irradiated animals and their unirradiated partners strongly suggests that bystander effects (in partner animals) are not the same as abscopal effects (in the irradiated animal). Furthermore, the presence of a tumour in the right brain alters the magnitude of both abscopal and bystander effects in the tissues from the directly irradiated animal and in the unirradiated partners which did not contain tumours, meaning the type of signal was different.

Keywords: Radiation-induced bystander effects, Synchrotron microbeam radiation, Fischer rats, F98 glioma

4.2 INTRODUCTION

Non-targeted effects including bystander (effects in unirradiated cells receiving signals from irradiated cells) and abscopal effects (effects in unirradiated tissues following irradiation of a different tissue in a distant location) are known to occur following both low and high doses of radiation and other stressors both *in vitro* and *in vivo*. Most *in vivo* data involve shielding part of an animal and are complicated by systemic factors such as blood and endocrine factors, making it difficult to resolve mechanistic questions related for example to the role of the immune system or the inflammatory response in the process (Blyth & Sykes, 2011; Mancuso et al., 2012; Munro, 2009; Tomita & Maeda, 2014). While the existence of both bystander effects and abscopal effects are widely accepted, they remain poorly understood. By definition abscopal effects occur in vivo, usually as a result of targeted radiotherapy to another part of the body (Kaminski et al., 2005; Zeng, Harris, Lim, Drake, & Tran, 2013). Bystander effects have been demonstrated in vitro in numerous cell lines across all species groups and in vivo in rodent models (Carmel Mothersill et al., 2014), fish (C Mothersill, Smith, Hinton, Aizawa, & Seymour, 2009; O'Dowd et al., 2006), amphibians (Audette-Stuart & Yankovich, 2011) and yeast (MotherSill & Seymour, 2012). In much of the literature, abscopal and bystander effects are thought to share common mechanisms and to be mediated by similar signals (Mancuso et al., 2012; Rastogi, Coates, Lorimore, & Wright, 2012) . However most bystander

research is conducted using low doses of mainly low LET radiation delivered to the entire organism or cell culture, while abscopal effects are detected following high doses of targeted radiotherapy to precise areas of the body which usually contain tumour tissue (Blyth & Sykes, 2011; Mancuso et al., 2012; G. Yang et al., 2008). This makes it difficult to ascertain whether common mechanisms are involved or whether both mechanism are related.

A further limitation of research in the field is that in vivo “bystander” experiments usually use shielding of part of the body to demonstrate effects in non-irradiated areas (Ilnytskyy, Koturbash, & Kovalchuk, 2009; Koturbash, Zemp, Kolb, & Kovalchuk, 2011; Mancuso et al., 2011). This means that the hematopoietic, neural, immune and endocrine systems could be irradiated and could either pass through the unirradiated area (blood and endocrine effectors) or share common neuronal connections leading to detection of distant effects. Additionally, scatter and out of field doses may contribute sufficient radiation to trigger bystander effects, which have thresholds in the 2-3mGy dose range (Benadjaoud et al., 2012; Butterworth et al., 2014; Liu et al., 2006, 2007; Ruben et al., 2014; G Schettino et al., 2003; Giuseppe Schettino, Folkard, Michael, & Prise, 2005; Shields, Vega-Carrascal, Singleton, Lyng, & McClean, 2014). It is unclear whether an animal that received the estimated scatter dose as whole body irradiation, is a sufficient control to cover these possibilities for reasons which will be discussed later in the paper. Clearly there could be confusion in

determining separate mechanisms involved in bystander and abscopal effects in vivo.

In the past we have successfully used an approach where non-irradiated companion animals are placed in close proximity to irradiated animals. Our group have conducted several experiments with irradiated fish (Carmel Mothersill et al., 2006; Carmel Mothersill, Smith, Agnihotri, & Seymour, 2007; Smith, Wang, Bucking, Mothersill, & Seymour, 2007) sharing aquarium water with unirradiated fish, and Surinov's group in Russia (Isaeva & Surinov, 2007; Surinov, Isaeva, & Dukhova, 2004) and our group (Carmel Mothersill et al., 2014) have also demonstrated communication between irradiated mice and their cage mates with subsequent signal expression in the non-irradiated animals. These experiments parallel in vitro "medium transfer" experiments in that the unirradiated animals receiving signals from irradiated animals were never anywhere near the radiation source and never had any part of their bodies exposed to X-rays. This precludes systemic effects due to the circulating blood, or endocrine or neural components being affected by exposure of part of the body to irradiation. The fish experiments involved whole body exposure to very low X-ray doses and confirmed a role of serotonin and calcium in the production of the bystander signal in vivo (Saroya, Smith, Seymour, & Mothersill, 2009; Singh et al., 2011) as was seen in vitro (F. M. Lyng, Maguire, McClean, Seymour, & Mothersill, 2006; Carmel Mothersill,

Saroya, Smith, Singh, & Seymour, 2010; Poon, Agnihotri, Seymour, & Mothersill, 2007).

The development of microbeam radiation therapy (MRT) using synchrotron generated kilovoltage energy X-rays is based on the concept that sparing of normal tissues will occur in the dose valleys between the peak dose tracks (Blattmann et al., 2005; Elke Bräuer-Krisch et al., 2010; D. N. Slatkin, Spanne, Dilmanian, & Sandborg, 1992). MRT, a still experimental form of spatially fractionated radiotherapy, has been developed for the treatment of small and otherwise intractable brain and spinal cord tumours (F Avraham Dilmanian et al., 2003; F. a Dilmanian et al., 2005; F.A. Dilmanian et al., 2002; Laissue et al., 2013; Serduc et al., 2009; D N Slatkin, Spanne, Dilmanian, Gebbers, & Laissue, 1995). Bystander effects are thought to play a role in the dose valleys where the absorbed X-ray dose is generally lower than in the peak dose zones by more than one order of magnitude (F. Avraham Dilmanian et al., 2007; Cristian Fernandez-Palomo et al., 2013; Smith et al., 2013). However the precise nature and role of these effects is unclear especially since the valley dose greatly exceeds the threshold of 2-3mGy established for the induction of bystander signalling processes in low dose in vitro irradiation (Liu et al., 2006, 2007; G Schettino et al., 2003). The current experiments were performed using the biomedical beamline ID17 at the European Synchrotron Radiation Facility (ESRF) in Grenoble as part of a wider study of the use of microbeam and pencil beam therapy in the treatment of malignant brain tumours in small animal models (C

Fernandez-Palomo et al., 2013; Cristian Fernandez-Palomo et al., 2013; Carmel Mothersill et al., 2014; Schültke et al., 2008, 2013; Smith et al., 2013).

Preliminary experiments (Carmel Mothersill et al., 2014), with normal tumour-free rats, have shown that bystander signals were being communicated from irradiated rats to unirradiated rats. Tissues from the unirradiated rats when cultured, gave rise to conditioned medium, which reduced the clonogenic survival of reporter cells. However the influence of tumour tissue on this process was not examined.

There is evidence from earlier in vitro experiments by our group, that some tumour cells – particularly those which are radioresistant or have mutant or dysfunctional p53 do not produce death inducing bystander signals (Carmel Mothersill & Seymour, 1998; Carmel Mothersill et al., 2011; Carmel Mothersill, Seymour, & Joiner, 2002; Ryan, Seymour, Joiner, & Mothersill, 2009). Two glioma cell lines which serve as experimental models for glioma in rodents were considered for these experiments; the F98 glioma cell line, which was developed in the Fisher rat and the C6 glioma cell line, which was originally developed in the Wistar rat (Barth & Kaur, 2009; L. Ko, Koestner, & Wechsler, 1980a, 1980b). The C6 line was used in the preliminary experiments (Carmel Mothersill et al., 2014). There is a divergence of opinion in the literature concerning the p53 status of F98 cells with one author claiming they have wild type and another claiming mutant

status (Schlegel et al., 1999; Senatus et al., 2006). The consensus at present is that the line F98 contains mutant p53. C6 cells are reported to have wild type p53 (Asai et al., 1994; Barth & Kaur, 2009; Strigari et al., 2014; S.-H. Yang et al., 2014). The results of several studies suggest that activating p53 expression using various drugs enhances apoptotic death after radiation exposure in both these cell lines, presumably because of dysfunctional operation of up-stream or downstream elements of the pathways involving p53 which should be activated following radiation exposure (Adam et al., 2006; Bencokova et al., 2008; Biston et al., 2004; Ikeda et al., 2001; Senatus et al., 2006; Tada et al., 1998). For these experiments the decision was made to use the F98 glioma cell line in Fisher rats so that the effect of a p53 mutant tumour could be examined.

In the experiments to be described here, F98 cells were stereotactically inoculated into the brain of Fisher rats. The rats were irradiated using the microbeam synchrotron radiation at the ESRF after which they were put in cages with unirradiated rats for 48hrs. Samples from all rats and various controls were taken and examined in a reporter assay for evidence of bystander signal production.

4.3 METHODS

4.1.1 Animals:

Male adult Fisher rats in the weight range 260-280g (Charles River, France) were used as the animal model in our experiments. Animals were housed and cared for prior to the experiments by the ESRF Animal Facility in accordance with French and Canadian Animal Care Protocols.

4.1.2 Tumour Inoculation:

The F98 glioma cell line was selected for our studies because of its mutant p53 status and because it shares a wide range of characteristics with the highly malignant human brain tumour glioblastoma multiforme (GBM) (Barth & Kaur, 2009). Once injected into the brain, F98 glioma cells rapidly proliferate forming a solid, highly invasive malignant tumour, delineated by a rim of activated astrocytes and small groups of infiltrating tumour cells (Barth & Kaur, 2009). This tumour model has been used in multiple studies involving conventional radiotherapy and synchrotron radiation.

For these experiments, F98 cells were obtained from ATCC and maintained in T75 cm^2 flasks using Dulbecco's Modified Eagle Medium (Gibco, France) supplemented with 10% FBS (Gibco, France) and 5ml Penicillin-Streptomycin (Gibco, France). Cells from a 90% confluent culture were detached by incubation with 20 ml of calcium and magnesium free Hank's Balanced Salt Solution (Gibco, France) for 20 minutes at 37°C in an atmosphere of 5% CO_2 in

air. The cell suspension was centrifuged at 1000 rpm for 4 min, the pellet was re-suspended in 1ml of fresh growth medium and cells were counted using a haemocytometer.

Fisher rats were subjected to general anaesthesia (2 – 2.5 % isofluorane in 2 L/min compressed air) and placed in a stereotactic frame. An incision of 1 to 1.5 cm length was made on the scalp following the sagittal midline. A burr hole was placed in the skull over the right hemisphere, 3 mm to the right from the sagittal midline and 3 mm posterior from the coronal suture. Then 100,000 F98 cells suspended in 10 μl were slowly injected into the brain 3 mm below the cortical surface over 4 minutes, using an automated syringe pump (KDS 320, GENEQ). Once the injection was finished and the needle removed, the hole was sealed with bone wax and the incision was closed. Rats were maintained for 7 days to allow tumour development.

4.1.3 Irradiation:

In preparation for the irradiation, rats were deeply anesthetised using 3% isofluorane in 2L/min compressed air and maintained with an intraperitoneal injection of a Ketamine-Xylazine cocktail (Ketamine: Xylazine = 1: 0.625; Ket 1000 and Paxman from Virback France). Animals were transported from the animal facility to the biomedical beam line ID17 in less than 5 minutes. Each irradiation group had 5 rats, which were individually positioned on the goniometer and the corresponding radiation dose for its treatment group was applied in

anterior –posterior position to the tumour location in the right cerebral hemisphere by off-setting one edge of the irradiation field 2mm towards the right from the midline (Figure 4.1). The left non-irradiated cerebral hemispheres and the urinary bladder served as fields for study of abscopal effects in the directly irradiated animals. After irradiation, all rats were put in individual cages with a marked, unirradiated normal (tumour free) rat, meaning that 5 irradiated rats were matched with 5 non-irradiated rats for the study of bystander effects.

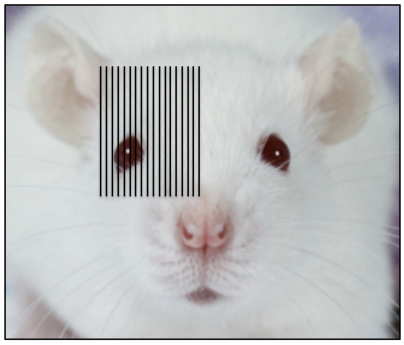

Figure 4.1 Graphical representation of the incident synchrotron microbeam.

Animals irradiated in MRT mode were exposed in a single treatment session to 20 or 200 Gy skin-entry doses. The skin entrance dose corresponds to the peak dose at 3 mm depth and is determined as described hereafter. Absolute dose measurements for preclinical experiments are performed using a pinpoint ion chamber (PTW 31014) in a solid water phantom (Gammex) to measure the dose rate in Gy/s/mA for a 2 cm x 2 cm field size at 2 cm depth. All

corrections, like for temperature and pressure, the polarization between the electrodes, the calibration of the electrometer, a correction factor for our energy spectrum and the ion recombination correction according to the IAEA 398 protocol are included.

The peak skin entrance dose is then converted with the help of Monte Carlo pre-calculated output factors depending on the desired beam-size chosen for the irradiation. In order to translate the experimentally determined dose rate within the MRT GUI (Graphical user interface) into a vertical displacement to extend the 520 micron height microbeams into an array of 50 quasi-parallel 14mm high rectangular planar microbeams, the MRT goniometer speed is calculated to deliver the desired peak entrance dose, depending on the electron current (mA) in the storage ring. A 10mm wide array of multichromatic beamlets was generated by a multislit collimator (E Bräuer-Krisch et al., 2009) with a mean energy of 105 keV by filtering the white Synchrotron beam with several filters including 1.5 mm of Aluminium and 1 mm of Copper. The typical dose rate during these experiments was ~14,000 Gy/s. The valley dose is computed by Monte Carlo calculations and more recently a Treatment Planning System (TPS) with an analytical approach is used to calculate the valley dose based on CT data from a rat applying the irradiation parameters in these experiments (Bartzsch, Lerch, Petasecca, Bräuer-Krisch, & Oelfke, 2014; Bartzsch & Oelfke, 2013). Benchmarking of the calculated dose is still ongoing, but results with Gafchromic

film dosimetry confirm an agreement within 10% between the computed and measured valley dose values (Bartzsch & Tag, 2014).

Although multi-directional treatment is more successful in increasing survival, the geometry of the unidirectional beam works better for understanding bystander effects. Unidirectional irradiation creates a less complicated 3D geometrical pattern of dose peaks and dose valleys within the brain tissue than bidirectional irradiation and therefore makes it easier to study how the normal tissue between the microbeams is involved in the induction of bystander effects.

In order to determine whether scatter radiation places a role in the induction of bystander and abscopal responses, 5 rats and 5 cage mates were selected as scatter controls. A PTW semiflex ion chamber (PTW, Freiburg, Germany) was used to measure the scatter dose received at the urinary bladder after brain irradiation with 200Gy delivered in MRT mode. The dose at the site of the urinary bladder was calculated as 3.31 mGy for MRT. An X-ray generator was adapted with different additional filters to obtain an adequate dose rate, in order to deliver the whole body dose of 3.31 mGy to the rats. HD-610 and MD-55 Gafchromic Films (ISP Advanced Materials, http://online1.ispcorp.com/) were used to verify all irradiation doses and modalities applied.

Untreated controls stayed in the ESRF animal facility and never left the cage. One group received anaesthesia before euthanasia (sham control) and another group received no anaesthesia to exclude potential effects of the anaesthetic. These control rats were also paired with cage mates and were held

two to a cage similar to the other experimental groups. We previously demonstrated that a sham irradiation did not induce abscopal effects or affect the protein expression of brain compared to controls (Smith et al., 2013).

All irradiated rats were transported back to the ESRF animal facility after irradiation. At 48hrs after irradiation, the animals were deeply anesthetised, beheaded and dissected.

4.1.4 Dissections and Sampling for Explant Culture:

Dissection of the brain was performed in a biosafety cabinet. Two pieces of brain tissue (approximately 5mm x 5mm x 3mm) were taken from both the right and the left cerebral hemispheres using sterile instruments. The tissue sample from the right (irradiated) hemisphere was taken from the centre of the irradiation array and the sample from the left (unirradiated) hemisphere was taken from the corresponding contralateral location. Samples were placed in a 5ml sterile tube containing 1mL of Roswell Park Memorial Institute (RPMI 1640, Gibco) growth medium, supplemented with 10% FBS, 5ml of Penicillin-Streptomycin (Gibco), 5ml of L-glutamine (Gibco), 0.5 mg/ml of Hydrocortisone (Sigma-Aldrich), and 12.5 ml of 1M HEPES buffer solution (Gibco). Samples were immediately transported on ice to the tissue culture laboratory to be prepared for explant culture. The remaining brain tissue was snap-frozen in liquid nitrogen and stored at -80°C for proteomic studies. The entire extracted urinary bladder was also

placed in a sterile 5ml tube containing 1ml of complete growth medium and used to set up tissue explant cultures.

4.1.5 Explant tissue culture and culture medium harvest:

Explant tissue culture was performed in the biosafety level 2 laboratory of the ESRF biomedical beamline. Brain and urinary bladder tissues were cut in 3 equal-size pieces of approximately $2mm^3$ in a biosafety cabinet. The pieces were plated as single explants in the centre of a $25cm^2$ growth area in a 50 ml volume flask (Falcon), containing 2ml of complete growth medium. Flasks were then placed in a tissue culture incubator set at 37°C, with an atmosphere of 5% CO_2 in air and 95% humidity left undisturbed for 24hrs. Growth medium from each of the three explant pieces (total approximately 5ml) was harvested 24 hours later by pouring it off into a sterile plastic container. This was then filtered through a sterile 0.22 μm filter (Acrodisc Syringe Filter with HT Tuffryn Membrane, Pall Life Sciences) to ensure that cells or other debris were not present in the harvested medium, and placed in a 7mL tube. Conditioned growth medium was kept in 4°C until all media were collected and then transported to McMaster University for clonogenic reporter bioassays.

4.1.6 Clonogenic Reporter Cell Line:

HaCaT cells have been used as reporters for explanted tissue assays by our group in Canada and earlier in Ireland for over 15 years (C Mothersill et al.,

2001). The cell line consists of epithelial cells, which became immortal spontaneously. They were derived originally from normal human skin from a patient with a melanoma (Boukamp et al., 1999) and have been used in a wide range of experiments due to their reliable and stable response to bystander signals. They show a reduction of around 40% in colony survival in response to addition of autologous irradiated cell conditioned medium (ICCM) over a wide range of donor cell radiation doses (Carmel Mothersill & Seymour, 1997). HaCaT cells have 3 p53 point mutations; 1 in codon 179 of exon 5 on one allele, and 2 consecutive mutations in codons 281 and 282 of exon 8 on the other allele (Lehman et al., 1993). In spite of its mutations, data show that p53 in HaCaT cells remains functional with respect to inducing apoptosis (Henseleit et al., 1997). In our hands they behave like wild-type cells with respect to bystander effect reporting. Unlike true p53 mutant or null cells where signal is produced but the cells cannot respond to signal. This leads us to suspect that the critical p53 function in determining whether response to bystander signals happens, is located in the wild type codons.

The HaCaT cells were cultured in T75 flasks (Falcon) with RPMI 1640 supplemented as above. Once the cells reached about 90-95% confluence they were detached using 1:1 (v:v) solution of 0.02 % Trypsin/EDTA (1mM) (Gibco) and Dulbecco's Phosphate-Buffered Solution (1x) (Gibco). The concentration of cells was determined using a Coulter Counter (Beckman Coulter model Z_n).

4.1.7 Clonogenic HaCaT cell reporter bioassay:

Upon arrival at McMaster University, the conditioned medium harvested in France was transferred into 25cm^2 flasks containing the HaCaT reporter cells. Reporter flasks were seeded with 500 cells and set up 6 hours prior to the medium transfer from T75 flasks which were 90-95% confluent. Plating efficiency and medium transfer controls were also set up. The flasks were then placed in an incubator for 10-12 days to allow for colony formation using the Puck and Marcus technique (Puck & Marcus, 1956). Once colonies reached a suitable size they were stained using 2mL of a 1:4 solution of Carbol Fuchsin in water.

Colonies were counted using a 50 cells threshold and the percentage survival fraction was calculated using the plating efficiency (PE) of the reporter cells as shown below:

Equation 4.1 Survival Fraction

$$Survival\ Fraction = \frac{PE\ of\ treated\ cells}{PE\ of\ control\ cells}\ x\ 100$$

4.1.8 Fura-2 measurements to determine intracellular free calcium in HaCaT cells:

The cells were seeded in glass bottomed dish (MatTek) at a density of approximately 500,000 cells and incubated at 37°C and 5% CO_2 for 18-24 hours prior to measurement to achieve 50% confluence. Cells were washed 3 times with buffer (130mM NaCl, 5 mM KCl, 1 mM Na2HPO4, 1 mM CaCl2, 1 mM

MgCl2 and 25 mM Hepes (pH 7.4)) followed by incubation with 1ml of 8.2μM Fura-2/AM (aminopolycarboxylic acid which binds to free intracellular calcium)(Sigma) at 37°C for 30min. Cells were washed 3 times with buffer to remove residual Fura-2/AM and 300μL of fresh buffer added to the dish for imaging. An Olympus 1X81 microscope was used with a 40X oil objective and Fura filter cube with 510nm emission. Fura-2 was excited at 380 and 340nm and the ratio images were recorded every 4s for 5 minutes with addition of 100μl of ICCM or control media after a stable baseline was reached approaching 30s. All measurements were conducted in the dark at room temperature.

4.1.9 Statistical Analysis:

Data are presented as standard deviation of the mean for the specific n value of each experiment. Significance between and within groups was determined using the Tukey multi-comparison test after a two-way ANOVA. In all cases p values ≤ 0.05 were selected as significant. Pearson correlations and linear regressions were done using SPSS and Prism 6.0.

4.4 RESULTS

The clonogenic assay reports the bystander signal strength measured as the ability of the signals to reduce the clonogenic survival of the well characterised keratinocyte cell line. Figure 4.2 shows the signal strength from

the directly irradiated right brain (4.1a) of normal and tumour bearing rats receiving 20 and 200Gy irradiation from the MRT beam and the abscopal effects in the left brain (4.1b) and distant bladder (4.1c) in these animals. All the data are normalised to the relevant sham control. There was no significant effect of the anaesthesia on clonogenic survival (non-significant p-values for right brain, left brain and urinary bladder respectively: $p = 0.495$; $p = 0.989$; $p= 0.993$). The statistical analyses were therefore performed between the sham irradiated and the irradiated groups. Because of the high doses used in these experiments and the reported low threshold dose for triggering bystander signalling in vitro (Liu et al., 2006, 2007; G Schettino et al., 2003), a control group was included which received a whole body dose equivalent to the highest calculated scatter dose of 3.31mGy after delivery of 200Gy to the head. The data in figure 4.2a show that there was no statistically significant difference between the different controls. There is however a significant difference in signal strength between the controls and the healthy rats which received direct irradiation of 20Gy or 200Gy to the right brain. The effect on clonogenic survival after 200Gy is similar to that seen after 20Gy MRT. The effect of direct irradiation to the tumour bearing right brain (Fig. 4.2d) is much more visible showing a very strong effect on clonogenic survival of the reporter cells after 20Gy and an even stronger suppression of clonogenic growth after 200Gy irradiation. Looking at the effects of signals from the left brain hemisphere (Fig. 2b), which received only a scatter dose, it is apparent that the only statistically significant effect is from the healthy animals

where the tumour bearing right brain received 20Gy MRT. The 200Gy signals from the healthy animal are weaker but still present although not statistically significant. No significant effects are seen from the tumour bearing rats (Fig. 4.2e) when compared to their own controls. The data for the distant urinary bladder are shown in Figure 2c. Once again there are no significant effects on clonogenic survival seen in the scatter group. The signals from the bladders of the 20Gy irradiated healthy animals are not significantly different to those in the control group. However again the 20Gy group signals are stronger than those in the 200Gy group. When the tumour bearing animals are considered (Fig. 4.2f), it appears that relative to their own controls, the signals from the bladders of the groups receiving irradiation to the right brain are actually stimulating clonogenic survival.

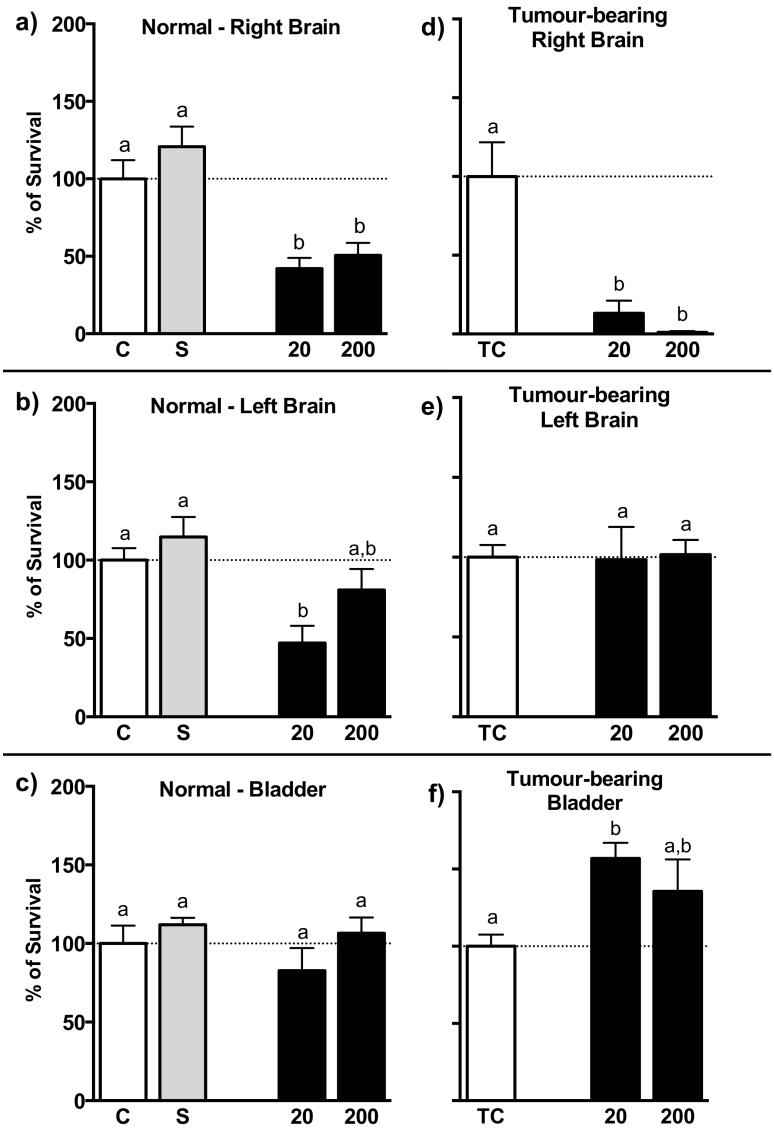

Figure 4.2 shows clonogenic survival induced by normal and tumour-bearing rats. C=Control; S=Scatter; TC=Tumour control; black bars: irradiated rats (20=20Gy; 200=200Gy). Letters a, b & c indicate significant differences between groups. Error bars show SEM.

Figure 4.3 shows the results of the true bystander animals, which merely shared a cage for 48hrs with irradiated rats. All the unexposed companion cage mates were tumour free but were paired with either a tumour bearing (d-f) or a tumour free animal (a-c). In Figure 4.3a the clonogenic survival of reporter cells receiving signals from the right brain of the unirradiated cage mates is presented. Clearly the effect in the unexposed companion cage mate's right brain tissue is much stronger when they are paired with tumour free irradiated rats. When viewed in comparison to their own control, there are no significant differences in signal strength when the irradiated rats have tumours (Fig. 4.3d). The same pattern is seen when signal strength is monitored in left brain cage mate tissue (Fig. 4.3b, 4.3e). In the unexposed companion cage mates bladder (Fig. 4.3c), signal strength is not significantly different from the controls when the irradiated rat was tumour free but the tumour bearing rat bladder (Fig. 4.3f) signals stimulate the clonogenic survival of the reporter cells as was seen with the directly irradiated rats.

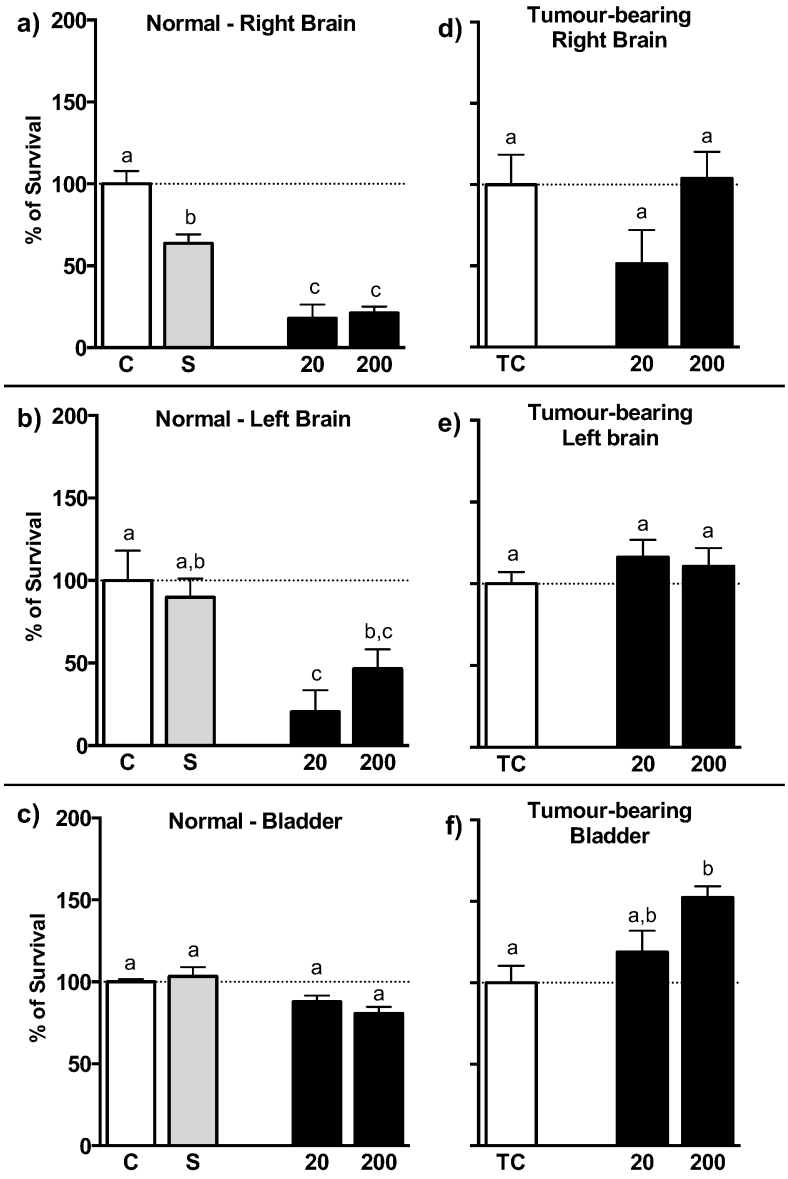

Figure 4.3 shows clonogenic survival induced by unexposed companion cage mates (Bystander rats). C=Control; S=Scatter; TC=Tumour control; 20=20Gy; 200=200Gy. Letters a, b & c indicate significant differences between groups. Error bars show SEM.

To better understand the overall influence of the presence of the tumour, figure 4.4 shows a comparison of the clonogenic survival of normal and tumour-bearing rats relative to the unirradiated normal control. Direct-irradiated normal rats (Fig. 4.4a) shows clearly the escalating decline in signal strength after 20 and 200 Gy, which is significantly different from the normal control group in the right and left hemispheres after 20 Gy, and only in the right hemisphere after 200 Gy. Direct-irradiated rats harbouring tumour showed a similar pattern of escalating decline in signal strength at 20 and 200 Gy, but it was only significant in the right brain hemisphere. The unirradiated tumour control rats clearly induced a decrease in survival in the reporter cell line relative to the unirradiated normal control rats, and this decrease was significant in both right brain hemisphere and bladder.

The bystander animals (Fig. 4.4b) paired with normal irradiated rats show similar escalating decrease in survival at 20 and 200Gy, which is significant in all brain tissues but not in bladder. The bystander animals paired with tumour-bearing rats followed almost the same pattern as their direct-irradiated mates but the only significant group was 20Gy. The unirradiated tumour control rats also seemed to influence their cage mates but the decrease in clonogenicity was not significant.

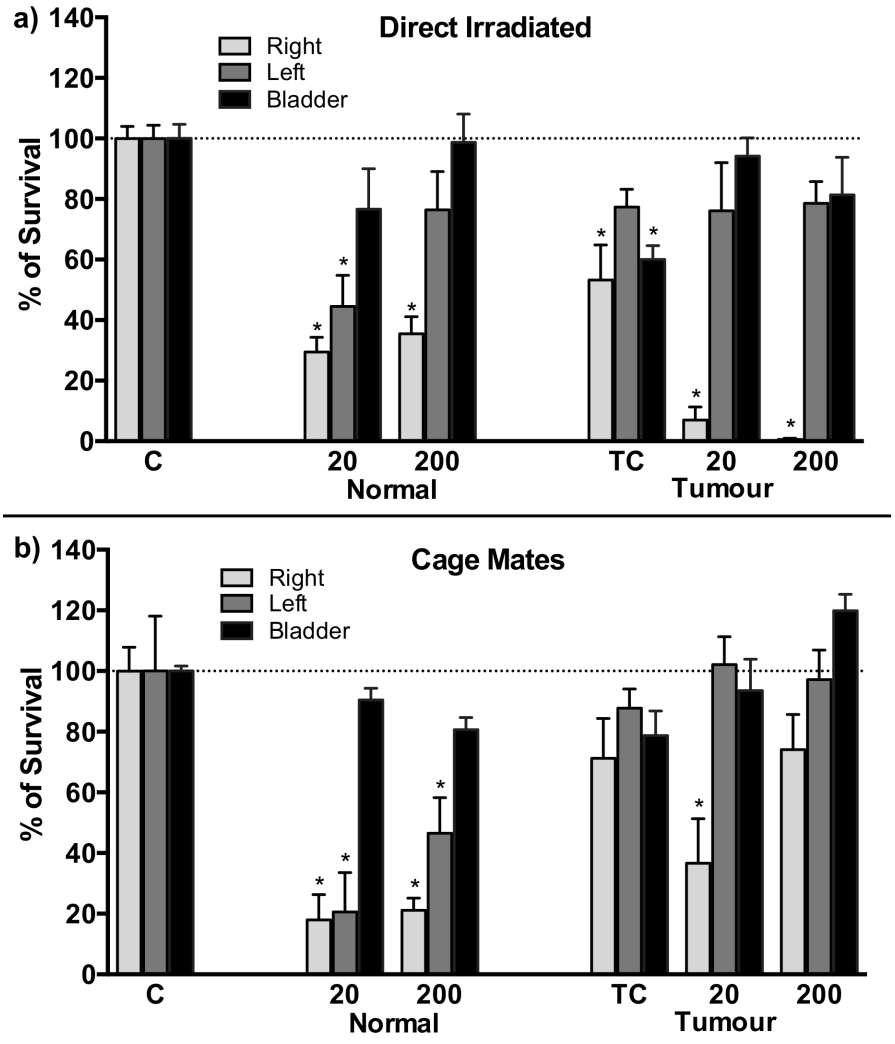

Figure 4.4 Comparison of clonogenic survival relative to the normal control. Letter "a" correspond to direct-irradiated rats, while "b" refers to the unirradiated Cage Mates or bystander animals. "*" Indicates significant difference against the unirradiated normal control rat. Error bars indicate SEM.

The results of the calcium flux analysis are presented in figure 4.5(a-f) and 4.6(a-f) and on table 1. The data show the relationship between the amount of clonogenic cell death in reporters and the calcium flux seen in the reporter cells. This is because calcium flux (measured as area under the curve) is usually considered to be the trigger, which induces the response pathway in the reporter cells. In figures 4.5 & 4.6 the data were modelled with a linear regression between % of cell death and Calcium flux induced by ICCM from irradiated and cage mate rats. Table 1 shows the Pearson Correlation statistics for these data. The conditioned medium from explants of the right brains of both normal and tumour bearing rats which were directly irradiated show weak positive, but statistically significant, correlations between the amount of clonogenic cell death of the reporter cells and the calcium flux in these cells. The right and left brains of unirradiated companion cage mates of normal rats without tumour show a very weak inverse correlation for right brain and a weak direct correlation for left brain which are statistically significant. Companion cage mates paired with animals with tumour show a negative significant correlation only in the left brains. None of the bladder tissues showed any significant effects.

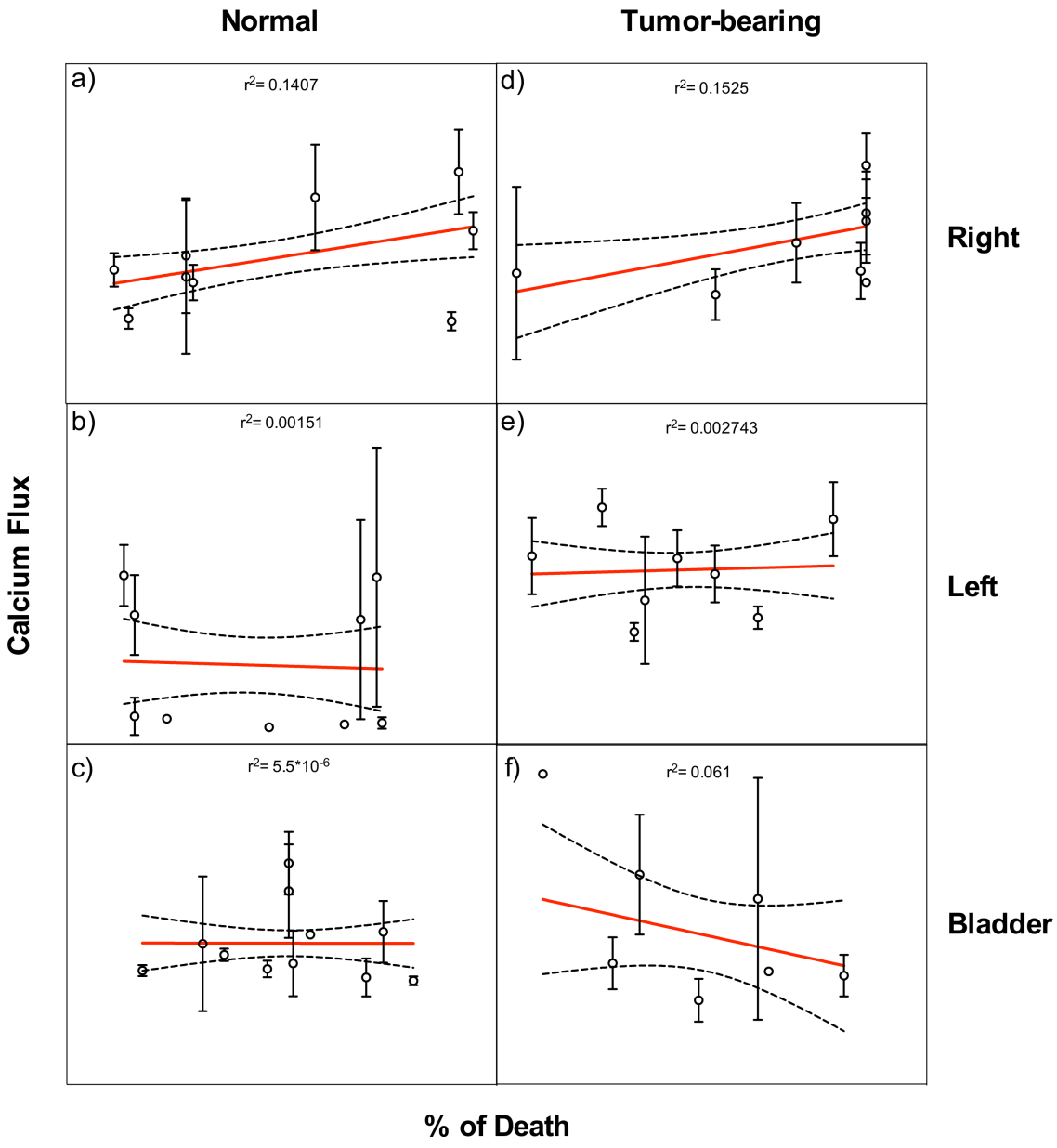

Figure 4.5 Scatter plots showing the relationship between Calcium Flux and % of death produced by ICCM from Irradiated Rats. *Red line corresponds to the linear regression with a 95% of confidence intervals.*

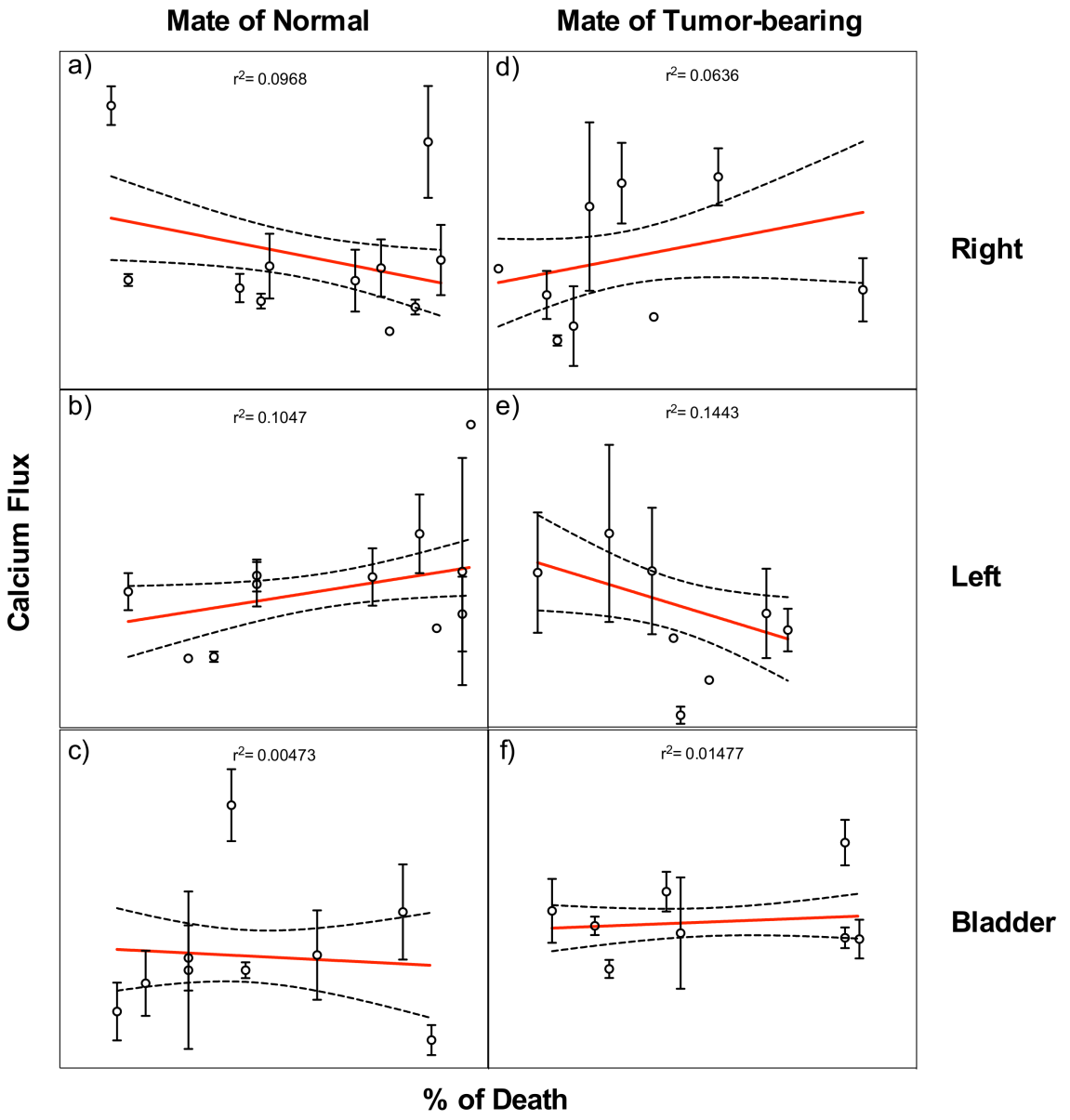

Figure 4.6 Scatter plots showing the relationship between Calcium Flux and % of death produced by ICCM from Cage Mate rats. *Red line corresponds to the linear regression with a 95% of confidence intervals.*

Table 4.1 Pearson Correlation between % of cell death and Calcium flux induced by ICCM from irradiated and cage mate rats.
(* Correlation is significant at the 0.05 level)

Group	Tissue	Pearson Correlation	Significance (2-tailed)
Normal	Right	* 0.375	(0.014)
	Left	-0.39	(0.817)
	Bladder	-0.02	(0.988)
Tumour	Right	* 0.39	(0.022)
	Left	0.052	(0.769)
	Bladder	-0.249	(0.264)
Mate of Normal	Right	* -0.311	(0.037)
	Left	* 0.324	(0.047)
	Bladder	-0.069	(0.681)
Mate of Tumour	Right	0.252	(0.164)
	Left	* -0.38	(0.042)
	Bladder	0.122	(0.501)

4.5 DISCUSSION

A key aim of this work was to look at the strength and type of signalling occurring within irradiated rats and between irradiated rats and their unexposed companion cage mates. This work builds on previously published data and preliminary results showing that there is a distinction between the effects of direct irradiation in an organism or a cell culture and the effects of the signals passed by that organism or cell culture to others. Clearly if the mechanisms of radiation

action are to be fully understood, it is important to understand the signalling (indirect effects as well as the direct effects). The early literature on bystander effects was almost all concerned with effects in vitro (Carmel Mothersill & Seymour, 2001). These were mostly negative effects and the bystander effect was considered to be an extension of the negative effects of radiation. When in vivo experiments confirmed remote cell killing, transformation or mutational effects (Chai et al., 2013; Hatzi et al., 2013; Sugihara, Murano, Nakamura, & Tanaka, 2013), the consensus was that bystander effects were bad and represented a spreading of the damage induced by the direct dose deposition. However there were also reports of adaptive responses (M. Ko, Lao, Kapadia, Elmore, & Redpath, 2006; Nenoi, Wang, & Vares, 2014; Staudacher et al., 2010). These particularly occurred in cells treated with medium from irradiated cells before being themselves irradiated (Maguire, Mothersill, McClean, Seymour, & Lyng, 2007). However, the groups using microbeams also occasionally reported adaptive and protective effects. Further extension of this to fish swimming with irradiated fish and tadpoles swimming with irradiated tadpoles (Audette-Stuart et al., 2011; Carmel Mothersill et al., 2006, 2007) suggested that bystander effects could be positive as well as negative. We consider this to be an important key for understanding radiotherapy outcomes.

The data presented here for normal Fisher rats and their cage mates contradicts those published for normal Wistar rats (Carmel Mothersill et al.,

2014). In the Wistar rat study, the abscopal effects in the directly irradiated rats became weaker as the distance from the targeted brain tissue increased. This was not attributable to declining scatter dose because the highest scatter dose calculated was given to rats as a whole body X-ray dose and had no effect. However in the unexposed companion cage mate group all the tissues in these unexposed animals produced the same response in the reporter cells. We thus deduced that the signals from the exposed animals produced a common systemic effect in the cage mates. The proteomic evidence (C Mothersill et al., n.d.) suggests this effect is protective. Similar proteomic data suggesting upregulation of protective proteins, were obtained with fish where irradiated fish were partnered with unirradiated fish (Smith et al., 2007). In the experiments reported here however, while there are small effects induced in the left brain from the group in which the right brain was directly exposed to 20Gy, there are no significant signals from the bladder suggesting a weak or absent abscopal signalling mechanism. In the cage mates, it appears that the induced signalling is strongest in the right brain, weaker in the left brain and absent in the bladder. This suggests tissue specific signals rather than the homogeneous effect seen in the Wistar rats. We conclude that there must be a strain difference and draw attention to studies with CBA and C57Bl6 mice (Carmel Mothersill et al., 2005) where similar strain differences in production of and response to bystander signals were observed.

What is very evident in this study is that the presence of an F98 glioma in the directly irradiated rats prevents or counteracts the signalling in the other tissues of the rat and also in all the tissues of the tumour-free unexposed companions (cage mates). Moreover, instead of stimulating growth, the non-irradiated rats harbouring tumour induced a decrease in survival relative to the normal controls. This suggests that the presence of a tumour does not boost reporter cell growth; but it rather seems to counteract the bystander signal. In fact, in the bladder tissues, the inducible effect in both directly irradiated and cage mates that seems stimulatory is always under or near the 100% value of survival observed in the unirradiated normal control tissues (Fig. 4.4). Since the tumour develops from F98 cells implanted in the brain rather than evolving naturally over time within a supporting microenvironment, it is hard to argue that the animal's microenvironment is incapable of producing the signal. Most of the brain in the track of the microbeam is composed of normal tissue and in the healthy rats this produced signals. While strain and cell line differences in bystander signalling support the existence of a neutral or blocking effect associated with genomic instability (Chinnadurai, Paul, & Venkatachalam, 2013), cancer phenotype (Akudugu, Azzam, & Howell, 2012), cancer susceptibility (Lorimore, Mukherjee, Robinson, Chrystal, & Wright, 2011), mutant p53 (E Bräuer-Krisch et al., 2009; Kalanxhi & Dahle, 2012; Lorimore, Rastogi, Mukherjee, Coates, & Wright, 2013) and radioresistance (Kashino, Suzuki, Kodama, Watanabe, & Prise, 2013; C. Mothersill, Seymour, & Seymour, 2006; Carmel Mothersill, Seymour, & Seymour,

2004), this appears to be the first report that bystander effects in the healthy cage mate and abscopal effects in unirradiated tissues of the directly exposed rats can both be blocked by the presence of a glioma in the brain of the rat receiving direct irradiation. Our conclusion is that the presence of tumour in the brain actively counteracts signal production.

The work raises a number of key questions about non-targeted effects in vivo. First and most important is the lack of cell killing by bystander signals when the tumour is present in the directly irradiated brain. We hypothesize that this likely stems from anti-death signals expressed by the tumour rather than absence of signals. Possibly the stress response pathways such as mitogen-activated protein kinases (MAPK), which are normally activated in response to radiation or receipt of bystander signals and which lead to apoptosis are somehow actively neutralised. Previous research (Carmel Mothersill et al., 2011) has demonstrated that mutant p53 cells are not able to respond to bystander signals although in the situation where p53 is mutated or knocked out, they can produce signals. In the model used here, the host rat is p53 wild type but the inoculated tumour is mutant. Thus it is necessary to postulate that the secretion of a systemic signal from the tumour capable of “disarming” the stress sensing mechanisms in the normal cells would bypass apoptosis in the cells damaged by radiation. While it might be plausible to predict this in the directly irradiated rats it is difficult to see why this would be communicated to unirradiated healthy cage mates. However,

in the literature, reference can be found to have whole body irradiation causing the secretion of volatiles, which make the irradiated rats socially unattractive to the cage mates (Frey et al., 2014). The effects of the volatiles caused the cage mates to develop compromised immune responses (Abramova & Surinov; Mukherjee, Coates, Lorimore, & Wright, 2014). Possibly, the irradiation of tumour tissue releases different volatiles, which neutralise the other bystander effects on the normal tissues as part of a strategy of the tumour to evade being attacked by the immune system. A key to resolving these questions might be to estimate the relative volume of tumour to normal tissue irradiated in the microbeam protocol.

Analysis of calcium flux in cells in vitro (Liu et al., 2007; Fiona M Lyng, Seymour, & Mothersill, 2002) suggested that a sharp transient calcium flux triggered the response in cells receiving bystander signals. Investigation of various stress pathways suggested a role for the MAPK pathway leading to induction of apoptosis (F. M. Lyng et al., 2006). Therefore in these experiments calcium flux was measured to see if the flux was associated with reporter cell death where the tissue generating signals had or had not been directly irradiated. By analyzing the directly irradiated rats and the unirradiated companion cage mates, we hoped to distinguish bystander effects in the companions from abscopal effects in the irradiated animal and to confirm a role for the presence of tumour cells in determining the response. As expected, the data in Table 1 do show positive correlations between death and calcium flux where the right brain

tissue was directly irradiated. However the left brain and bladder tissues in the directly irradiated rats show no correlations either for normal or tumour bearing tissues. This suggests that the abscopal effects seen in these tissues are not related to the calcium flux pathway. Moreover, the calcium data versus % of cell death from table 1 shows significant positive correlations for the right brain, while the linear regressions show very low r^2 values. Therefore, the biological significance of a linear relationship across all doses may be spurious, a conclusion that extends to the cage mate data. The calcium flux versus fractional cell death data for the cage mates are much more difficult to explain. While companions of irradiated normal rats showed significant negative and positive correlations for the right and left brain respectively, the companion cage mate showed a reverse effect when a tumour was present in the irradiated rat. These observations suggest that the tumour modifies the pathways for abscopal and bystander effects. The data for the bladder explants from directly irradiated and unirradiated companion animals where the clonogenic survival suggested that the signals in the conditioned medium caused a significant positive growth stimulating effect reveal no correlation between calcium flux and growth stimulation. This again suggests that the pathways involved in the abscopal and bystander effects in these experiments do not involve the calcium flux which is associated with the directly irradiated right brain tissues. It is likely that downstream secondary signalling pathways are induced and that primary calcium

signalling is confined to the tissue that actually received direct radiation energy deposition.

Another interesting result is the strain difference between Wistar and Fisher rats. While strain differences in radiation response are well known as was discussed earlier, this is a strain difference not just involving the tissues of the directly irradiated rats but involving the communication between the irradiated rats and their cage mates and the level of induced response in the cage mates. In one case the response is the same in all tissues of the cage mates but in the other it varies in the same way as the abscopal response. This could have major implications for research into individual radiosensitivity.

Finally since synchrotron microbeam irradiation is mainly tested in the treatment of aggressive brain tumours giving the therapist the opportunity to focus a very high X-ray dose in a small tissue volume in such a way that maximal protection of normal tissue is achieved, it is important to consider the implications of our findings for this type of therapy. Early use of MRT considered the peak and valley doses to be key to achieving normal tissue sparing (F. Avraham Dilmanian et al., 2007; Tomita, Maeda, Maezawa, Usami, & Kobayashi, 2010). The existence of bystander effects was well known and communication of bystander signals was considered in the field but not at all understood. In our experiments the peak entrance dose of 200Gy to cells in the path of the beam is associated

with a 20Gy dose in the “valleys” between the microbeam tracks. Both these doses are several orders of magnitude larger than the in vitro threshold doses of around 2-3mGy for triggering bystander effects. Bystander effects are known to saturate at a dose of about 0.5 Gy and a further increase in dose (at least up to 10 Gy) does not increase the level of signal (Seymour & Mothersill, 2000). This means that bystander signalling will be saturated in the dose valleys as well as in the peak dose zones. In terms of impacts therefore, the question arises whether bystander signals from normal tissue are amplifying the harmful effects of radiation or are enabling beneficial effects and whether counter effects expressed by irradiated tumour cells (peak or valley) are having any effect. Both harmful and beneficial effects have been reported but the factors determining which response occurs are not known. Clearly more work is needed using biomarkers for damage such as γ-H2AX already identified as a useful marker (Sokolov, Dickey, Bonner, & Sedelnikova, 2007) and for repair/protective effects using markers such as 53bp1 which indicate induction of repair (Tartier, Gilchrist, Burdak-Rothkamm, Folkard, & Prise, 2007).

In conclusion, the work reported here suggests that the presence of tumour tissue in the irradiated brain can modulate the abscopal effect in other organs of the directly irradiated animal and modify bystander response in unirradiated companion cage mates. The data taken together with earlier studies also suggest strain differences in these in vivo bystander responses. The

implications for targeted radiotherapy using MRT are unknown and in need of further study.

4.6 ACKNOWLEDGEMENTS

We thank Dr. Géraldine Le Duc as leader of the ID 17 animal facility and Ms. Hélène Bernard for taking care of our animals during the irradiation experiments at the ESRF. Dr. E. Schültke holds an EU Marie Curie Reintegration Grant (PIRG-GA-2010-268250). The work has been performed with the support of the TD1205 "SYRA3" COST Action project and the ESRF funded proposal MD 736. We acknowledge support from the Canada Research Council Canada Research Chairs programme, The National Science and Engineering Research Council of Canada's Discovery Grant Programme and the Government of Chile for funding Cristian Fernandez-Palomo's PhD studies through Becas Chile.

4.7 REFERENCES

Abramova, M. R., & Surinov, B. P. Attractive and immunosuppressive properties of volatile secretions induced in mice separately and combine influence of ionizing radiation and cyclophosphamide. *Radiatsionnaia Biologiia, Radioecologiia / Rossiiskaia Akademiia Nauk*, *50*(1), 74–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20297684

Adam, J.-F., Joubert, A., Biston, M.-C., Charvet, A.-M., Peoc'h, M., Le Bas, J.-F., … Elleaume, H. (2006). Prolonged survival of Fischer rats bearing F98 glioma after iodine-enhanced synchrotron stereotactic radiotherapy. *International Journal of Radiation Oncology, Biology, Physics*, *64*(2), 603–11. doi:10.1016/j.ijrobp.2005.09.004

Akudugu, J. M., Azzam, E. I., & Howell, R. W. (2012). Induction of lethal bystander effects in human breast cancer cell cultures by DNA-incorporated Iodine-125 depends on phenotype. *International Journal of Radiation Biology*, *88*(12), 1028–38. doi:10.3109/09553002.2012.683511

Asai, A., Miyagi, Y., Sugiyama, A., Gamanuma, M., Hong, S. H., Takamoto, S., … Kuchino, Y. (1994). Negative effects of wild-type p53 and s-Myc on cellular growth and tumorigenicity of glioma cells. Implication of the tumor suppressor genes for gene therapy. *Journal of Neuro-Oncology*, *19*(3), 259–68. doi:10.1007/BF01053280

Audette-Stuart, M., Kim, S. B., McMullin, D., Festarini, a, Yankovich, T. L., Carr, J., & Mulpuru, S. (2011). Adaptive response in frogs chronically exposed to low doses of ionizing radiation in the environment. *Journal of Environmental Radioactivity*, *102*(6), 566–73. doi:10.1016/j.jenvrad.2011.03.001

Audette-Stuart, M., & Yankovich, T. (2011). Bystander effects in bullfrog tadpoles. *Radioprotection*, *46*(6), S497–S497. doi:http://dx.doi.org/10.1051/radiopro/20116770

Barth, R. F., & Kaur, B. (2009). Rat brain tumor models in experimental neuro-oncology: the C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. *Journal of Neuro-Oncology*, *94*(3), 299–312. doi:10.1007/s11060-009-9875-7

Bartzsch, S., Lerch, M., Petasecca, M., Bräuer-Krisch, E., & Oelfke, U. (2014). Influence of polarization and a source model for dose calculation in MRT. *Medical Physics*, *41*, 041703. doi:10.1118/1.4867858

Bartzsch, S., & Oelfke, U. (2013). A new concept of pencil beam dose calculation for 40-200 keV photons using analytical dose kernels. *Medical Physics*, *40*, 111714. doi:10.1118/1.4824150

Bartzsch, S., & Tag, J. (2014, December 11). *Microbeam Radiation Therapy – physical and biological aspects of a new cancer therapy and development of a treatment planning system*. Retrieved from http://archiv.ub.uni-heidelberg.de/volltextserver/17714/1/Arbeit_Gesamt.pdf

Benadjaoud, M. A., Bezin, J., Veres, A., Lefkopoulos, D., Chavaudra, J., Bridier, A., … Diallo, I. (2012). A multi-plane source model for out-of-field head scatter dose calculations in external beam photon therapy. *Physics in Medicine and Biology*, *57*(22), 7725–39. doi:10.1088/0031-9155/57/22/7725

Bencokova, Z., Pauron, L., Devic, C., Joubert, A., Gastaldo, J., Massart, C., … Foray, N. (2008). Molecular and cellular response of the most extensively used rodent glioma models to radiation and/or cisplatin. *Journal of Neuro-Oncology*, *86*(1), 13–21. doi:10.1007/s11060-007-9433-0

Biston, M.-C., Joubert, A., Adam, J.-F., Elleaume, H., Bohic, S., Charvet, A.-M., … Balosso, J. (2004). Cure of Fisher rats bearing radioresistant F98 glioma treated with cis-platinum and irradiated with monochromatic synchrotron X-rays. *Cancer Research*, *64*(7), 2317–23. doi:10.1158/0008-5472.CAN-03-3600

Blattmann, H., Gebbers, J.-O., Bräuer-Krisch, E., Bravin, A., Le Duc, G., Burkard, W., … Laissue, J. a. (2005). Applications of synchrotron X-rays to radiotherapy. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 17–22. doi:10.1016/j.nima.2005.03.060

Blyth, B. J., & Sykes, P. J. (2011). Radiation-induced bystander effects: what are they, and how relevant are they to human radiation exposures? *Radiation Research*, *176*(2), 139–57. doi:10.1667/RR2548.1

Boukamp, P., Popp, S., Bleuel, K., Tomakidi, E., Bürkle, A., & Fusenig, N. E. (1999). Tumorigenic conversion of immortal human skin keratinocytes (HaCaT) by elevated temperature. *Oncogene*, *18*(41), 5638–45. doi:10.1038/sj.onc.1202934

Bräuer-Krisch, E., Requardt, H., Brochard, T., Berruyer, G., Renier, M., Laissue, J. a, & Bravin, a. (2009). New technology enables high precision multislit collimators for microbeam radiation therapy. *The Review of Scientific Instruments*, *80*(7), 074301. doi:10.1063/1.3170035

Bräuer-Krisch, E., Serduc, R., Siegbahn, E. a, Le Duc, G., Prezado, Y., Bravin, A., … Laissue, J. A. (2010). Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. *Mutation Research*, *704*(1-3), 160–6. doi:10.1016/j.mrrev.2009.12.003

Butterworth, K. T., Redmond, K. M., McMahon, S. J., Cole, a J., McCarthy, H. O., O'Sullivan, J. M., … Prise, K. M. (2014). Conventional in vivo irradiation procedures are insufficient to accurately determine tumor responses to non-uniform radiation fields. *International Journal of Radiation Biology*, 1–16. doi:10.3109/09553002.2014.980468

Chai, Y., Calaf, G. M., Zhou, H., Ghandhi, S. a, Elliston, C. D., Wen, G., … Hei, T. K. (2013). Radiation induced COX-2 expression and mutagenesis at non-targeted lung tissues of gpt delta transgenic mice. *British Journal of Cancer*, *108*(1), 91–8. doi:10.1038/bjc.2012.498

Chinnadurai, M., Paul, S. F. D., & Venkatachalam, P. (2013). The effect of growth architecture on the induction and decay of bleomycin and X-ray-induced bystander response and genomic instability in lung adenocarcinoma cells and blood lymphocytes. *International Journal of Radiation Biology*, *89*(2), 69–78. doi:10.3109/09553002.2012.726397

Dilmanian, F. a, Qu, Y., Liu, S., Cool, C. D., Gilbert, J., Hainfeld, J. F., … McDonald, J. W. (2005). X-ray microbeams: Tumor therapy and central nervous system research. *Nuclear Instruments & Methods in Physics Research. Section A, Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 30–37. doi:10.1016/j.nima.2005.03.062

Dilmanian, F. A., Button, T. M., Le Duc, G., Zhong, N., Peña, L. A., Smith, J. A. L., … Ren, B. (2002). Response of rat intracranial 9L gliosarcoma to microbeam radiation therapy. *Neuro-Oncology*, *4*(1), 26. doi:10.1016/j.ejrad.2008.04.055

Dilmanian, F. A., Morris, G. M., Zhong, N., Bacarian, T., Hainfeld, J. F., Kalef-Ezra, J., … Rosen, E. M. (2003). Murine EMT-6 carcinoma: high therapeutic efficacy of microbeam radiation therapy. *Radiation Research*, *159*(5), 632–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12710874

Dilmanian, F. A., Qu, Y., Feinendegen, L. E., Peña, L. A., Bacarian, T., Henn, F. A., … McDonald, J. W. (2007). Tissue-sparing effect of x-ray microplanar beams particularly in the CNS: is a bystander effect involved? *Experimental Hematology*, *35*(4 Suppl 1), 69–77. doi:10.1016/j.exphem.2007.01.014

Fernandez-Palomo, C., Bräuer-Krisch, E., Trippel, M., Schroll, C., Requardt, H., Bartzsch, S., … Schültke, E. (2013). DNA double strand breaks in the acute phase after synchrotron pencilbeam irradiation. *Journal of Instrumentation*, *8*(07), C07005–C07005. doi:10.1088/1748-0221/8/07/C07005

Fernandez-Palomo, C., Schültke, E., Smith, R., Bräuer-Krisch, E., Laissue, J., Schroll, C., … Mothersill, C. (2013). Bystander effects in tumor-free and tumor-bearing rat brains following irradiation by synchrotron X-rays. *International Journal of Radiation Biology*, *89*(6), 445–53. doi:10.3109/09553002.2013.766770

Frey, B., Rubner, Y., Kulzer, L., Werthmöller, N., Weiss, E.-M., Fietkau, R., & Gaipl, U. S. (2014). Antitumor immune responses induced by ionizing irradiation and further immune stimulation. *Cancer Immunology, Immunotherapy : CII*, *63*(1), 29–36. doi:10.1007/s00262-013-1474-y

Hatzi, V. I., Laskaratou, D. a, Mavragani, I. V, Nikitaki, Z., Mangelis, A., Panayiotidis, M. I., … Georgakilas, A. G. (2013). Non-targeted radiation effects in vivo: A critical glance of the future in radiobiology. *Cancer Letters*. doi:10.1016/j.canlet.2013.11.018

Henseleit, U., Zhang, J., Wanner, R., Haase, I., Kolde, G., & Rosenbach, T. (1997). Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *The Journal of Investigative Dermatology*. doi:10.1111/1523-1747.ep12340708

Ikeda, J., Tada, M., Ishii, N., Saya, H., Tsuchiya, K., Okaichi, K., … Van Meir, E. G. (2001). Restoration of endogenous wild-type p53 activity in a glioblastoma cell line with intrinsic temperature-sensitive p53

induces growth arrest but not apoptosis. *International Journal of Cancer. Journal International Du Cancer*, *94*(1), 35–43. doi:10.1002/ijc.1431

Ilnytskyy, Y., Koturbash, I., & Kovalchuk, O. (2009). Radiation-induced bystander effects in vivo are epigenetically regulated in a tissue-specific manner. *Environmental and Molecular Mutagenesis*, *50*(2), 105–13. doi:10.1002/em.20440

Isaeva, V. G., & Surinov, B. P. (2007). [Postradiation volatile secretion and development of immunosupression effectes by laboratory mice with various genotype]. *Radiatsionnaia Biologiia, Radioecologiia / Rossiĭskaia Akademiia Nauk*, *47*(1), 10–6. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17387990

Kalanxhi, E., & Dahle, J. (2012, October). The role of serotonin and p53 status in the radiation-induced bystander effect. *International Journal of Radiation Biology*. doi:10.3109/09553002.2012.711919

Kaminski, J. M., Shinohara, E., Summers, J. B., Niermann, K. J., Morimoto, A., & Brousal, J. (2005). The controversial abscopal effect. *Cancer Treatment Reviews*, *31*(3), 159–72. doi:10.1016/j.ctrv.2005.03.004

Kashino, G., Suzuki, K., Kodama, S., Watanabe, M., & Prise, K. M. (2013). Increased susceptibility to delayed genetic effects of low dose X-irradiation in DNA repair deficient cells. *International Journal of Radiation Biology*, *89*(4), 295–300. doi:10.3109/09553002.2013.752596

Ko, L., Koestner, a., & Wechsler, W. (1980a). Characterization of cell cycle and biological parameters of transplantable glioma cell lines and clones. *Acta Neuropathologica*, *51*(2), 107–111. doi:10.1007/BF00690451

Ko, L., Koestner, A., & Wechsler, W. (1980b). Morphological Characterization of Nitrosourea-induced Glioma Cell Lines and Clones *. *Acta Neuropathologica*, *51*, 23–31.

Ko, M., Lao, X.-Y., Kapadia, R., Elmore, E., & Redpath, J. L. (2006). Neoplastic transformation in vitro by low doses of ionizing radiation: role of adaptive response and bystander effects. *Mutation Research*, *597*(1-2), 11–7. doi:10.1016/j.mrfmmm.2005.08.013

Koturbash, I., Zemp, F., Kolb, B., & Kovalchuk, O. (2011). Sex-specific radiation-induced microRNAome responses in the hippocampus, cerebellum and frontal cortex in a mouse model. *Mutation Research*, *722*(2), 114–8. doi:10.1016/j.mrgentox.2010.05.007

Laissue, J. a, Bartzsch, S., Blattmann, H., Bräuer-Krisch, E., Bravin, A., Dalléry, D., … Slatkin, D. N. (2013). Response of the rat spinal cord to X-ray microbeams. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, *106*(1), 106–11. doi:10.1016/j.radonc.2012.12.007

Lehman, T. A., Modali, R., Boukamp, P., Stanek, J., Bennett, W. P., Welsh, J. A., … Rogan, E. M. (1993). p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis*, *14*(5), 833–839.

Liu, Z., Mothersill, C. E., McNeill, F. E., Lyng, F. M., Byun, S. H., Seymour, C. B., & Prestwich, W. V. (2006). A dose threshold for a medium transfer bystander effect for a human skin cell line. *Radiation Research*, *166*(1 Pt 1), 19–23. doi:10.1667/RR3580.1

Liu, Z., Prestwich, W. V, Stewart, R. D., Byun, S. H., Mothersill, C. E., McNeill, F. E., & Seymour, C. B. (2007). Effective target size for the induction of bystander effects in medium transfer experiments. *Radiation Research*, *168*(5), 627–30. doi:10.1667/RR1014.1

Lorimore, S. a, Mukherjee, D., Robinson, J. I., Chrystal, J. a, & Wright, E. G. (2011). Long-lived inflammatory signaling in irradiated bone marrow is genome dependent. *Cancer Research*, *71*(20), 6485–91. doi:10.1158/0008-5472.CAN-11-1926

Lorimore, S. a, Rastogi, S., Mukherjee, D., Coates, P. J., & Wright, E. G. (2013). The influence of p53 functions on radiation-induced inflammatory bystander-type signaling in murine bone marrow. *Radiation Research*, *179*(4), 406–15. doi:10.1667/RR3158.2

Lyng, F. M., Maguire, P., McClean, B., Seymour, C., & Mothersill, C. (2006). The Involvement of Calcium and MAP Kinase Signaling Pathways in the Production of Radiation-Induced Bystander Effects. *Radiation Research*, *165*(4), 400–409. doi:10.1667/RR3527.1

Lyng, F. M., Seymour, C., & 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*(4), 365–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893237

Maguire, P., Mothersill, C., McClean, B., Seymour, C., & Lyng, F. M. (2007). Modulation of radiation responses by pre-exposure to irradiated cell conditioned medium. *Radiation Research*, *167*(4), 485–92. doi:10.1667/RR0159.1

Mancuso, M., Pasquali, E., Giardullo, P., Leonardi, S., Tanori, M., Di Majo, V., … Saran, A. (2012). The radiation bystander effect and its potential implications for human health. *Current Molecular Medicine*, *12*(5), 613–24. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22452594

Mancuso, M., Pasquali, E., Leonardi, S., Rebessi, S., Tanori, M., Giardullo, P., … Saran, a. (2011). Role of connexin43 and ATP in long-range bystander radiation damage and oncogenesis in vivo. *Oncogene*, *30*(45), 4601–8. doi:10.1038/onc.2011.176

Mothersill, C., Bristow, R. G., Harding, S. M., Smith, R. W., Mersov, A., & Seymour, C. B. (2011). A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells. *International Journal of Radiation Biology*, *87*(11), 1120–5. doi:10.3109/09553002.2011.610866

Mothersill, C., Bucking, C., Smith, R., Agnihotri, N., Oneill, a, Kilemade, M., & Seymour, C. (2006). Communication of radiation-induced stress or bystander signals between fish in vivo. *Environmental Science & Technology*, *40*(21), 6859–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17144322

Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., … Seymour, C. (2014). Transmission of signals from rats receiving high doses of microbeam radiation to cage mates: an inter-mammal bystander effect. *Dose-Response : A Publication of International Hormesis Society*, *12*(1), 72–92. doi:10.2203/dose-response.13-011.Mothersill

Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S., & Wright, E. (2005). Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. *Radiation Research*, *163*(4), 391–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15799694

Mothersill, C., Rea, D., Wright, E. G., Lorimore, S. A., Murphy, D., Seymour, C. B., & O'Malley, K. (2001). Individual variation in the production of a "bystander signal" following irradiation of primary cultures of normal human urothelium. *Carcinogenesis*, *22*(9), 1465–1471. doi:10.1093/carcin/22.9.1465

Mothersill, C., Saroya, R., Smith, R. W., Singh, H., & Seymour, C. B. (2010). Serum serotonin levels determine the magnitude and type of bystander effects in medium transfer experiments. *Radiation Research*, *174*(1), 119–23. doi:10.1667/RR2036.1

Mothersill, C., & Seymour, C. (1997). Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology*, *71*(4), 421–7. doi:10.1080/095530097144030

Mothersill, C., & Seymour, C. (2001). Radiation-induced bystander effects: past history and future directions. *Radiation Research*, *155*(6), 759–767. doi:10.1667/0033-7587(2001)155[0759:RIBEPH]2.0.CO;2

MotherSill, C., & Seymour, C. (2012). Changing paradigms in radiobiology. *Mutation Research/Reviews in Mutation Research*, *750*(2), 85–95. doi:10.1016/j.mrrev.2011.12.007

Mothersill, C., & Seymour, C. B. (1998). Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiation Research*, *149*(3), 256–262. doi:10.2307/3579958

Mothersill, C., Seymour, C. B., & Joiner, M. C. (2002). Relationship between radiation-induced low-dose hypersensitivity and the bystander effect. *Radiation Research*, *157*(5), 526–32. doi:10.1667/0033-7587(2002)157[0526:RBRILD]2.0.CO;2

Mothersill, C., Seymour, R. J., & Seymour, C. B. (2004). Bystander effects in repair-deficient cell lines. *Radiation Research*, *161*(3), 256–63. doi:10.1667/RR3136

Mothersill, C., Seymour, R. J., & Seymour, C. B. (2006). Increased radiosensitivity in cells of two human cell lines treated with bystander medium from irradiated repair-deficient cells. *Radiation Research*, *165*(1), 26–34. doi:10.1667/RR3488.1

Mothersill, C., Smith, R. W., Agnihotri, N., & Seymour, C. B. (2007). Characterization of a radiation-induced stress response communicated in vivo between zebrafish. *Environmental Science & Technology*, *41*(9), 3382–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17539553

Mothersill, C., Smith, R. W., Hinton, T. G., Aizawa, K., & Seymour, C. B. (2009). Communication of radiation-induced signals in vivo between DNA repair deficient and proficient medaka (Oryzias latipes). *Environmental Science & Technology*, *43*(9), 3335–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19534155

Mothersill, C., Smith, R., Wang, J., Fernandez-Palomo, C., Fazzari, J., Schültke, E., … Seymour, C. (n.d.). Proteomic analysis of brains from rats receiving high doses of microbeam irradiation and their cage mates. *International Journal of Radiation Biology*, *Submitted*.

Mukherjee, D., Coates, P. J., Lorimore, S. a, & Wright, E. G. (2014). Responses to ionizing radiation mediated by inflammatory mechanisms. *The Journal of Pathology*, *232*(3), 289–99. doi:10.1002/path.4299

Munro, A. J. (2009). Bystander effects and their implications for clinical radiotherapy. *Journal of Radiological Protection : Official Journal of the Society for Radiological Protection*, *29*(2A), A133–42. doi:10.1088/0952-4746/29/2A/S09

Nenoi, M., Wang, B., & Vares, G. (2014). In vivo radioadaptive response: A review of studies relevant to radiation-induced cancer risk. *Human & Experimental Toxicology*. doi:10.1177/0960327114537537

O'Dowd, C., Mothersill, C., 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 gamma radiation. *Radiation Research*, *166*(4), 611–7. doi:10.1667/RR0606.1

Poon, R. C. C., Agnihotri, N., Seymour, C., & Mothersill, C. (2007). Bystander effects of ionizing radiation can be modulated by signaling amines. *Environmental Research*, *105*(2), 200–11. doi:10.1016/j.envres.2006.12.003

Puck, T. T., & Marcus, P. I. (1956). Action of x-rays on mammalian cells. *The Journal of Experimental Medicine*, *103*(5), 653–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10380286

Rastogi, S., Coates, P. J., Lorimore, S. a, & Wright, E. G. (2012). Bystander-type effects mediated by long-lived inflammatory signaling in irradiated bone marrow. *Radiation Research*, *177*(3), 244–50. doi:10.1667/RR2805.1

Ruben, J. D., Smith, R., Lancaster, C. M., Haynes, M., Jones, P., & Panettieri, V. (2014). Constituent Components of Out-of-Field Scatter Dose for 18-MV Intensity Modulated Radiation Therapy Versus 3-Dimensional Conformal Radiation Therapy: A Comparison With 6-MV and Implications for Carcinogenesis. *International Journal of Radiation Oncology, Biology, Physics*, *90*(3), 645–53. doi:10.1016/j.ijrobp.2014.05.052

Ryan, L. a, Seymour, C. B., Joiner, M. C., & Mothersill, C. E. (2009). Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. *International Journal of Radiation Biology*, *85*(1), 87–95. doi:10.1080/09553000802635062

Saroya, R., Smith, R., Seymour, C., & Mothersill, C. (2009). Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism. *Dose-Response : A Publication of International Hormesis Society*, *8*(3), 317–30. doi:10.2203/dose-response.09-043.Saroya

Schettino, G., Folkard, M., Michael, B. D., & Prise, K. M. (2005). Low-Dose Binary Behavior of Bystander Cell Killing after Microbeam Irradiation of a Single Cell with Focused C K X Rays. *Radiation Research*, *163*(3), 332–336. doi:10.1667/RR3319

Schettino, G., Folkard, M., Prise, K. M., Vojnovic, B., Held, K. D., & Michael, B. D. (2003). Low-dose studies of bystander cell killing with targeted soft X rays. *Radiation Research*, *160*(5), 505–11. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14565833

Schlegel, J., Piontek, G., Kersting, M., Schuermann, M., Kappler, R., Scherthan, H., … Mennel, H. (1999). The p16/Cdkn2a/Ink4a gene is frequently deleted in nitrosourea-induced rat glial tumors. *Pathobiology : Journal of Immunopathology, Molecular and Cellular Biology*, *67*(4), 202–6. doi:28073

Schültke, E., Juurlink, B. H. J., Ataelmannan, K., Laissue, J., Blattmann, H., Bräuer-Krisch, E., … Fourney, D. (2008). Memory and survival after microbeam radiation therapy. *European Journal of Radiology*, *68*(3 SUPPL.), 142–146. doi:10.1016/j.ejrad.2008.04.051

Schültke, E., Trippel, M., Bräuer-Krisch, E., Renier, M., Bartzsch, S., Requardt, H., … Nikkhah, G. (2013). Pencilbeam irradiation technique for whole brain radiotherapy: technical and biological challenges in a small animal model. *PloS One*, *8*(1), e54960. doi:10.1371/journal.pone.0054960

Senatus, P. B., Li, Y., Mandigo, C., Nichols, G., Moise, G., Mao, Y., … Fine, R. L. (2006). Restoration of p53 function for selective Fas-mediated apoptosis in human and rat glioma cells in vitro and in vivo by a p53 COOH-terminal peptide. *Molecular Cancer Therapeutics*, *5*(1), 20–8. doi:10.1158/1535-7163.MCT-05-0181

Serduc, R., Bouchet, A., Bräuer-Krisch, E., Laissue, J. A., Spiga, J., Sarun, S., … Le Duc, G. (2009). Synchrotron microbeam radiation therapy for rat brain tumor palliation-influence of the microbeam width at constant valley dose. *Physics in Medicine and Biology*, *54*(21), 6711–24. doi:10.1088/0031-9155/54/21/017

Seymour, C. B., & Mothersill, C. (2000). Relative contribution of bystander and targeted cell killing to the low-dose region of the radiation dose-response curve. *Radiation Research*, *153*(5 Pt 1), 508–11. doi:10.1667/0033-7587(2000)153

Shields, L., Vega-Carrascal, I., Singleton, S., Lyng, F. M., & McClean, B. (2014). Cell Survival and DNA Damage in Normal Prostate Cells Irradiated Out-of-Field. *Radiation Research*, *182*(5), 499–506. doi:10.1667/RR13777.1

Singh, H., Saroya, R., Smith, R., Mantha, R., Guindon, L., Mitchel, R. E. J., … Mothersill, C. (2011). Radiation induced bystander effects in mice given low doses of radiation in vivo. *Dose-Response : A*

Publication of International Hormesis Society, *9*(2), 225–42. doi:10.2203/dose-response.09-062.Singh

Slatkin, D. N., Spanne, P., Dilmanian, F. a, Gebbers, J. O., & Laissue, J. A. (1995). Subacute neuropathological effects of microplanar beams of x-rays from a synchrotron wiggler. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(19), 8783–8787. doi:10.1073/pnas.92.19.8783

Slatkin, D. N., Spanne, P., Dilmanian, F. A., & Sandborg, M. (1992). Microbeam radiation therapy. *Medical Physics*, *19*(6), 1395–1400. doi:10.1118/1.596771

Smith, R. W., Wang, J., Bucking, C. P., Mothersill, C. E., & Seymour, C. B. (2007). Evidence for a protective response by the gill proteome of rainbow trout exposed to X-ray induced bystander signals. *Proteomics*, *7*(22), 4171–80. doi:10.1002/pmic.200700573

Smith, R. W., Wang, J., Schültke, E., Seymour, C. B., Bräuer-Krisch, E., Laissue, J. A., … Mothersill, C. E. (2013). Proteomic changes in the rat brain induced by homogenous irradiation and by the bystander effect resulting from high energy synchrotron X-ray microbeams. *International Journal of Radiation Biology*, *89*(2), 118–27. doi:10.3109/09553002.2013.732252

Sokolov, M. V., Dickey, J. S., Bonner, W. M., & Sedelnikova, O. A. (2007). gamma-H2AX in bystander cells: not just a radiation-triggered event, a cellular response to stress mediated by intercellular communication. *Cell Cycle (Georgetown, Tex.)*, *6*(18), 2210–2. doi:10.4161/cc.6.18.4682

Staudacher, A. H., Blyth, B. J., Lawrence, M. D., Ormsby, R. J., Bezak, E., & Sykes, P. J. (2010). If bystander effects for apoptosis occur in spleen after low-dose irradiation in vivo then the magnitude of the effect falls within the range of normal homeostatic apoptosis. *Radiation Research*, *174*(6), 727–31. doi:10.1667/RR2300.1

Strigari, L., Mancuso, M., Ubertini, V., Soriani, A., Giardullo, P., Benassi, M., … Bossi, G. (2014). Abscopal effect of radiation therapy: Interplay between radiation dose and p53 status. *International Journal of Radiation Biology*, *90*(3), 248–55. doi:10.3109/09553002.2014.874608

Sugihara, T., Murano, H., Nakamura, M., & Tanaka, K. (2013). In vivo partial bystander study in a mouse model by chronic medium-dose-rate γ-ray irradiation. *Radiation Research*, *179*(2), 221–31. doi:10.1667/RR3081.1

Surinov, B. P., Isaeva, V. G., & Dukhova, N. N. (2004). Postirradiation volatile secretions of mice: syngeneic and allogeneic immune and behavioral effects. *Bulletin of Experimental Biology and Medicine*, *138*(4), 384–6. doi:10.1007/s10517-004-0018-z

Tada, M., Matsumoto, R., Iggo, R. D., Onimaru, R., Shirato, H., Sawamura, Y., & Shinohe, Y. (1998). Selective sensitivity to radiation of cerebral glioblastomas harboring p53 mutations. *Cancer Research*, *58*(9), 1793–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9581814

Tartier, L., Gilchrist, S., Burdak-Rothkamm, S., Folkard, M., & Prise, K. M. (2007). Cytoplasmic irradiation induces mitochondrial-dependent 53BP1 protein relocalization in irradiated and bystander cells. *Cancer Research*, *67*(12), 5872–9. doi:10.1158/0008-5472.CAN-07-0188

Tomita, M., & Maeda, M. (2014). Mechanisms and biological importance of photon-induced bystander responses: do they have an impact on low-dose radiation responses. *J Radiat Res*, 1–15. doi:10.1093/jrr/rru099

Tomita, M., Maeda, M., Maezawa, H., Usami, N., & Kobayashi, K. (2010). Bystander cell killing in normal human fibroblasts is induced by synchrotron X-ray microbeams. *Radiation Research*, *173*(3), 380–5. doi:10.1667/RR1995.1

Yang, G., Mei, T., Yuan, H., Zhang, W., Chen, L., Xue, J., … Wang, Y. (2008). Bystander/abscopal effects induced in intact Arabidopsis seeds by low-energy heavy-ion radiation. *Radiation Research*, *170*(3), 372–80. doi:10.1667/RR1324.1

Yang, S.-H., Wang, S.-M., Syu, J.-P., Chen, Y., Wang, S.-D., Peng, Y.-S., … Kung, H.-N. (2014). Andrographolide induces apoptosis of C6 glioma cells via the ERK-p53-caspase 7-PARP pathway. *BioMed Research International*, *2014*, 312847. doi:10.1155/2014/312847

Zeng, J., Harris, T. J., Lim, M., Drake, C. G., & Tran, P. T. (2013). Immune modulation and stereotactic radiation: improving local and abscopal responses. *BioMed Research International*, *2013*, 658126. doi:10.1155/2013/658126

Chapter 5

5 INVESTIGATION OF ABSCOPAL AND BYSTANDER EFFECTS IN IMMUNECOMPROMISED MICE AFTER EXPOSURE TO PENCILBEAM AND MICROBEAM SYNCHROTRON RADIATION.

Cristian Fernandez-Palomo, Elisabeth Schültke, Elke Bräuer-Krisch, Jean Laissue, Hans Blattman, Colin Seymour, Carmel Mothersill

---

This research project was design by the first author, Mothersill and Schültke. Irradiations were performed by: the first author, Schültke, Bräuer-Krisch, and Seymour. Tissue harvesting, processing and clonogenic assays were performed by: Laissue, Blatmann, the first author, and Mothersill. The first author performed data collection and analysis. The manuscript was written by the first author & Mothersill.

The manuscript was submitted to the Journal of Health Physics on July 30th, 2015

5.1 ABSTRACT

Out-of-field effects are of considerable interest in radiotherapy. The mechanisms are poorly understood but are thought to involve signalling processes which induce responses in non-targeted cells and tissues. The immune response is thought to play a role. The goal of this research was to study the induction of abscopal effects in the bladders of immunocompromised NUDE mice after irradiating their brains using pencil Beam (PB) or microbeam (MRT) irradiation at the European Synchrotron Radiation Facility (ESRF) in Grenoble France. Athymic nude mice injected with F98 glioma cells into their right cerebral hemisphere 7 days earlier were treated with either MRT or PB. After recovery times of 2, 12 and 48h, the urinary bladders were extracted and cultured as tissue explants for 24h. The growth medium containing the potential signalling factors was harvested, filtered and transferred to HaCaT reporter cells to assess their clonogenic survival and calcium signalling potential. The results show that in the tumour free-mice both treatment modalities produce strong bystander/abscopal signals using the clonogenic reporter assay, however the calcium data does not support a calcium channel mediated mechanism.

The presence of tumour reduces or reverses the effect. PB produced significantly stronger effects in the bladders of tumour bearing animals kept for 48hrs before tissue harvest. We conclude that immune-compromised mice do produce signals which can alter the response of unirradiated reporter cells, however the mechanisms involve may be different.

Keywords: Synchrotron, Bystander, Abscopal, Athymic

5.2 INTRODUCTION

Current research on bystander effects i.e. effects detected in unirradiated cells receiving signals from irradiated cells is mainly conducted in vitro using two different methods; the microbeam irradiation of part of a cell culture or by allowing a non-irradiated culture to receive physical or medium borne signals from an irradiated culture. (Mothersill & Seymour, 1997; Prise, 1998; Zhou et al., 2000). Apart from the important mechanistic questions relating to how these effects occur, the other key research area concerns potential impacts of these so-called non-targeted effects in radiotherapy (Burdak-Rothkamm & Prise, 2009; Sun et al., 2014). Efforts to study pure bystander effects in vivo are complicated because irradiation of a part of the body does not preclude blood, endocrine and neural systems which receive irradiation from causing systemic effects confounding the mechanisms (Koturbash, Zemp, Kolb, & Kovalchuk, 2011; Mancuso et al., 2008). There is a considerable old literature on abscopal or out-of-field effects in radiobiology and radiotherapy. These date back to the early 20th century (Murphy and Morton 1915; Mothersill and Seymour 2012) but were officially named by RH Mole who coined the term “abscopal” in 1953 (Mole, 1953). In recent years the lines between in vivo bystander and abscopal effects have become blurred and the terms are often used interchangeably leading to assumptions that mechanisms identified in vitro may apply in vivo (Mothersill and Seymour 2012). A key system which is considered to be very relevant is the immune system. This has been investigated following irradiation in partially exposed animals in vivo

(Koturbash et al., 2008; Mancuso et al., 2008) and inflammatory responses in distant tissues have been detected. It has also been investigated in mice, which received total body irradiation to ablate the bone marrow followed by injection with opposite sex bone marrow stem cells. The repopulating cells showed high levels of genomic instability which could only have come from signals in the irradiated microenvironment (Watson et al. 2000; Watson et al. 2001). In vitro work on components of the immune system confirm a role for the inflammatory *response* in what is described as a bystander effect, although it is not clear if signal production requires an intact immune response capability system (Holyoake et al., 2001). Also the question of whether a functional immune system is necessary to produce bystander/abscopal effects in vivo still requires clarification. To address some of these issues, we participated in experiments using athymic (Nude) mice at the European Synchrotron Research Facility (ESRF) microbeam facility in Grenoble in France. Synchrotron microbeam radiation has been employed in the development of innovative methods to treat brain cancer. Efforts are focused on improving the Microbeam Radiation Therapy (MRT) developed originally at the Brookhaven National Laboratory in New York. ESRF and other international facilities are focusing on this task. Teams of scientists consistently show that MRT yields a higher therapeutic index when compared with broad beam irradiation when treating aggressive tumours such as the intracerebral rat F98 glioma (Biston et al. 2004, Schültke et al. 2008), or 9L gliosarcoma (Laissue et al. 1998; Dilmanian et al. 2002; Bouchet et al. 2013).

MRT has also been used for the palliation of mice bearing the aggressive murine squamous cell carcinomas VII (Miura et al. 2006) and the treatment of mammary tumours in mice (Crosbie et al 2010). MRT uses an array of <100 μm wide quasi-parallel rectangular beamlets, created by a high flux of synchrotron X-rays that are spatially fractionated by the insertion of a multislit collimator (Slatkin et al. 2010) . This configuration exposes the tissue to either peak-doses deposited by the photons of the microbeams or valley-doses resulting from scattered photons that hit the tissue between the microbeams (Blattman et al. 2005). The dose variation between peaks and valleys depends on the configuration and size of the beam array but mostly range between 2-10% of the peak dose (Bräuer-Krisch et al. 2009). In terms of volume of tissue exposed, valley doses would be measured in between 4 to 8 fold more brain tissue than peak doses, thus explaining the high normal tissue tolerance observed by this treatment. Tissue tolerance is also expressed by the ratio between peak-to-valley doses or (PVDR), which is a critical factor in decreasing the dose to sensible areas, such as the hippocampus (Slatkin et al. 2010).

The efficacy of MRT over broad beam has been attributed to the effects of high valley doses given in a single fraction and reinforced by the peak doses. The valley dose -is sufficiently high to damage the tumour microvasculature but low enough to avoid great deleterious effects in the normal tissue counterpart (Bräuer-Krisch et al. 2010) . Recently, research has also shown that MRT seems to stimulate the immune system by regulating an early expression of a vast

network of mediators such as growth factors, cytokines and lymphokines (Bouchet et al. 2013) . Furthermore, a comparison between MRT and broad beam revealed different molecular pathways involving the recruiting of tumour-associated immune cells (Yang et al. 2014). Dilmanian et al. (2007) have suggested that 6 hours after irradiation dying cells at the beam's edge seem to signal neighbouring cells and promote the fast disappearance of hit cells and structural damage. Data from our team have shown that bystander signals seem to extend the area where development of γ-H2AX foci can be seen from the microbeam's edge into the valley dose areas after peak doses of 350Gy (Fernandez-Palomo et al. 2015). Also, abscopal or radiation-induced out-of-field tissue/organ effects have been investigated by our group. For instance, proteomic analysis of the unirradiated left brain hemisphere in normal Wistar rats suggests that the MRT-induced proteome may result in protective effects in the unirradiated brain (Smith et al. 2013) and unirradiated brain tissue and unirradiated urinary bladder tissue explants originated from right brain MRT-irradiated animals, induced bystander effects in our well established reported cell system (Fernandez-Palomo et al. 2013).

While MRT research has so far been focused on the treatment of primary brain tumours, research led by Schültke and the design of a new treatment technique called pencilbeam therapy (PB) aims also to treat secondary brain malignancies (Schültke et al. 2013) . The majority of the drugs typically used to treat metastatic growth are not able to cross the blood-brain barrier (Pardridge

2005). Thus, patients with multiple brain metastases are often selected for whole brain radiotherapy (WBRT) simply because it is the only option available. However, the most common problem reported after WBRT are chronic changes in the white matter, which are associated with stroke-like migraines (Kerklaan et al. 2011; Black et al. 2013; Armstrong et al. 2014) , cognitive deficits (Shi et al. 2009; Peiffer et al. 2014; Forbes et al. 2014) and dementia (DeAngelis et al. 1989; D'Ambrosio et al. 2007; Tallet et al. 2012). Following the principle of MRT, PB delivers extremely high doses of synchrotron X-rays along very narrow tracks to the tumour while sparing the normal tissue (Schültke et al. 2013). Since the tissue volume receiving peak doses is even smaller than with monoplanar MRT it is hoped that as a consequence the normal tissue tolerance is increased compared to monoplanar MRT. This could improve greatly cancer therapy outcomes after whole brain exposure. The work by Schültke et al. tested for motor and memory function after delivering PB to normal C57 BL/6J mice. Animals recovered well compared to controls after peak doses of 800 Gy and a peak center-to-center distance of 400 μm (Schültke et al. 2013). Also, in-situ brain studies using the γ-H2AX marker demonstrated that the geometry of PB was preserved after whole-brain irradiation and that DNA damage was correlated with the dose [Fernandez-Palomo et al, 2013a]. However, more preclinical studies are still needed in particular to investigate mechanisms and to access the role of bystander effects in the tissue sparing seen with MRT and the newer PB.

Previous experiments using rats (Fernandez-Palomo et al. 2013a, 2015b) had shown that bystander effects could be detected in the contralateral brain and bladder of rats receiving right brain irradiation using MRT. It was also shown that the presence of a tumour experimentally introduced into the brain could reduce the strength of the signals measured as the ability of culture media from the tissue to reduce the cloning efficiency of a reporter cell line, and the ability to cause a calcium flux in reporter cells – both well established assays (Lyng, Seymour, & Mothersill, 2000; Mothersill & Seymour, 1998). In the current experiments we measured signal strength from the bladder as a distant and truly abscopal organ in nude mice receiving whole brain exposure to either MRT or PB irradiation from the synchrotron.

5.3 MATERIALS AND METHODS

5.3.1 Animal Model:

Male adult athymic nude mice (Charles River, France), were used as animal models. The animals were housed and cared in the ESRF Animal Facility following a 12h light/dark cycle in agreement with French and Canadian Animal Care Protocols. The mice are immunodeficient because the absence of a thymus makes them poor in T-cells [Ikehara et al, 1984]. This strain originated in 1969, when Dr Rygaard paired the spontaneous hairless mutant "nude" mouse (which

showed absence of thymus) with the NMRI strain (which had high viability and fertility) [Rygaard, 1969]. This finally creating a suitable mouse model for hosting tumour xenografts.

5.3.2 Tumour Inoculation:

The tumour cell line selected was the F98 glioma, which shows mutant p53 and shares a wide range of characteristics with the glioblastoma multiforme (GBM) (Barth and Kaur, 2009) . The F98 glioma cells have an infiltrative pattern of growth in the brain and they are weakly immunogenic (Tzeng et al. 1969) . This tumour model is often used in studies involving conventional radiotherapy and synchrotron radiation (Gil et al. 2011; Desmarais et al. 2012, 2015].

For these experiments, F98 cells were obtained from ATCC and grown in 75 cm^2 flasks containing 25 ml of Dulbecco's Modified Eagle Medium (GIBCO, France), supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin. A 90% confluent flask was selected and cells were detached by incubation with 20 ml of calcium and magnesium-free Hank's Balanced Salt Solution (Gibco, France), during 20 minutes at 37°C in an atmosphere of 5% CO_2 in air. The cell suspension is centrifuged at 1000 rpm for 5 min, the pellet re-suspended in 1ml of fresh culture medium and counted using a haemocytometer. Athymic mice are anaesthetized using 1.5% isofluorane in 2 L/min compressed air, before and during the surgical procedure. A vertical

incision of 1.5 to 2 cm is made on the skin following the sagittal plane. A hole is then drilled in the skull above the right hemisphere 3 mm to the right of the midline and 3 mm posterior to the coronal suture. Then a volume of 2 µl of culture medium containing 10,000 F98 cells is carefully inoculated 2.5 mm into the brain and the incision is closed 2 minutes after inoculation. Animals are supervised until they recovered and the housed for seven days to allow for tumour development.

5.3.3 Irradiations:

In preparation for the irradiations, mice were deeply anaesthetised using a cocktail of Ketamine/Xylazine (1 ml of 10% ketamine, 0,5ml of 2% xylazine, 8.5 ml non-saline solution) at 0.01 ml/g. Each irradiation group had 3 male mice and 1 female, which were individually positioned on the goniometer and received the radiation dose corresponding to its group. MRT and PB were delivered laterally to the whole brain in a right to left direction. Animals were allowed to recover for 2, 12 and 48 hours after irradiation and the urinary bladder was used for the study of abscopal effects while the brains served as material for other studies not shown in this paper.

Animals were exposed to valley doses of 0.5 Gy and 2.5 Gy in a single treatment session. Peak entry doses were adjusted accordingly to achieve the valley doses. The beam size of 8 mm high and 20 mm wide remained constant at

all times. Animals irradiated with MRT were exposed to 22 Gy or 110 Gy skin-entry doses, while the animals receiving PB were exposed to 200Gy and 1000Gy respectively. The beam was set in a lateral view by locating its right side 4mm toward the nose from the eye, and from that point it extended 20 mm to the left (Figure 5.1). The peak entry doses correspond to the dose at 3 mm depth. They were calculated prior to the experiments using a solid water phantom (Gammex) and a pinpoint ion-chamber (PRW 31014). The peak entry and valley doses are then converted with Monte Carlo pre-calculated output factors into the desired beam size. Valley doses are also calculated using a Treatment Planning System based on CT data from previous experiments on rats. Although benchmarking of the estimated doses is still in progress, Gafchromic film dosimetry agrees within 10% with the computed valley doses (Bartzsch and Tag, 2014) .

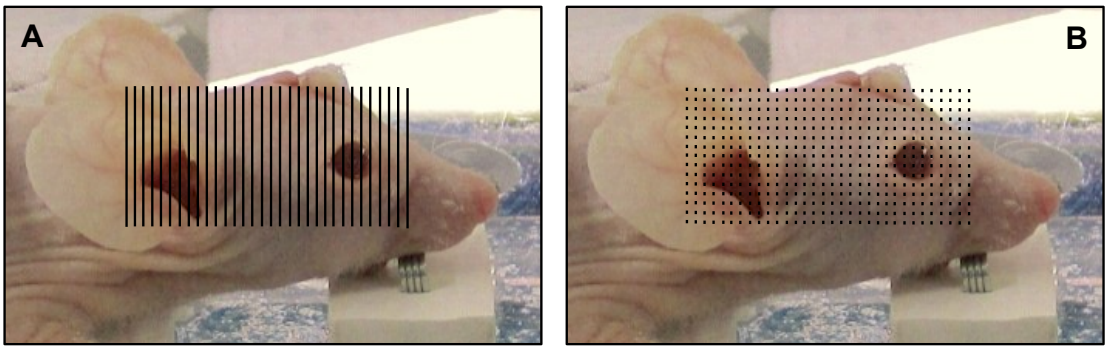

Figure 5.1 Schematic representation of the irradiation modalities.
This figure graphically shows the geometrical differences between the two arrays studied. Athymic Nude mice were exposed to whole brain irradiation of A) MRT or B) PB. The dimensions of actual beam delivered were 8 mm high & 20 mm wide. MRT: Microbeam Radiation Therapy; PB: Pencilbeam Therapy

The array of multichromatic pencil beams was generated by a multislit collimator with a mean energy of 105 keV (Bräuer-Krisch et al. 2009). The typical dose rate during these experiments was ~14,000 Gy/sec. MRT was composed by rectangular quasi-parallel 50 μm wide microbeams with intermediate gaps of 400 μm. The PB array was formed by square quasi-parallel 50x50 μm microbeams, with 400 μm of intermediate distance. Untreated controls for both normal and tumour-bearing animals were included. HD-610 and MD-55 Gafchromic Films (ISP Advanced Materials) were used to verify all irradiation doses and modalities applied. After irradiation, mice were taken back to the ESRF animal facility for recovery in their correspondent treatment groups.

Previous studies show that scatter radiation does not play a role in the induction of abscopal and bystander effect after Synchrotron irradiation. However, we decided to control for scatter because this is the first time that bystander effects have been studied after PB. The scatter dose was measured at the level of the urinary bladder after 200Gy MRT, which is equivalent to 1000 Gy PB. The dose at the site was 1.36 mGy. Thus, an X-ray generator was used to deliver a whole-body dose of 1.36 mGy after adequate adjustment of the dose rate. All mice were moved back to the ESRF animal facility after irradiation. Untreated controls remained in the ESRF animal facility and never left the cage.

5.3.4 Sampling, explant tissue culture and culture medium harvest:

All animals received anaesthesia before euthanasia. Urinary bladders were extracted 2, 12 and 48 hours after irradiation. Immediately they were single-placed in 5 ml sterile tubes containing 1mL of Roswell Park Memorial Institute growth medium (RPMI 1640, Gibco, Canada). Supplemented with 10% FBS, 5ml of Penicillin-Streptomycin (Gibco, Canada), 5ml of L-glutamine (Gibco, Canada), 0.5 mg/ml of Hydrocortisone (Sigma-Aldrich, Canada), and 12.5 ml of 1M HEPES buffer solution (Gibco, Canada). Tissue samples were put on ice and immediately transported to the biosafety level 2 laboratory of the ESRF biomedical beamline.

Urinary bladders were cut into 3 equal-size pieces of approximately 2 mm^3 in a biosafety cabinet. The pieces were individually placed in the centre of a 25 cm^2 growth area flask (Falcon), containing 2 ml of the complete growth medium previously described. Flasks were then placed in an incubator set at 37°C, 5% CO_2 in air and left undisturbed during 24h to allow for the release of bystander signals. The irradiated-tissue conditioned medium (ITCM) was harvested at 24 hours, filtered using a sterile 0.22 μm filter (Acrodisc Syringe Filter with HT Tuffryn Membrane, Pall Life Sciences), and placed in a sterile 7mL tube. The collected media was kept at 4ºC in the dark and then transported to McMaster University for clonogenic reporter bioassays.

5.3.5 Clonogenic Reporter Cell Line:

The cell line selected as the reporter was the human epithelial HaCaT, which has been used by our group at McMaster University for several years due to the line's reliable and stable response to bystander signals (Mothersill & Seymour, 1997). The cell line was originally derived by Dr Petra Boukamp in Germany and was given to us as a kind gift. The line was developed from normal human skin that surrounded a melanoma and became immortal spontaneously (Boukamp et al, 1999) . Although, HaCaT cells have 3 p53 point mutations (Lehman et al. 1993), data show that the line remains functional with respect to inducing apoptosis and reproductive death and behaves as though wild-type p53 were present (Henseleit et al. 1997). In our laboratory HaCaTs also behave like wild-type cells in terms of the bystander effect response. The HaCaT cells were maintained in a 75 cm^2 grown area flask (Falcon) with RPMI 1640 supplemented as previously indicated. Cells 95% confluent were detached using 1:1 (v:v) solution of 0.02 % Trypsin/EDTA (1mM) (Gibco, Canada) and Dulbecco's Phosphate-Buffered Solution (1x) (Gibco, Canada). The number of cells was measured using an automatic cell counter (Beckman Coulter).

5.3.6 Clonogenic reporter bioassay:

Flasks containing 90-95% confluent HaCaT cells were selected for the experiments. Reporter flasks of 25 cm^2 growth area were seeded with 500 cells 6

hours before the medium transfer. Then the conditioned medium harvested in France was transferred into the reporter flasks. Plating efficiency and medium transfer controls were also set up. All flasks were then transferred into an incubator and left undisturbed for about 10-12 days to allow for colony formation using the technique developed by Puck and Marcus (1956) . Colonies were then stained using a 1:4 (v/v) solution of Carbol Fuchsin (Ricca Chemical Company) in water. Colonies with more than 50 cells were scored as survivors and the survival fraction was calculated using the plating efficiency (PE) of the reporter cells as described in previous research (Fernandez-Palomo et al. 2011, 2015).

Equation 5.1Survival Fraction

$$Plating\ Efficiency\ (PE) = \frac{\#\ of\ Colonies}{\#\ of\ Cells\ seeded} \times 100$$

5.3.7 Ratiometric calcium measurements

The following protocol was initially developed by Dr. Fiona Lyng, DIT, Dublin, Ireland (ref). 100,000 HaCaT cells were seeded in Glass Bottom Dishes (MatTek Corporation) containing 2 ml of cell culture media RPMI-1640 (Gibco, Oakville, Canada) supplemented with 10% FBS, and placed in a incubator at 37° C in an atmosphere of, 5% CO_2 in air for 24 hours. For calcium measurements,

the culture medium was discarded and the cells were washed gently three times with Hank's Balanced Salt Solution (HBSS) with calcium and magnesium (Cat#: 14025-092, Gibco, Oakville, Canada), supplemented with 25 mM of HEPES (Gibco, Oakville, Canada). The HBSS was discarded and cells were loaded with 200 ml of 8.4 mM of Fura-2/AM (Sigma-Aldrich, Milwaukee, USA), for 1 hour at room temperature in the dark. This protocol avoided the compartmentalization of the dye within the cellular organelles, which was a problem if cells were placed in an incubator at 37° C. At the end of the loading time, the Fura-2/AM was discarded, the cells were washed three times with HBSS, and 300 ml of the same buffer was added to the dish. Cells were observed with a x40 oil objective on an Olympus inverted fluorescent microscope (Olympus Canada, Richmond Hill, Canada) and images were captured with a CCD Cool-Snap HQ camera (Photometrics, Tucson, Arizona). For the measurements, 100 ml of conditioned medium (or test medium) is added onto the cells 90 seconds after acquisition starts. Fura 2/AM emits light at 510 nm when is excited at 380 nm and 340 nm. The ratio of emissions between those wavelengths correlates with the calcium flux through the cellular membrane which we have shown to be the earliest measurable response to the presence of bystander signal in the test medium (Lyng et al, 2000). After obtaining the calcium concentrations plotted as a function of time post addition of the test medium, , the data from each of ten randomly selected cells were analyzed and the area under the curve was calculated for each cell and meaned to give an average result per sample. Each

sample was repeated three times and there were 3 samples available to test from each bladder

5.3.8 Statistical Analysis

Survival fractions are presented as a standard deviation of the mean. Significance between and within groups was determined using the Tukey multi-comparison test after a two-way ANOVA. Data was defined as significant when p values were ≤ 0.05. Graphs were plotted using the Prism 6.0 software.

5.4 RESULTS

5.4.1 Clonogenic assay

Figure 5.2 shows the clonogenic survival of reporter HaCaT cells grown in ITCM originated from the urinary bladder of nude mice. This figure focused exclusively on comparing the effects of ITCM from normal and tumour-bearing mice. The data shows that the presence of the F98 glioma in the right hemisphere but in the absence of radiation significantly increased the survival of the reporter cells compared to the control group (p-value = 0.0061). Similarly, clonogenic survival was also increased when ITCM originated from scatter animals, which also had a tumour but were exposed to a whole body dose of 1.36 mGy (p-value = 0.0011). Comparing the Tumour Control and Scatter groups we

can also observe that scatter radiation does not play a significant role modifying the clonogenic survival.

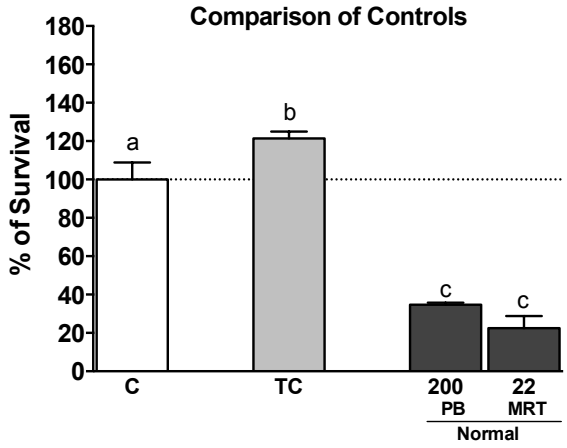

Figure 5.2 Comparison of clonogenic survival of HaCaT cells grown in ITCM from the control groups. The figure shows the clonogenic survival of reporter HaCaT cells grown ITCM originated the urinary bladders of the nude mice. Irradiated animals received a whole-brain exposure. The mice from the Tumour Control group were not irradiated. White bar = Normal animals. Gray bars = animals inoculated with F98 glioma cells. Black bars = Normal irradiated animals (22=22Gy; 200=200Gy). C=Normal Control, TC=Tumour control, PB=Pencil beam, MRT=Microbeam radiation. Letters a, b & c indicate significant differences between groups. n = 4, controls=4; error bars indicate SEM.

As a matter of comparison, figure 5.2 also includes reporter cells grown in ITCM originated from tumour free mice. ITCM from animal exposed to valley doses of 0.5 Gy, which correspond to 200 Gy for PB and 22 Gy for MRT, significantly decreased the survival of the reporter cells compared to the control group (p-value = < 0.0001 for PB; and p < 0.0001 for MRT).

Figure 5.3 shows the clonogenic survival of the reporter HaCaT cells receiving ITCM from urinary bladders harvested at 2, 12 and 48 hours after irradiation. The data generated 2 hours after irradiation (Fig 5.3A) indicate that ITCM from normal mice exposed to 200 Gy PB or 22 Gy MRT significantly reduced the survival of the reporter cells (p-value = < 0.0001 for PB; p= < 0.0001 for MRT). However, the difference between the two modalities was not significant in this case. When mice harbouring F98 tumours were irradiated with the same doses as above ITCM from these animals did not significantly reduce the survival of the reporter cells. However, the difference between PB and MRT was now significant (p-value = 0.04) with the PB inducing stronger signals. ITCM from animals exposed to 1000 Gy PB significantly reduced the clonogenic survival of the reporter cells (p-value = 0.01), while the 110 Gy MRT group did not. The PB and MRT modalities were also statistically different in this subgroup (p-value = 0.001).

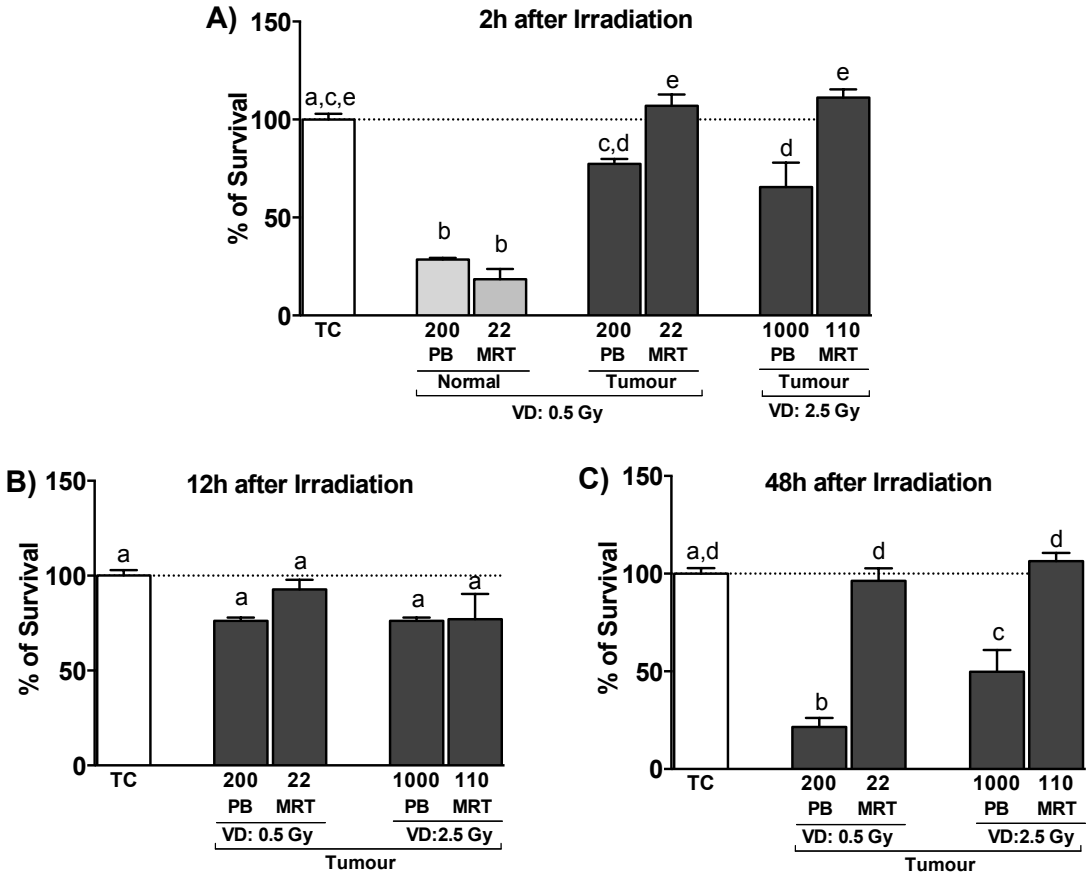

Figure 5.3 Clonogenic survival of HaCaT cells grown in ITCM from synchrotron irradiated nude mice.

ITCM was originated from the urinary bladders of the synchrotron irradiated nude mice. Animals received a whole-brain irradiation. Normal and tumour bearing mice were exposed to peak doses of 200Gy and 1000 Gy for PB and 22Gy and 110 Gy for MRT in order to achieve constant valley doses of 0.5Gy and 2.5 Gy respectively. White bar = Normal animals. Gray bars = tumour-free irradiated animals. Black bars = tumour-bearing irradiated animals. C=Control, TC=Tumour control, PB=Pencil beam, MRT=Microbeam radiation therapy, VD=Valley dose Letters a, b & c indicate significant differences between groups. n = 3, controls =4, error bars indicate SEM.

Signals from bladders harvested 12 hours after irradiation (Fig 5.3B) did not induce any significant decrease in reporter survival. However, ITCM from mice dissected 48 h after irradiation (Fig 5.3C) significantly reduced the survival of the reporters compared to the control; regardless of the ITCM originating from animals exposed to 200 Gy PB (p-value < 0.0001) or 1000 Gy PB (p-value < 0.0001). Conversely, none of the MRT groups had an effect on the HaCaT cells. When comparing each valley dose sub-group, 200 Gy PB was significantly different to 22 Gy MRT (p-value < 0.0001), as well as for 1000 Gy PB and 110 Gy MRT (p-value < 0.0001).

Figure 5.4 shows the results for the calcium flux assay for the ITCM obtained from the bladder tissue of healthy control, the tumour control and the healthy animals whose brains were exposed to PB or an equivalent MRT dose. The data are presented as bar charts representing the total area under the curve for the flux from the point of addition of the ITCM to the return of the calcium spike to the baseline. While there are differences in area under the curve none of these are significant although the trend is similar to that seen in Figure 5.2 for the clonogenic endpoint. Figure 5.5 shows a comparison of the data for the PB and MRT irradiated tumour-bearing animals. These animals were sacrificed 2, 12 and 48hrs post irradiation of the brain. The signals from these bladders are very variable and show no significant effects or trends.

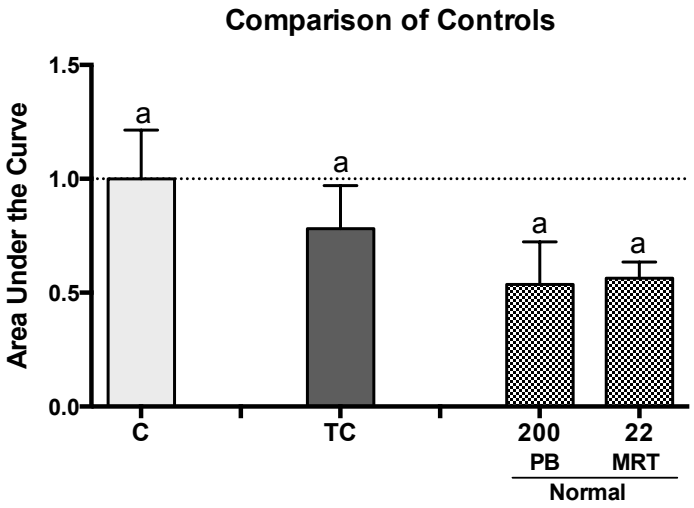

Figure 5.4 Clonogenic survival of HaCaT cells grown in ITCM from synchrotron irradiated nude mice.
ITCM was originated from the urinary bladders of the synchrotron irradiated nude mice. Animals received a whole-brain irradiation. Normal and tumour bearing mice were exposed to peak doses of 200Gy and 1000 Gy for PB and 22Gy and 110 Gy for MRT in order to achieve constant valley doses of 0.5Gy and 2.5 Gy respectively. White bar = Normal animals. Gray bars = tumour-free irradiated animals. Black bars = tumour-bearing irradiated animals. C=Control, TC=Tumour control, PB=Pencil beam, MRT=Microbeam radiation therapy, VD=Valley dose Letters a, b & c indicate significant differences between groups. n = 3; Controls=4, error bars indicate SEM.

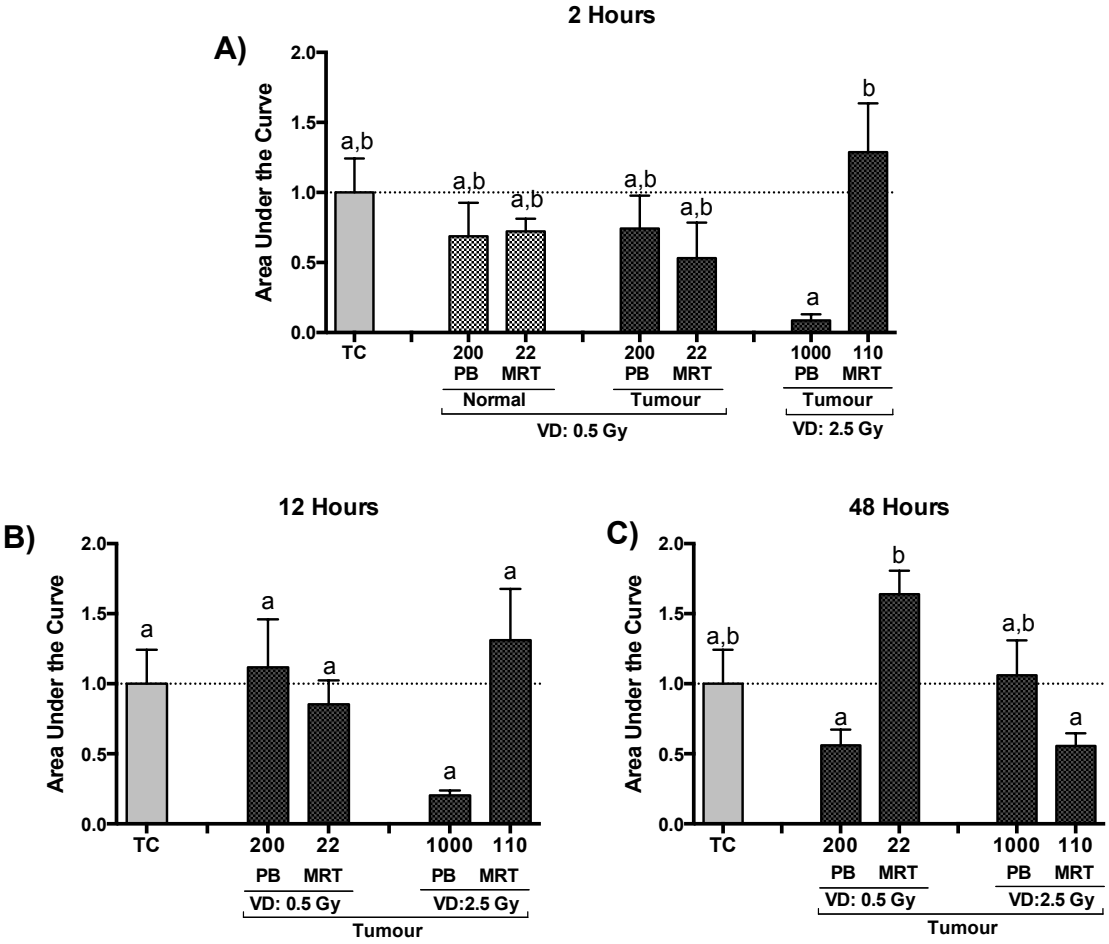

Figure 5.5 Calcium Fluxes of reporter HaCaT cells.
The figure shows the calcium fluxes of reporter HaCaT cells exposed to ITCM originated from the urinary bladders of nude mice. Irradiated animals received a whole-brain exposure. 200=200Gy; 1000=1000Gy; 22=22Gy; 110=110Gy. TC=Tumour control (no radiation); Normal=Tumour-free irradiated animals; Tumour=tumour-bearing irradiated animal, PB=Pencilbeam, MRT=Microbeam radiation**,** VD=Valley dose. Letters a, b & c indicate significant differences between groups. n=3; controls=4, error bars indicate SEM.

5.5 DISCUSSION

The data presented in this paper result from an attempt to determine if the bystander/abscopal effects seen in rats could also be seen in athymic mice which have a compromised immune system. The data suggest that in normal nude mice, which did not have a tumour implanted in the brain, irradiation of the brain leads to the generation of signals capable of reducing the clonogenic survival in our reporter assay. However the calcium flux data while supporting this trend, was not significant. This was mainly due to extremely high variability in the calcium flux response from cell to cell which made the errors very high. Such high variability, could suggest that the calcium flux pathway which we have established to be important in rodents, fish and a wide variety of cell lines (Mothersill and Seymour 2012), may not be critical for the production of a bystander response in these immune compromised mice. The fact that the clonogenic reporter cells did “see” a signal from the normal bladders, suggests signal production occurred at some level but the transduction of the response may not depend on calcium. In regard to the comparison between PB and MRT modalities, both had similar effects on normal tissue. This might be expected because the doses were calculated to irradiate similar tissue volumes and to lead to similar valley doses. However in the tumour bearing groups there was no bystander effect after MRT at any time point post irradiation. When harvested after 48hrs the signal from the PB irradiated animals is significant however this trend was not seen at all in the 12hr group and was present but weaker at 2hrs

post irradiation and only statistically significant following the high dose exposure. The finding in this study, that the presence of tumour in the animal led to no or significantly weaker signals being “seen” by the reporter cells in the clonogenic assay supports the observations in the rat experiments using F98 cells (Fernandez-Palomo et al. 2015). We are not aware of any other reports of bystander effects being weaker or ablated when tumour is present in the animal but very early work by our group (Mothersill & Seymour, 1998) using human normal urothelium and urothelium from transitional cell carcinoma (TCC) patients did show a loss of signal or a switch to pro-survival signals in the irradiated tissues from the TCC group.

To conclude, this study suggests that signals are produced by immune compromised mice but the pathways involved in signal transduction may be different. The question of whether abscopal effects following irradiation using PB or MRT in tumour bearing animals are beneficial or harmful remains open because the presence of tumour in the animal appears to weaken these signals even when these are measured in distant tissue without tumour.

5.6 ACKNOWLEDGEMENTS

Funding for this work was received from the Natural Sciences and Engineering Council (NSERC) Discovery Grants programme, The Chilean Government – BecasChile (support for Fernandez-Palomo) and the ESRF Grenoble (support for European partners during beamtime). E. Schültke held a Marie-Curie-Reintegration Grant of the EU (PIRG-GA-2010-268250).

5.7 REFERENCES

Armstrong, A. E., Gillan, E., & Dimario, F. J. (2014). SMART Syndrome (Stroke-Like Migraine Attacks After Radiation Therapy) in Adult and Pediatric Patients. *J Child Neurol*, *29*(3), 336–341. doi:10.1177/0883073812474843

Barth, R. F., & Kaur, B. (2009). Rat brain tumor models in experimental neuro-oncology: the C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. *Journal of Neuro-Oncology*, *94*(3), 299–312. doi:10.1007/s11060-009-9875-7

Bartzsch, S., & Tag, J. (2014, December 11). *Microbeam Radiation Therapy – physical and biological aspects of a new cancer therapy and development of a treatment planning system*. Retrieved from http://archiv.ub.uni-heidelberg.de/volltextserver/17714/1/Arbeit_Gesamt.pdf

Biston, M.-C., Joubert, A., Adam, J.-F., Elleaume, H., Bohic, S., Charvet, A.-M., … Balosso, J. (2004). Cure of Fisher rats bearing radioresistant F98 glioma treated with cis-platinum and irradiated with monochromatic synchrotron X-rays. *Cancer Research*, *64*(7), 2317–23. doi:10.1158/0008-5472.CAN-03-3600

Black, D. F., Morris, J. M., Lindell, E. P., Krecke, K. N., Worrell, G. a., Bartleson, J. D., & Lachance, D. H. (2013). Stroke-like migraine attacks after radiation therapy (SMART) syndrome is not always completely reversible: A case series. *American Journal of Neuroradiology*, *34*(12), 2298–2303. doi:10.3174/ajnr.A3602

Blattmann, H., Gebbers, J.-O., Bräuer-Krisch, E., Bravin, A., Le Duc, G., Burkard, W., … Laissue, J. a. (2005). Applications of synchrotron X-rays to radiotherapy. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 17–22. doi:10.1016/j.nima.2005.03.060

Bouchet, A., Lemasson, B., Christen, T., Potez, M., Rome, C., Coquery, N., … Serduc, R. (2013). Synchrotron microbeam radiation therapy induces hypoxia in intracerebral gliosarcoma but not in the normal brain. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, *108*(1), 143–8. doi:10.1016/j.radonc.2013.05.013

Bouchet, A., Sakakini, N., El Atifi, M., Le Clec'h, C., Brauer, E., Moisan, A., … Pelletier, L. (2013). Early gene expression analysis in 9L orthotopic tumor-bearing rats identifies immune modulation in molecular response to synchrotron microbeam radiation therapy. *PloS One*, *8*(12), e81874. doi:10.1371/journal.pone.0081874

Boukamp, P., Popp, S., Bleuel, K., Tomakidi, E., Bürkle, A., & Fusenig, N. E. (1999). Tumorigenic conversion of immortal human skin keratinocytes (HaCaT) by elevated temperature. *Oncogene*, *18*(41), 5638–45. doi:10.1038/sj.onc.1202934

Bräuer-Krisch, E., Requardt, H., Brochard, T., Berruyer, G., Renier, M., Laissue, J. a, & Bravin, a. (2009). New technology enables high precision multislit collimators for microbeam radiation therapy. *The Review of Scientific Instruments*, *80*(7), 074301. doi:10.1063/1.3170035

Bräuer-Krisch, E., Serduc, R., Siegbahn, E. a, Le Duc, G., Prezado, Y., Bravin, A., … Laissue, J. A. (2010). Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. *Mutation Research*, *704*(1-3), 160–6. doi:10.1016/j.mrrev.2009.12.003

Burdak-Rothkamm, S., & Prise, K. M. (2009). New molecular targets in radiotherapy: DNA damage signalling and repair in targeted and non-targeted cells. *European Journal of Pharmacology*, *625*(1-3), 151–5. doi:10.1016/j.ejphar.2009.09.068

Crosbie, J. C., Anderson, R. L., Rothkamm, K., Restall, C. M., Cann, L., Ruwanpura, S., … Rogers, P. a W. (2010). Tumor cell response to synchrotron microbeam radiation therapy differs markedly from cells in normal tissues. *International Journal of Radiation Oncology, Biology, Physics*, *77*(3), 886–94. doi:10.1016/j.ijrobp.2010.01.035

D'Ambrosio, D. J., Cohen, R. B., Glass, J., Konski, A., Buyyounouski, M. K., & Feigenberg, S. J. (2007). Unexpected dementia following prophylactic cranial irradiation for small cell lung cancer: Case report. *Journal of Neuro-Oncology*, *85*(1), 77–79. doi:10.1007/s11060-007-9384-5

DeAngelis, L. M., Delattre, J. Y., & Posner, J. B. (1989). Radiation-induced dementia in patients cured of brain metastases. *Neurology*, *39*(6), 789–96. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2725874

Desmarais, G., Charest, G., Fortin, D., Bujold, R., Mathieu, D., & Paquette, B. (2015). Cyclooxygenase-2 inhibitor prevents radiation-enhanced infiltration of F98 glioma cells in brain of Fischer rat. *International Journal of Radiation Biology*, 1–10. doi:10.3109/09553002.2015.1043756

Desmarais, G., Fortin, D., Bujold, R., Wagner, R., Mathieu, D., & Paquette, B. (2012). Infiltration of glioma cells in brain parenchyma stimulated by radiation in the F98/Fischer rat model. *International Journal of Radiation Biology*, *88*(8), 565–74. doi:10.3109/09553002.2012.692495

Dilmanian, F. A., Button, T. M., Le Duc, G., Zhong, N., Peña, L. A., Smith, J. A. L., … Ren, B. (2002). Response of rat intracranial 9L gliosarcoma to microbeam radiation therapy. *Neuro-Oncology*, *4*(1), 26. doi:10.1016/j.ejrad.2008.04.055

Fernandez-Palomo, C. (2011). *Radiobiological Response of Healthy and Tumour-bearing Rat Brains to Synchrotron Microbeam Radiation*. McMaster University. Retrieved from http://digitalcommons.mcmaster.ca/opendissertations/6433/

Fernandez-Palomo, C., Bräuer-Krisch, E., Laissue, J., Vukmirovic, D., Blattmann, H., Seymour, C., … Mothersill, C. (2015). Use of synchrotron medical microbeam irradiation to investigate radiation-induced bystander and abscopal effects in vivo. *Physica Medica : PM : An International Journal Devoted to the Applications of Physics to Medicine and Biology :*

Official Journal of the Italian Association of Biomedical Physics (AIFB), 1–12. doi:10.1016/j.ejmp.2015.03.004

Fernandez-Palomo, C., Bräuer-Krisch, E., Trippel, M., Schroll, C., Requardt, H., Bartzsch, S., … Schültke, E. (2013). DNA double strand breaks in the acute phase after synchrotron pencilbeam irradiation. *Journal of Instrumentation*, *8*(07), C07005–C07005. doi:10.1088/1748-0221/8/07/C07005

Fernandez-Palomo, C., Mothersill, C., Bräuer-Krisch, E., Laissue, J., Seymour, C., & Schültke, E. (2015). γ-H2AX as a Marker for Dose Deposition in the Brain of Wistar Rats after Synchrotron Microbeam Radiation. *PLOS ONE*, *10*(3), e0119924. doi:10.1371/journal.pone.0119924

Fernandez-Palomo, C., Schültke, E., Smith, R., Bräuer-Krisch, E., Laissue, J., Schroll, C., … Mothersill, C. (2013). Bystander effects in tumor-free and tumor-bearing rat brains following irradiation by synchrotron X-rays. *International Journal of Radiation Biology*, *89*(6), 445–53. doi:10.3109/09553002.2013.766770

Forbes, M. E., Paitsel, M., Bourland, J. D., & Riddle, D. R. (2014). Early-Delayed, Radiation-Induced Cognitive Deficits in Adult Rats are Heterogeneous and Age-Dependent. *Radiation Research*, *71*, 60–71. doi:10.1667/RR13662.1

Gil, S., Sarun, S., Biete, A., Prezado, Y., & Sabés, M. (2011). Survival analysis of F98 glioma rat cells following minibeam or broad-beam synchrotron radiation therapy. *Radiation Oncology (London, England)*, *6*(1), 37. doi:10.1186/1748-717X-6-37

Henseleit, U., Zhang, J., Wanner, R., Haase, I., Kolde, G., & Rosenbach, T. (1997). Role of p53 in UVB-induced apoptosis in human HaCaT keratinocytes. *The Journal of Investigative Dermatology*. doi:10.1111/1523-1747.ep12340708

Holyoake, T. L., Freshney, M. G., Samuel, K., Ansell, J., Watson, G. E., Wright, E. G., … Pragnell, I. B. (2001). In vivo expansion of the endogenous B-cell compartment stimulated by radiation and serial bone marrow transplantation induces B-cell leukaemia in mice. *British Journal of Haematology*, *114*(1), 49–56. doi:10.1046/j.1365-2141.2001.02903.x

Ikehara, S., Pahwa, R. N., Fernandes, G., Hansen, C. T., & Good, R. a. (1984). Functional T cells in athymic nude mice. *Proceedings of the National Academy of Sciences of the United States of America*, *81*(3), 886–888.

Kerklaan, J. P., Lycklama Á Nijeholt, G. J., Wiggenraad, R. G. J., Berghuis, B., Postma, T. J., & Taphoorn, M. J. B. (2011). SMART syndrome: A late reversible complication after radiation therapy for brain tumours. *Journal of Neurology*, *258*(6), 1098–1104. doi:10.1007/s00415-010-5892-x

Koturbash, I., Kutanzi, K., Hendrickson, K., Rodriguez-Juarez, R., Kogosov, D., & Kovalchuk, O. (2008). Radiation-induced bystander effects in vivo are sex specific. *Mutation Research*, *642*(1-2), 28–36. doi:10.1016/j.mrfmmm.2008.04.002

Koturbash, I., Zemp, F., Kolb, B., & Kovalchuk, O. (2011). Sex-specific radiation-induced microRNAome responses in the hippocampus, cerebellum and frontal cortex in a mouse model. *Mutation Research*, *722*(2), 114–8. doi:10.1016/j.mrgentox.2010.05.007

Laissue, J. A., Geiser, G., Spanne, P. O., Dilmanian, F. a, Gebbers, J. O., Geiser, M., … Slatkin, D. N. (1998). Neuropathology of ablation of rat gliosarcomas and contiguous brain tissues using a microplanar beam of synchrotron-wiggler-generated X rays. *International Journal of Cancer. Journal International Du Cancer*, *78*(5), 654–60. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med4&NEWS=N&AN=9808538

Lehman, T. A., Modali, R., Boukamp, P., Stanek, J., Bennett, W. P., Welsh, J. A., … Rogan, E. M. (1993). p53 mutations in human immortalized epithelial cell lines. *Carcinogenesis*, *14*(5), 833–839.

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2000). Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *British Journal of Cancer*, *83*(9), 1223–30. doi:10.1054/bjoc.2000.1433

Mancuso, M., Pasquali, E., Leonardi, S., Tanori, M., Rebessi, S., Di Majo, V., … Saran, A. (2008). Oncogenic bystander radiation effects in Patched heterozygous mouse cerebellum. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(34), 12445–12450. doi:10.1073/pnas.0804186105

Miura, M., Blattmann, H., Bräuer-Krisch, E., Bravin, A., Hanson, A. L., Nawrocky, M. M., … Laissue, J. A. (2006). Radiosurgical palliation of aggressive murine SCCVII squamous cell carcinomas using synchrotron-generated X-ray microbeams. *The British Journal of Radiology*, *79*(937), 71–5. doi:10.1259/bjr/50464795

Mole, R. H. (1953). Whole body irradiation; radiobiology or medicine? *The British Journal of Radiology*, *26*(305), 234–41. doi:10.1259/0007-1285-26-305-234

Mothersill, C., & Seymour, C. (1997). Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology*, *71*(4), 421–7. doi:10.1080/095530097144030

MotherSill, C., & Seymour, C. (2012). Changing paradigms in radiobiology. *Mutation Research/Reviews in Mutation Research*, *750*(2), 85–95. doi:10.1016/j.mrrev.2011.12.007

Mothersill, C., & Seymour, C. B. (1998). Cell-cell contact during gamma irradiation is not required to induce a bystander effect in normal human keratinocytes: evidence for release during irradiation of a signal controlling survival into the medium. *Radiation Research*, *149*(3), 256–262. doi:10.2307/3579958

Murphy, J. B., & Morton, J. J. (1915). The Effect Of Roentgen Rays On The Rate Of Growth Of Spontaneous Tumors In Mice. *The Journal of Experimental Medicine*, *22*(6), 800–803. doi:10.1084/jem.22.6.800

Pardridge, W. M. (2005). The blood-brain barrier: bottleneck in brain drug development. *NeuroRx : The Journal of the American Society for Experimental NeuroTherapeutics*, *2*(1), 3–14. doi:10.1602/neurorx.2.1.3

Peiffer, A. M., Creer, R. M., Linville, C., Olson, J., Kulkarni, P., Brown, J. A., … Brunso-Bechtold, J. E. (2014). Radiation induced cognitive impairment and altered diffusion tensor imaging in a juvenile rat model of cranial radiotherapy. *International Journal of Radiation Biology*, *90*(February), 1–22. doi:10.3109/09553002.2014.938278

Prise, K. M. (1998). Studies of bystander effects in human fibroblasts using a charged particle microbeam. *International Journal of Radiation Biology*, *74*(6), 793–798. doi:10.1080/095530098141087

Puck, T. T., & Marcus, P. I. (1956). Action of x-rays on mammalian cells. *The Journal of Experimental Medicine*, *103*(5), 653–66. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10380286

Rygaard, J. (2009). IMMUNOBIOLOGY OF THE MOUSE MUTANT “NUDE.” *Acta Pathologica Microbiologica Scandinavica*, *77*(4), 761–762. doi:10.1111/j.1699-0463.1969.tb04521.x

Schültke, E., Juurlink, B. H. J., Ataelmannan, K., Laissue, J., Blattmann, H., Bräuer-Krisch, E., … Fourney, D. (2008). Memory and survival after microbeam radiation therapy. *European Journal of Radiology*, *68*(3 SUPPL.), 142–146. doi:10.1016/j.ejrad.2008.04.051

Schültke, E., Trippel, M., Bräuer-Krisch, E., Renier, M., Bartzsch, S., Requardt, H., … Nikkhah, G. (2013). Pencilbeam irradiation technique for whole brain radiotherapy: technical and biological challenges in a small animal model. *PloS One*, *8*(1), e54960. doi:10.1371/journal.pone.0054960

Shi, L., Linville, M. C., Iversen, E., Molina, D. P., Yester, J., Wheeler, K. T., … Brunso-Bechtold, J. K. (2009). Maintenance of white matter integrity in a rat model of radiation-induced cognitive impairment. *Journal of the Neurological Sciences*, *285*(1-2), 178–184. doi:10.1016/j.jns.2009.06.031

Slatkin, D. N., Spanne, P., Dilmanian, F. A., & Sandborg, M. (1992). Microbeam radiation therapy. *Medical Physics*, *19*(6), 1395–1400. doi:10.1118/1.596771

Smith, R. W., Wang, J., Schültke, E., Seymour, C. B., Bräuer-Krisch, E., Laissue, J. A., … Mothersill, C. E. (2013). Proteomic changes in the rat brain induced by homogenous irradiation and by the bystander effect resulting from high energy synchrotron X-ray microbeams. *International Journal of Radiation Biology*, *89*(2), 118–27. doi:10.3109/09553002.2013.732252

Sun, R., Sbai, A., Ganem, G., Boudabous, M., Collin, F., Marcy, P.-Y., … Thariat, J. (2014). [Non-targeted effects (bystander, abscopal) of external beam radiation therapy: an overview for the clinician]. *Cancer Radiothérapie : Journal de La Société Française de Radiothérapie Oncologique*, *18*(8), 770–8. doi:10.1016/j.canrad.2014.08.004

Tallet, A. V, Azria, D., Barlesi, F., Spano, J.-P., Carpentier, A. F., Gonçalves, A., & Metellus, P. (2012). Neurocognitive function impairment after whole brain radiotherapy for brain metastases: actual assessment. *Radiation Oncology*, *7*(1), 77. doi:10.1186/1748-717X-7-77

Tzeng, J. J., Barth, R. F., Orosz, C. G., & James, S. M. (1991). Phenotype and functional activity of tumor-infiltrating lymphocytes isolated from immunogenic and nonimmunogenic rat brain tumors. *Cancer Research*, *51*(9), 2373–2378.

Watson, G. E., Lorimore, S. a, Macdonald, D. a, & Wright, E. G. (2000). Chromosomal instability in unirradiated cells induced in vivo by a bystander effect of ionizing radiation. *Cancer Research*, *60*(20), 5608–5611.

Watson, G. E., Pocock, D. a, Papworth, D., Lorimore, S. a, & Wright, E. G. (2001). In vivo chromosomal instability and transmissible aberrations in the progeny of haemopoietic stem cells induced by high- and low-LET radiations. *International Journal of Radiation Biology*, *77*(4), 409–417. doi:10.1080/09553000010028476

Yang, Y., Crosbie, J. C., Paiva, P., Ibahim, M., Stevenson, A., & Rogers, P. a. W. (2014). In Vitro Study of Genes and Molecular Pathways Differentially Regulated by Synchrotron Microbeam Radiotherapy. *Radiation Research*, *182*(6), 626–639. doi:10.1667/RR13778.1

Zhou, H., Randers-Pehrson, G., Waldren, C. a, Vannais, D., Hall, E. J., & Hei, T. K. (2000). Induction of a bystander mutagenic effect of alpha particles in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(5), 2099–104. doi:10.1073/pnas.030420797

Chapter 6

6 γ-H2AX AS A MARKER FOR DOSE DEPOSITION IN THE BRAIN OF WISTAR RATS AFTER SYNCHROTRON MICROBEAM RADIATION

Cristian Fernandez-Palomo, Carmel Mothersill, Elke Bräuer-Krisch, Jean Laissue, Colin Seymour, Elisabeth Schültke

---

This research project was design by Schültke and the first author of this thesis with major contributions from Mothersill and Bräuer-Krisch. The irradiations were performed by the first author, Schültke, Bräuer-Krisch, and Seymour. Tissue harvesting, processing and clonogenic assays were performed by Laissue, the first author, and Mothersill. Tissue immunofluorescence, data recollection and image analysis were performed by the first author. The manuscript was written by the first author and Mothersill; and edited by Schültke, Laissue and Bräuer-Krisch.

The manuscript was published in PloS ONE on March 23, 2015. Volume 10; Issue 3; pages 1-17; doi: 10.1371/journal.pone.0119924.

6.1 ABSTRACT

Objective: Synchrotron radiation has shown high therapeutic potential in small animal models of malignant brain tumours. However, more studies are needed to understand the radiobiological effects caused by the delivery of high doses of spatially fractionated x-rays in tissue. The purpose of this study was to explore the use of the γ-H2AX antibody as a marker for dose deposition in the brain of rats after synchrotron microbeam radiation therapy (MRT).

Methods: Normal and tumour-bearing Wistar rats were exposed to 35, 70 or 350 Gy of MRT to their right cerebral hemisphere. The brains were extracted either at 4 or 8 hours after irradiation and immediately placed in formalin. Sections of paraffin-embedded tissue were incubated with anti γ-H2AX primary antibody.

Results: While the presence of the C6 glioma does not seem to modulate the formation of γ-H2AX in normal tissue, the irradiation dose and the recovery versus time are the most important factors affecting the development of γ-H2AX foci. Our results also suggest that doses of 350 Gy can trigger the release of bystander signals that significantly amplify the DNA damage caused by radiation and that the γ-H2AX biomarker does not only represent DNA damage produced by radiation, but also damage caused by bystander effects.

Conclusion: In conclusion, we suggest that the γ-H2AX foci should be used as biomarker for targeted and non-targeted DNA damage after synchrotron radiation rather than a tool to measure the actual physical doses.

Keywords: Synchrotron Microbeam Radiation, γ-H2AX, Biodosimetry, C6 Glioma, Brain, Wistar rat, Bystander Effects

6.2 INTRODUCTION

Conventional radiotherapy has been very successful at treating a wide variety of cancers such as those of skin and breast, but it still remains poorly effective when targeting malignant brain tumours. Brain tumours account for 2% of all cancer in adults and represent the second cause of deaths in children after leukemia (World Health Organization, 2008). Unfortunately, the most frequent tumours - glioblastoma multiforme (GBM) in adults, and astrocytic tumours in children - are both the most aggressive and resistant to radiation therapy (Beygi, Saadat, Jazayeri, & Rahimi-Movaghar, 2013; Ricard et al., 2012). New treatments are required to improve prognosis.

Synchrotron radiation is a promising approach for brain radiotherapy. Research on microbeam radiation therapy (MRT) in the last two decades, initiated at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory (BNL), Upton, New York, was continued at the European Synchrotron Radiation Facility (ESRF) and at other international facilities. Results of this research have repeatedly and consistently shown, in the hands of many different teams of scientists, that single-fraction MRT yields a larger therapeutic index than does a single irradiation by a single broad beam, for aggressive tumours such as transplanted intracerebral rat 9L gliosarcomas or F98 tumours (Bräuer-Krisch et al., 2010; F.A. Dilmanian et al., 2002; J A Laissue et al., 1998); for transplanted subcutaneous murine mammary carcinomas (Crosbie et al., 2010); for the aggressive and invasive, extraordinarily radioresistant murine squamous cell

carcinoma VII (Miura et al., 2006), and for other tumours. The remarkable sparing by x-ray microbeams of normal tissues of vertebrates - particularly of the normal brain and spinal cord - has been extensively documented in suckling and adult rats (Audrey Bouchet et al., 2014; F Avraham Dilmanian et al., 2003; F. a Dilmanian et al., 2005; F.A. Dilmanian et al., 2002; J. a Laissue et al., 2013; Jean A. Laissue et al., 1999; Serduc et al., 2009; D N Slatkin, Spanne, Dilmanian, Gebbers, & Laissue, 1995), duck embryos (F A Dilmanian et al., 2001), and weanling piglets (Jean A Laissue et al., 2001). The skin also tolerates relatively high doses of x-rays delivered by microbeams (Priyadarshika, Crosbie, Kumar, & Rogers, 2011; Zhong, Morris, Bacarian, Rosen, & Avraham Dilmanian, 2003). Further, MRT-associated bystander effects have been identified (F. Avraham Dilmanian et al., 2007; Cristian Fernandez-Palomo et al., 2013; Smith et al., 2013), and gene expression analysis of intracerebral gliosarcomas in rats have identified MRT-induced immune modulations (Audrey Bouchet, Sakakini, et al., 2013) and cytostatic effects (A Bouchet et al., 2014).

Among the potential advantages of MRT over temporally fractionated, conventional radiotherapy, we note 1) the very short time required for treatment, i.e., 1–2 days for MRT rather than several weeks; animals positioned online in the MRT hutch of ID 17 at the ESRF can be imaged just before irradiation, and changing from imaging to irradiation takes less than a minute (Serduc, Berruyer, Brochard, Renier, & Nemoz, 2010); 2) the normal organ tolerance particularly of

the normal brain and spinal cord, which might allow re-irradiation; 3) the ability to treat tumours with radiobiologically higher doses, with possibly improved local control rates. Radiation oncologists familiar with MRT deem that it might enable the palliation of central nervous system malignancies in infants and young children who at present cannot be safely and effectively palliated by existing radiotherapies or by any other kinds of therapies (for instance, diffuse pontine gliomas).

From a technical point of view, synchrotron radiation is typically used as an array of microbeams. The high photon flux of synchrotron-generated x-rays is spatially fractionated by the insertion of a collimator, which produces an array of quasi-parallel microbeams with intermediate gaps (D. N. Slatkin, Spanne, Dilmanian, & Sandborg, 1992). This particular microbeam configuration exposes areas of tissue to either peak or valley doses. The former corresponds to the tissue regions where the primary dose is deposited by the photons of the microbeam, while the latter refers to the secondary dose deposited in the tissue regions between the microbeams by the scattered photons (Blattmann et al., 2005). The valley dose varies between approximately 2% to 10% of the peak dose, depending on the configuration and size of the beam array (Bräuer-Krisch et al., 2010). Microbeam widths typically vary from 20 to 100μm, with gaps of 200-400 μm wide. Having gap widths of 4 to 8 times greater than the width of microbeams, the volume of tissue affected by the valley dose would be 4^3-8^3

times larger than the tissue volume targeted by the peak dose. The ratio between the dose of the microbeams and the dose of the gaps is called peak-to-valley dose ratio (PVDR), which plays a major role in decreasing the dose to normal tissue. Thus PVDR is an important parameter in understanding the tissue tolerance to the high doses used in MRT (D. N. Slatkin et al., 1992).

There are several hypotheses concerning the biological basis of the tumouricidal effect of microbeams: Intracerebral transplantable tumours of rats are killed because the “valley dose” (i.e. the radiation leakage between the microbeams produced by Compton scattering, particularly in the transitional zones sited between peaks and valleys) is very high. High valley doses, given in a single dose fraction, augmented by the “dose spikes” from the “peak doses” of the microbeams, might be high enough to destroy the tumour’s abnormal microvasculature, but too low to destroy the microvasculature of normal tissues (Bräuer-Krisch et al., 2010). This may be the case in small animals where the valley dose is approximately the same in the tumour and in the normal tissues proximally and distally to the tumour. Conversely, in deep tumours of large animals, the valley dose would be higher in the normal tissues proximal to the tumour than in the tumour because of a much higher incident dose has to be applied to compensate for greater x-ray attenuation (Audrey Bouchet et al., 2010; Audrey Bouchet, Lemasson, et al., 2013). Also, the tissue within the valley regions is of particular interest because, depending on the peak dose and the

radiation geometry, the dose deposited can be as low as 0.5 Gy, which is relevant for the induction of bystander effects (F. Avraham Dilmanian et al., 2007).

Radiation-induced bystander effects (RIBE) are described as the extent of damage in cells that were not exposed to direct irradiation, but that after receiving signals from irradiated cells respond similarly as if they had been irradiated (Mothersill & Seymour, 1997; Nagasawa & Little, 1992). RIBE are relevant for MRT because 1) the tissue in the dose valleys may respond to signals released by the cells in the path of the microbeam (F. Avraham Dilmanian et al., 2007), and 2) the tissue in the dose valleys will also receive low doses of radiation (i. e. scatter) that may allow the cells to produce bystander signals to then induce bystander effects on distant organs. Studies trying to identify the bystander molecule have found the involvement of extracellular mediators and intracellular pathways. Within the former we can identify reactive oxygen species (ROS) (F M Lyng, Seymour, & Mothersill, 2000; Narayanan, Goodwin, & Lehnert, 1997), reactive nitrogen species (RNS) (Shao, Stewart, Folkard, Michael, & Prise, 2003), interleukin-8 (IL8) (Narayanan, LaRue, Goodwin, & Lehnert, 1999), tumour necrosis factor-α (TNF-α) (Iyer & Lehnert, 2000), transforming growth factor-β1 (TGF-β1) (Iyer & Lehnert, 2000), serotonin (Fazzari, Mersov, Smith, Seymour, & Mothersill, 2012; Saroya, Smith, Seymour, & Mothersill, 2009) and exosomes as the latest candidate (Al-Mayah, Irons, Pink, Carter, & Kadhim, 2012; Jella et al., 2014). Within the latter we find mitogen-activated protein kinases (MAPKs), the

NF-κB transcription factor, COX2, NOS2 (Iyer & Lehnert, 2000; Prise & O'Sullivan, 2009), mitochondrial disruptions (F.M. Lyng, Seymour, & Mothersill, 2001; Murphy, Nugent, Seymour, & Mothersill, 2005; Zhou, Ivanov, Lien, Davidson, & Hei, 2008), Ca^{2+} fluxes (Fiona M Lyng, Seymour, & Mothersill, 2002), and expression of apoptotic and cell cycle regulatory molecules like p53, $p21^{Waf1}$, p34, and MDM2 (Azzam, de Toledo, & Little, 2004; F. M. Lyng, Maguire, McClean, Seymour, & Mothersill, 2006; Mothersill & Seymour, 2004). Moreover, the latest research show that another candidate for bystander triggering factor is UV-photon emission from irradiated cells (Ahmad et al., 2013; Le, McNeill, Seymour, Rainbow, & Mothersill, 2014).

Our group has previously explored the occurrence of bystander effects in tissue from irradiated and non-irradiated rat brains (Cristian Fernandez-Palomo et al., 2013). The results of that study suggested that the yield of bystander signals was higher in Wistar rats harbouring C6 gliomas than in tumour-free rats. Moreover, protein formation after synchrotron radiation has also been explored, showing that the bystander-induced proteome may confer anti-tumorigenic properties that are based on ROS-induced apoptosis (Smith et al., 2013). The probability of bystander signals being communicated between animals was also investigated. Wistar rats received synchrotron radiation to their right cerebral hemisphere and were then paired with unexposed rats over 48 hours (Mothersill et al., 2014). The results showed that radiation damage was effectively communicated between animals through bystander signals.

A major challenge with synchrotron radiation is tracking and quantifying the dose deposition in the tumour and in normal tissue. One of the most reliable markers for DNA damage after radiotherapy is γ-H2AX. This molecule describes the phosphorylation of the H2AX histone on serine 139 (Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998). γ-H2AX was first demonstrated to appear as rapidly as 1 min after ionizing radiation exposure, reaching its maximum formation at 10 min (Rogakou et al., 1998). The authors concluded that γ-H2AX was directly related with double strand breaks (DSBs) at a formation rate of 1% per gray. However, recent studies have challenged that view. Work published by Costes et. al. shows that γ-H2AX is a DNA damage sensing protein that does not necessarily correlate with DSBs. Instead, it may operate as a rigid scaffold on regions of chromatin to keep broken DNA in place while allowing DNA repair enzymes to access the damaged site (Costes, Chiolo, Pluth, Barcellos-Hoff, & Jakob, 2010). γ-H2AX has also been evaluated as a biomarker to predict radiation sensitivity. Greve et al. used the γ-H2AX marker to predict the clinical radiosensitivity of patients after cancer treatment (Greve et al., 2012). Although they observed that peripheral blood lymphocytes extracted from patients irradiated with 2 Gy produced a maximum of H2AX phosphorylation 1 hour after irradiation, no satisfactory conclusion about radiation sensitivity could be made. Nevertheless, these studies agreed that γ-H2AX formation is a rapid and sensitive cellular response to radiation stress, which makes it an important marker of dose deposition.

The use of γ-H2AX after synchrotron radiation has been explored in monolayers of cells (Rothkamm et al., 2012), the skin of healthy mice (Crosbie et al., 2010) and in mice harbouring skin tumours (Crosbie et al., 2010). Our group started to look at the use of γ-H2AX in mouse brain after synchrotron pencilbeam irradiation, where we demonstrated a correlation between dose and the formation of γ-H2AX foci (C Fernandez-Palomo et al., 2013). The aim of the present work was to study the dose deposition of synchrotron radiation in the brain and cerebellum of rats after micro- and broad beams using the γ-H2AX marker under several conditions.

6.3 MATERIALS AND METHODS

6.3.1 Animal Model

Adult male Wistar rats were housed and cared for in a 12-hour light/dark cycle in a temperature-regulated animal facility at the European Synchrotron Radiation Facility (ESRF). All experiments were performed according to the guidelines of the French and German Councils on Animal Care. This study was approved by the Institutional Animal Care and Use Committees of both European participating institutions (Freiburg University Medical Center and ESRF, G10-87).

The C6 glioma cell line was selected for our studies because it shares a wide range of characteristics with the highly malignant human brain tumour glioblastoma multiforme (GBM) (Farrell, Stewart, & Del Maestro, 1987). Once

injected into the brain, C6 gliomas rapidly proliferate forming a solid malignant tumour (morphologically similar to GBM), delineated by a rim of active astrocytes, with small groups of tumour cells migrating along the blood vessels (Nagano, Sasaki, Aoyagi, & Hirakawa, 1993). C6 gliomas were originally produced as a result of exposing Wistar-furth rats to N-nitrosomethylurea, and then isolated and grown as a cell culture (Benda, Lightbody, Sato, Levine, & Sweet, 1968). This tumour model has been used in multiple studies involving conventional radiotherapy (Cho et al., 2012; Safdie et al., 2012; Sheehan et al., 2008) and synchrotron radiation (Balvay et al., 2009; Le Duc et al., 2000; Schültke et al., 2008).

For these experiments, C6 cells were obtained from the American Type Culture Collection and maintained in T75 cm^2 flasks using Dulbecco's Modified Eagle Medium (Gibco, France) supplemented with 10% FBS (Gibco, France) and 5ml Penicillin-Streptomycin (Gibco, France). Cells from a 90% confluent culture were detached by incubation with 20 ml of Hank's Balanced Salt Solution (Gibco, France) without calcium and magnesium for 20 minutes at 37°C in an atmosphere of 5% CO_2 in air. The cell suspension was centrifuged at 1000 rpm for 4 min, the pellet was re-suspended in 1ml of fresh growth medium and cells were counted using a haemocytometer.

Wistar rats were subjected to general anaesthesia (2 – 2.5 % isofluorane in 2 L/min compressed air) and placed in a stereotactic frame. An incision of 1 to 1.5 cm length was made on the scalp following the sagittal midline. A burr hole

was placed in the skull over the right hemisphere, 3 mm to the right from the sagittal midline and 3 mm posterior from the coronal suture. Then 100,000 C6 cells suspended in 10 μl were slowly injected into the brain 3 mm below the cortical surface over 4 minutes, using an automated syringe pump (KDS 320, GENEQ). Once the injection was finished and the needle removed, the hole was sealed with bone wax and the incision was closed. Rats were maintained for 7 days to allow tumour development.

6.3.2 Irradiation

Prior to irradiation, animals were deeply anesthetised using 3% isofluorane in 2L/min compressed air and an intraperitoneal injection of a Ketamine-Xylazine (61.5 /3.6 $mg.kg^{-1}$ i.p.). Synchrotron radiation was delivered to the right cerebral hemisphere in anterior-posterior direction in a single session using either a broad beam or a microbeam configuration (Figure 6.1). Broad beam refers to a synchrotron x-ray beam uniformly distributed within the irradiation field, resembling a conventional radiotherapy approach. Microbeam irradiation refers to a spatially fractionated synchrotron x-ray beam arranged in an array of alternating quasi-parallel microplanar beams and gaps. The broad beam was 14 mm high and 9.825 mm wide, and the doses delivered were 35, 70, or 350 Gy at the skin-entry level. The microbeam had a similar field size as the broad beam but it was composed of 50 alternating quasi-parallel rectangular microbeams of 25 μm width with a center-to-center distance of 200 μm. The peak doses delivered were the

same as for broad beam irradiation: 35, 70 or 350 Gy at the skin-entry level. Gafchromic Films (HD-810 and MD-V2-55 films from Nuclear Associates, NY, USA) were used to verify all irradiation doses and modalities applied.

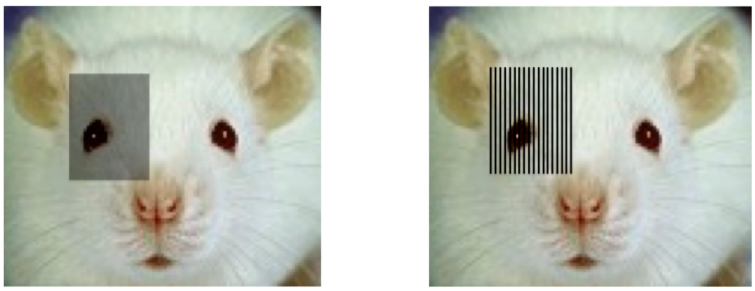

Figure 6.1 Schematic representation of a broad beam and a microbeam array

6.3.3 Tissue Preparation

Rats were anesthetised and decapitated either at 4 or 8 hours after irradiation for the extraction of the brains, which were kept in 10 % phosphate-buffered formalin overnight. The next morning, the brains were bisected into a superior and an inferior part in the horizontal plane. Both parts were placed in histology cassettes and immersed again in 10 % phosphate-buffered formalin overnight. The brains were then embedded in paraffin and sectioned into 3 μm thick tissue sections, which were mounted on microscopy slides and stored at +4°C.

6.3.4 Immunostaining

Tissue sections were deparaffinised and rehydrated through a series of alcohol and xylene washes. Vapour-based heat epitope retrieval was performed by putting the microscope slides in a citrate pH6 solution (Target retrieval solution, Dako, Germany, #S169984-2) at a 95°C for 40 minutes. The sections were then stained using the Shandon Sequenza Immunostaining Rack System. A coverplate was placed on the glass-slide to form a capillary gap that allows the staining reagents to flow through. The coverplate and slide assembly were placed in the rack and charged twice with PBS. Tissue sections were blocked with 100 μl of 1x PBS, 5% goat serum, and 0.3% triton X-100 buffer for 60 minutes at room temperature, followed by incubation with 1:100 dilution of γ-H2AX antibody (Abcam, Cambridge, UK) for 1 hour at room temperature. Slides were then rinsed 3 times with PBS and incubated with Alexa Fluor-488 (1:200 dilution), and DAPI for 1 hour at room temperature in the dark. Finally, slides were rinsed three times with PBS, the coverslips were placed using the Dako Fluorescent Mounting Medium and sealed with nail polish. Slides were stored flat at 4°C in the dark until image acquisition.

6.3.5 Image acquisition and analysis

Images were acquired in the Laboratory of Molecular Neurosurgery in the Freiburg Neurocenter using an Olympus AX70 fluorescence microscope. Images

were post processed using the Aperture 3.5.1 software, and analyzed at McMaster University.

Images were loaded into the Image Pro Plus 6.2.1 software, and three parameters were analyzed: 1) the width of the area covered by the γ-H2AX positive cells (radiation track) was measured in the cerebellum. 2) The intensity of fluorescence was measured through the use of intensity profile lines that were 100 μm long and traced perpendicular to the direction of the radiation tracks. The average values were then plotted as histograms using the Prism 6 software. 3) The number of γ-H2AX positive cells per micrometer-square was also detected. Images were analyzed by counting the number of γ-H2AX positive cells per micrometer area. A cell was counted as positive when it had strong fluorescence; cells with questionable positivity (weak or intermediate fluorescence) were defined as negative. The results were plotted accordingly using the Prism 6 software. Although a careful analysis of the results indicates that a comparison between the fluorescence intensities of peak and valley could enrich this study, it would require us to run the experiments again and we have no beam time to do this. For the aforementioned reason we highly recommend to any group running similar tests to consider a direct comparison between peak and valleys.

6.4 RESULTS

6.4.1 γ-H2AX foci as a biomarker for dose deposition

We analyzed the brain of Wistar rats after three different doses of synchrotron radiation (35, 75, and 350 Gy) using either microbeams or broad beams. The results demonstrated that the γ-H2AX antibody could effectively be used as a marker for dose deposition in the cerebral hemispheres and in the cerebellum. Brain images showing the exposure to 350 Gy of micro- and broad beams are shown in figures 6.2A and 6.2B respectively. The typical radiation tracks detected by the γ-H2AX antibody were observed in all brain samples exposed to synchrotron radiation. Because of the sharp dose gradient, the edges of the irradiation fields with both micro- and broad beams were well delineated. The cerebellum is shown in figures 6.2C and 6.2D. The higher cellular density of the granular cell layer allows a good visualization of the radiation tracks. Conversely, the cerebral hemispheres (6.2A & 6.2B) contain a lower amount of cell bodies and thus comparatively less DNA to be targeted. To complement the analysis, figure 6.2E shows the whole cerebellum after a microbeam exposure of 350 Gy. This image demonstrates the accuracy and precision of the synchrotron microbeam radiation technique. Due to an artefact of tissue distortion that occurs during the histological processing of the images, the radiation tracks do not always appear as perfectly straight lines.

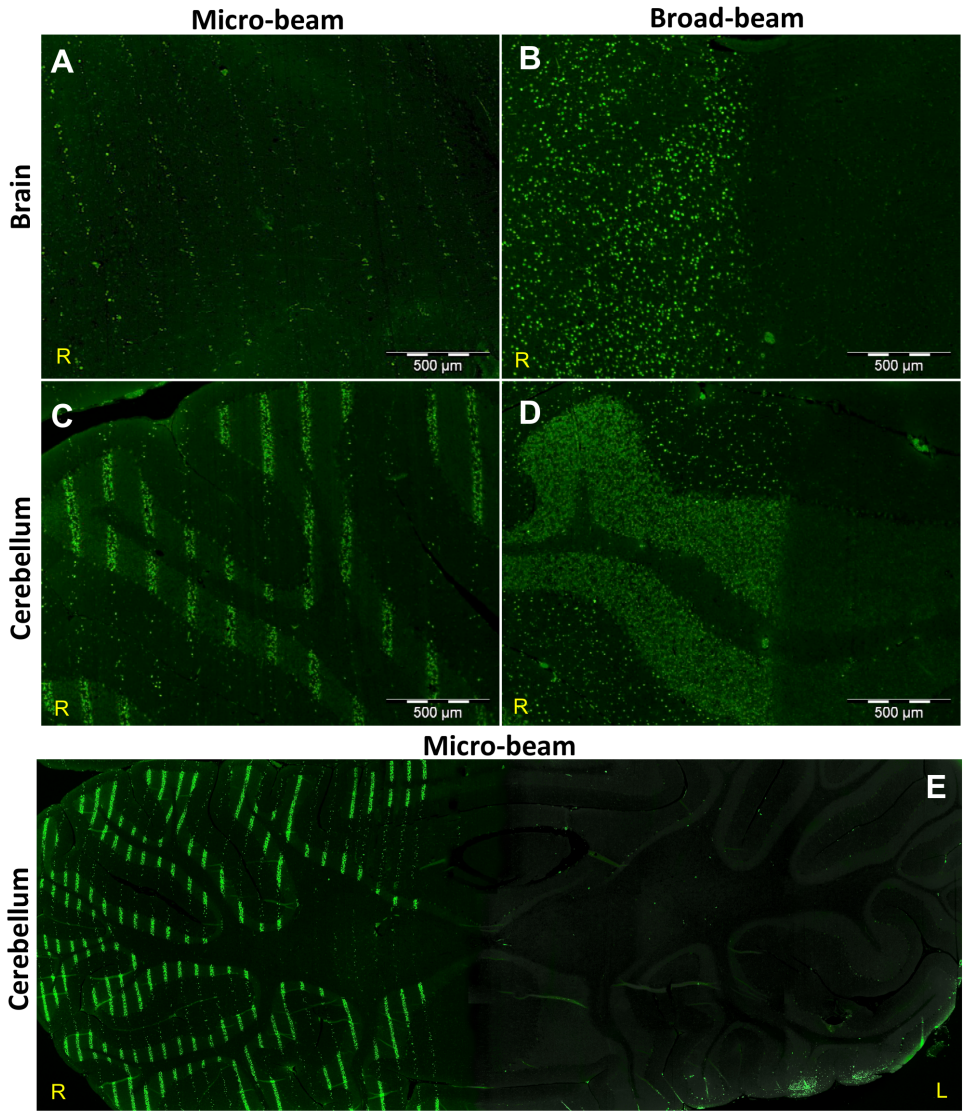

Figure 6.2γ-H2AX stain comparison between micro- and broad beam configurations. Horizontal sections of the irradiated right cerebral hemisphere and the cerebellum of Wistar rats. Images A - D were obtained from the irradiated right cerebral hemisphere and cerebellum of animals exposed to 350 Gy of either microbeam or broad beam; dissected 8 hours after irradiation. For the MRT array, the center-to-center distance was 200 µm. The position and intensity of the γ-H2AX marker (green) correlate with the deposition of the peak synchrotron doses. A) Radiation tracks of the microbeams in the right cerebral hemisphere. B) Right cerebral hemisphere after broad beam irradiation C) Radiation tracks of the microbeams in the cerebellum. D) Cerebellum after broad beam irradiation. E) Image reconstruction of several horizontal sections through the whole cerebellum after exposure of the right hemisphere to microbeams of 350 Gy; dissected at 4 hours after irradiation.

The comparison of γ-H2AX staining in 2 animals of the same cohort shown in figure 6.3 demonstrates the reliability of this stain in detecting unintended irradiation patterns due to technical problems during the irradiation process. In figure 6.3A, problems with the lateral translation of the collimator resulted in unequal spacing of the microbeams within the irradiation field in one of the animals, while figure 6.3B shows the correct delivery of the intended radiation pattern. This example indicates that the γ-H2AX marker can also be used to verify the accuracy of the delivery of the microbeams in situ.

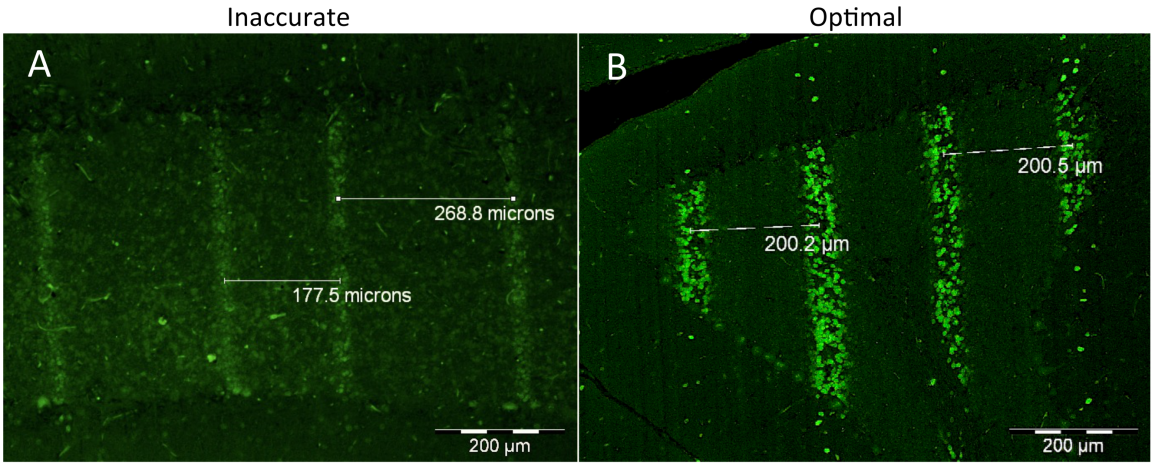

Figure 6.3 Comparison of radiation tracks produced by the same intended microbeam configuration (center-to-center distance of 200 µm). *By design the collimator spatially fractionates the microbeams with a center-to-center distance of 400 µm; to generate a center-to-center distance of 200 µm the collimator moves laterally followed by a second passage of the animal through the beam. A) Variable center-to-center distance of the microbeams due to inaccurate lateral translation of the collimator (35 Gy, 4 hours after irradiation). B) Accurate delivery of the microbeams. (350 Gy, 8 hours after irradiation).*

6.4.2 Factors that influence the formation of γ-H2AX foci

Figure 6.4 shows the width of the radiation path in micrometers, formed in the cerebellum by the phosphorylation of the H2AX histone to serine 139. Figure 6.4A shows how the dose significantly modifies the width of the tracks in normal rats. The plotted values indicate that 1) 35 Gy produced radiation tracks that match the width of the original microbeam and 2) 350 Gy increased the width of the tracks almost twice the width of the original microbeam. Moreover, the width of the radiation track significantly increased (P value < 0.01) from 4 to 8 hours after 350 Gy, while that phenomenon did not occur after 35 Gy (P value = 0.9265).

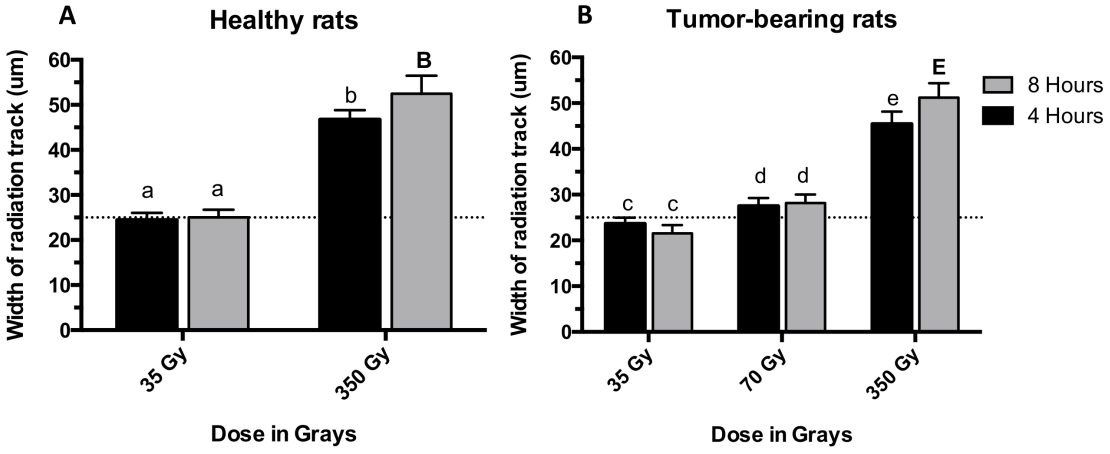

Figure 6.4 Mean thickness of the radiation tracks in the cerebellum. Microbeam irradiation was given to both normal (A) and tumour-bearing rats (B). The width of the microbeams was 25 μm, which is represented by the dotted line. Animals were exposed to 35, 70, or 350 Gy to their right cerebral hemisphere. Four and 8 hours indicate the two dissection times after irradiation. Different letters and different letter cases indicate significant differences between groups and within each group respectively. Error bars show SD.

Figure 6.4B shows that in tumour-bearing rats the irradiation dose affected the width of the radiation tracks similarly as in normal rats. While 35 Gy produced tracks of almost 25 μm width, 70 Gy resulted in slightly higher values producing tracks of 27 μm. The irradiation dose of 350 Gy produced once again an increase in the width of the microbeam tracks to almost twice the original width of the 25 μm microbeams. Furthermore, there was a statistically significant increase (P value < 0.01) in the width of the microbeam tracks between 4 and 8 hours after irradiation while no significance was found after 35 Gy (P value = 0.1563) and 70 Gy (P value = 0.9727). To summarize one can state that normal and tumour-bearing animals seem to have the same degree of response to both the irradiation dose and the time interval between irradiation and dissection.

Figure 6.5 demonstrates that the γ-H2AX fluorescence intensity in the cerebellum is directly related to irradiation dose. The histograms in figure 6.5A correspond to those from normal animals indicating that the dose is directly correlated with the intensity of the fluorescence. Additionally, the time interval between irradiation and dissection does not seem to have a major impact on the 350 Gy group, but it shows a clear difference after 35 Gy. Figure 6.5B shows that the dose is also correlated with the strength of the fluorescence in tumour-bearing animals. Here, dissection times show clear differences after 35 and 350 Gy irradiation. We attribute these differences to a possible migration of the irradiated cells towards the valley areas, which is explained in the discussion

during the analysis of figure 6.6. The presence or absence of a tumour does not seem to have a direct impact on the intensity of the fluorescence.

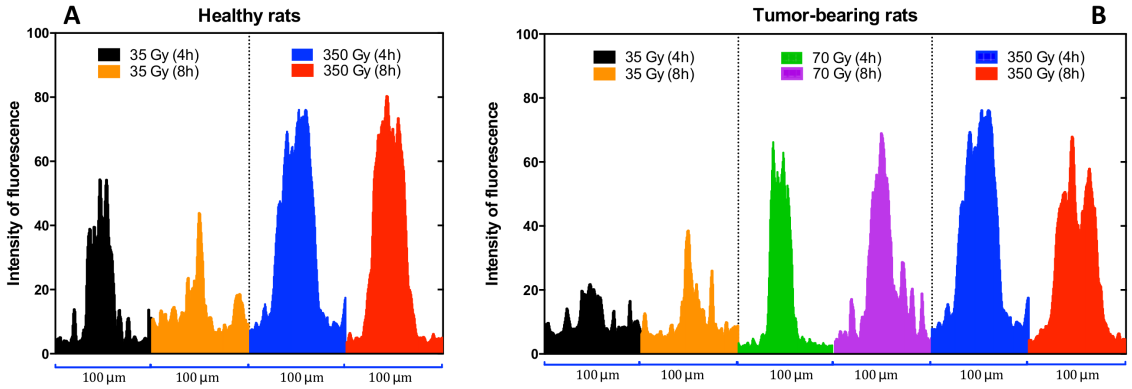

Figure 6.5 Intensity of the fluorescence. Masured through a 100 µm profile line traced perpendicular to the direction of the microbeams peak radiation path. *A) Intensity profile of normal rats. B) Intensity profile in tumour-bearing animals.*

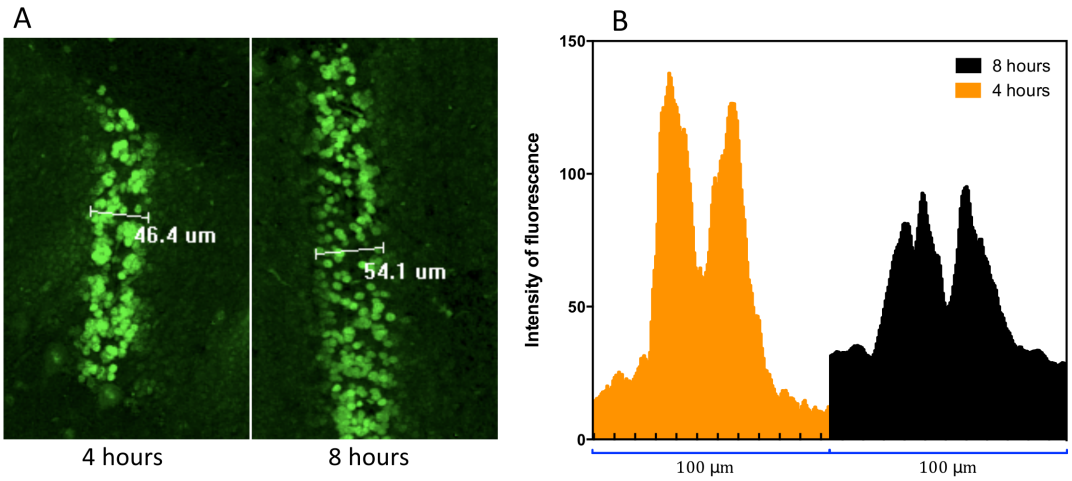

Figure 6.6 Different width of the radiation paths. A) Microbeam tracks as outlined by γ-H2AX stain at 4 and 8 hours after a 350 Gy irradiation. The high dose delivered resulted in an increase in the width of the microbeam track over time. B) Intensity of the fluorescence of the areas depicted in A.

The number of γ-H2AX positive cells per micrometer area was assessed and the results are shown in figure 6.7. There was a direct correlation between the number of positive cells per unit area and the radiation dose. The presence or absence of a tumour does not seem to affect this relationship. Although all the groups showed inverse correlation between the number of γ-H2AX positive cells per unit area and the time elapsed after irradiation, this observation was statistically significant (P value < 0.05) only in the normal animals.

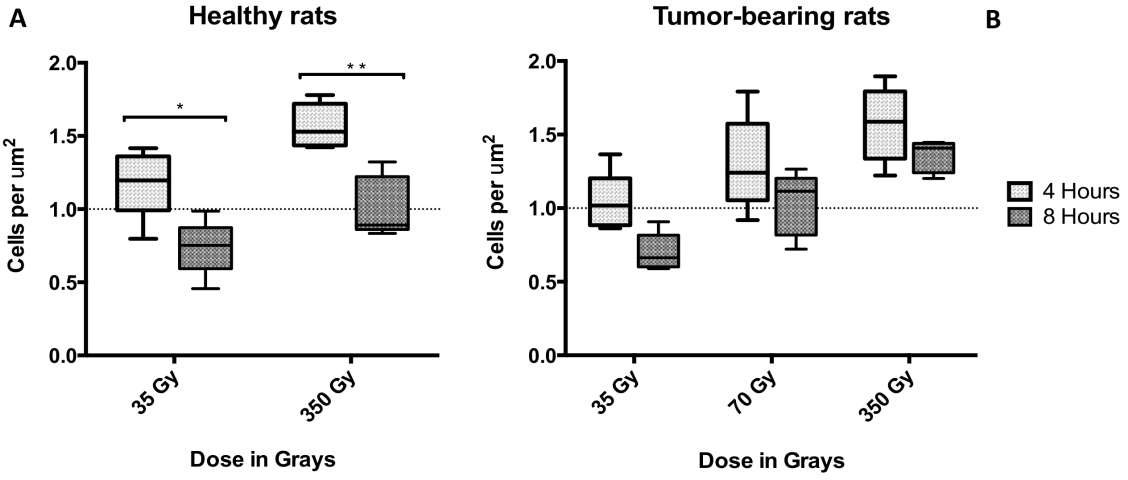

Figure 6.7 Number of γ-H2AX positive cells per unit area. The measurements were performed in the granular layer of the cerebellum. A) Shows the number of cells in normal rats while B) shows the number of cells in tumour-bearing animals. Stars show significant differences between 4 and 8 hours. Error bars correspond to SD.

6.5 DISCUSSION

The purpose of this work was to study the dose deposition by synchrotron radiation in the brain of Wistar rats using the phosphorylation of the H2AX histone as a biomarker. The conditions explored were 1) different survival times after irradiation to evaluate the dynamics of the γ-H2AX formation over time, 2) different doses of micro- and broad beam synchrotron radiation, and 3) the presence or absence of C6 glioma in the right (irradiated) cerebral hemisphere.

The γ-H2AX antibody stain positively reflected the deposition of the absorbed dose in the brain. The marker clearly outlined the paths of the microbeams and distinguished the irradiated hemisphere from the non-irradiated hemisphere. Our results are in accordance with observations made after synchrotron irradiation of fibroblast monolayers (Sprung, Cholewa, Usami, Kobayashi, & Crosbie, 2011) and EMT-6.5 tumours, normal skin, and hair follicle in mouse (Crosbie et al., 2010). The fluorescence observed after the delivery of the broad beam covers a large continuous volume of irradiated tissue in comparison to the much smaller tissue volumes traversed by the microbeams (Figure 2). The intensity of the fluorescence is stronger in the cerebellum than in the cerebral hemispheres because of the high cellular density of the granular cell layer. It was noted that the irradiation tracks outlined by the y-H2AX biomarker are not always perfectly parallel. This artefact is related to the histology technique. Several authors have described this phenomenon, attributing it to both

the process of paraffin embedding and to the distortion of thin tissue sections mounted on glass slides (Deverell, Bailey, & Whimster, 1989; Dobrin, 1996).

We also studied whether the presence of a tumour could modify the response of brain tissue to synchrotron radiation and lead to a different degree of γ-H2AX formation. Various authors have discussed the phenomenon called tumour-induced bystander effects, which is explained as changes in naïve cells that share the same milieu with cancer cells (Martin et al., 2011; Redon et al., 2010). The tumour microenvironment is established by the interaction between tumour, tumour stroma, the surrounding normal tissues, and the extracellular matrix (Atkinson, 2013). This distinctive microenvironment is rich in pro-inflammatory cytokines that contribute to an influx of macrophages and consequent angiogenesis as well as helping the tumour to escape from the immune system (Atkinson, 2013; Charles, Holland, Gilbertson, Glass, & Kettenmann, 2012; Gajewski, Schreiber, & Fu, 2013; Persano, Rampazzo, Basso, & Viola, 2013; Russell & Brown, 2013). Those reasons lead us to hypothesize that the presence of the C6 glioma in the right hemisphere of the brain could modulate the formation of γ-H2AX foci after synchrotron microbeam irradiation through tumour-induced bystander signals. However, our results indicated that there was no significant difference in γ-H2AX intensity, whether animals were normal or harboured a tumour.

When looking at the effect of the dose, our γ-H2AX data indicate that there is a direct correlation between the peak radiation dose and the width of the radiation tracks (Figure 4), the intensity of the fluorescence (Figure 5), and the number of immunoreactive cells per unit area (Figure 7). Our results are in accordance with work published by Rothkamm et al. (Rothkamm et al., 2012) in which they observed that the intensity and width of the γ-H2AX stripes increased with dose in fibroblasts and the skin of BALB/c mice. Furthermore, microdosimetry calculations published by Spiga et al. (Spiga, Siegbahn, Bräuer-Krisch, Randaccio, & Bravin, 2007) also correlated increases in the peak dose with increases in width of the radiation track, which is attributed to the increase in size of the transition zones. Transition or intermediate zones are those where the dose drastically changes from peak to valley levels (Blattmann et al., 2005; Spiga et al., 2007). As the peak dose increases the transition zone increases towards the valley areas, which increases the width of the radiation tracks and may explain our results. In vitro studies developed by Kashino et al. (Kashino et al., 2009) demonstrated that the number of foci per cell was also correlated to the dose in C6 cells. Furthermore, work developed in vivo in subcutaneous tumours and normal skin showed the same correlation between the intensity of γ-H2AX and the dose (Crosbie et al., 2010). The results of this present study further support the data acquired previously by our group (C Fernandez-Palomo et al., 2013) in which the brain of nude mice was exposed to synchrotron microbeams and pencil beams.

When evaluating the dynamic of γ-H2AX formation at 4 and 8 hours after irradiation, our results indicated a decrease in the number of γ-H2AX positive cells per unit area over time. This is in accordance with published work in vitro (Kashino et al., 2009) and in vivo (Crosbie et al., 2010), where the amount of positive cells per unit area was also assessed after synchrotron microbeam irradiation. Those investigations concluded that the DNA repair mechanisms are the reason of the decrease and our findings shown in figure 7 are in agreement with those conclusions. Interestingly, when looking at Figure 4 in detail, our data show an increase of immunoreactivity over time after 350 Gy, which could be explained by the occurrence of bystander effects within the transition zone and towards the valley area. Although our study shows a connection between bystander effects and synchrotron microbeam radiation therapy, we are not focusing on exploring the nature of the bystander molecule. That would require a larger experiment and the allocation of beam time in the synchrotron is very competitive and thus limited. Instead we focused on demonstrating that bystander effects (no matter the nature of the molecules) can trigger an increase in γ-H2AX signal that is independent of the direct hit by radiation. This idea is supported by work developed from Kashino et al (Kashino et al., 2009) who demonstrated that microplanar beams induced DSBs through bystander effects. Moreover work developed in 3-D tissue models by Sedelnikova et al (Sedelnikova et al., 2007) shows irradiated cells continuously producing DNA-

damaging agents which reached a plateau by the first day after alpha-particle microbeam irradiation. Our work shows that the γ-H2AX induction does not only depend on the direct hit by synchrotron radiation, it may as well depend on the actual absolute peak dose triggering different signals in the valley. As previously explained, as the dose increases the transition zone increases towards the tissue immediately adjacent to the nominal width of the microbeam tracks. This 'transition tissue' of ≈10-20 μm wide for our MRT spectrum, is therefore exposed to a dose-gradient that may generate a population of cells prone to produce and respond to bystander signals. This would ultimately amplify over time the amount of DNA damage caused by radiation, explaining why the width of the radiation track observed by the γ-H2AX foci increases from 4 to 8 hours. Therefore, our results do not reflect the physical PVDR, instead they suggest that the γ-H2AX should be used to simply observe and describe what is happening after different conditions using the physical dose rather than trying to measure PVDRs.

Although bystander experiments involving the use of microbeams are well described in the literature, a distinction needs to be made between our experiments and those using single versus multiple microbeams, as well as those performed in cell cultures versus animals. The use of a single microbeam is characterized for focusing the radiation to either a single cell or parts of the cells (Maeda, Kobayashi, Matsumoto, Usami, & Tomita, 2013; Maeda, Tomita, Usami, & Kobayashi, 2010; Tomita, Kobayashi, & Maeda, 2012; Tomita, Maeda,

Maezawa, Usami, & Kobayashi, 2010) or to multiple spots in a small area of cells (Anderson et al., 2014; Autsavapromporn et al., 2013; Mutou-Yoshihara, Funayama, Yokota, & Kobayashi, 2012) . Those cell culture systems allow for well-controlled experimental settings for the study of mechanisms of transmission of bystander signals and the molecules involved. Our synchrotron work is much more complex because, 1) we use a precise array of multiple rectangular microbeams that aim to target cells within 25μm with very high doses, leaving intermediate gaps of 200μm were cells are also exposed but to a much lower dose level, and 2) we perform our experiments on animals because we are aiming at developing a potential treatment for glioma. Although these differences present obstacles for the study of the bystander effect mechanism, our work provides the first evidence of active involvement of bystander effects in the study and development of innovative methods for brain radiotherapy using synchrotron radiation in vivo.

Figure 6 shows a comparison of two images 4 and 8 hours after the rats underwent microbeam radiation with a peak dose of 350 Gy. If observed carefully, clusters of cells have increased their relative distance from one another over time. Tofilon and Fike explain that during the first hours after radiation exposure the tissue response is characterized by incipient cell death, and secondary processes - such as bystander effects or inflammation - that generate a persistent oxidative stress environment which will contribute to tissue injury and

enhanced cytokine gene expression (Tofilon & Fike, 2000). Moreover, it is mentioned that those processes are directly related to the absorbed dose. Figure 6B clearly shows deep decrease in the fluorescence in the center of the profile. The combined analysis of figures 6A and 6B suggests that irradiated cells are migrating from the center toward the valley areas. However this claim needs further studies.

In summary, the exploration of γ-H2AX as a biomarker for dose deposition in the brain after synchrotron microbeam radiation brought a number of interesting findings. First, we were able to show a direct correlation between irradiation dose and the formation of γ-H2AX foci in the brain. There was a direct correlation between the width of the radiation track and the dissection time after 350 Gy, while there was no significant change in the width of the microbeam tracks at lower irradiation doses. This suggests that radiation-induced bystander effects are produced from the cells reached by both the high-peak doses and the dose-gradient of the transition zone. The release of these signals would amplify the DNA damage produced by the synchrotron radiation, which would translate in wider γ-H2AX tracks. Secondly, the presence of C6-Glioma does not seem to induce tumour-bystander effects that could modify the degree of γ-H2AX formation. Third, the availability of technical equipment for reliable accurate translation of the targeted animal or human patient is a critical prerequisite for an optimal delivery of synchrotron microbeam radiation. In conclusion, we suggest

that the γ-H2AX foci should be used as biomarker for targeted and non-targeted DNA damage after synchrotron radiation rather than a tool to measure the actual physical doses.

6.6 ACKNOWLEDGEMENTS

The authors want to acknowledge the support of Christian Schroll during the irradiation of the animals and the assistance of Viviana Sverdlick (Department of Neuropathology, Freiburg University Medical Center) during the immunohistofluorescence staining.

6.7 REFERENCES

Ahmad, S. B., McNeill, F. E., Byun, S. H., Prestwich, W. V, Mothersill, C., Seymour, C., … Fernandez, C. (2013). Ultra-Violet Light Emission from HPV-G Cells Irradiated with Low Let Radiation From (90)Y; Consequences for Radiation Induced Bystander Effects. *Dose-Response : A Publication of International Hormesis Society*, *11*, 498–516. doi:10.2203/dose-response.12-048.Ahmad

Al-Mayah, A. H. J., Irons, S. L., Pink, R. C., Carter, D. R. F., & Kadhim, M. A. (2012). Possible role of exosomes containing RNA in mediating nontargeted effect of ionizing radiation. *Radiation Research*, *177*(5), 539–45. doi:10.1667/RR2868.1

Anderson, D. L., Mirzayans, R., Andrais, B., Siegbahn, E. A., Fallone, B. G., & Warkentin, B. (2014). Spatial and temporal distribution of γH2AX fluorescence in human cell cultures following synchrotron-generated X-ray microbeams: lack of correlation between persistent γH2AX foci and apoptosis. *Journal of Synchrotron Radiation*, *21*(Pt 4), 801–10. doi:10.1107/S1600577514011424

Atkinson, M. J. (2013). Radiation treatment effects on the proteome of the tumour microenvironment. *Advances in Experimental Medicine and Biology*, *990*, 49–60. doi:10.1007/978-94-007-5896-4_3

Autsavapromporn, N., Suzuki, M., Funayama, T., Usami, N., Plante, I., Yokota, Y., … Murakami, T. (2013). Gap junction communication and the propagation of bystander effects induced by microbeam irradiation in human fibroblast cultures: the impact of radiation quality. *Radiation Research*, *180*(4), 367–75. doi:10.1667/RR3111.1

Azzam, E., de Toledo, S., & Little, J. (2004). Stress Signaling from Irradiated to Non-Irradiated Cells. *Current Cancer Drug Targets*, *4*(1), 53–64. doi:10.2174/1568009043481641

Balvay, D., Troprès, I., Billet, R., Joubert, A., Péoc'h, M., Cuenod, C. A., & Le Duc, G. (2009). Mapping the zonal organization of tumor perfusion and permeability in a rat glioma model by using dynamic

contrast-enhanced synchrotron radiation CT. *Radiology*, *250*(3), 692–702. doi:10.1148/radiol.2501071929

Benda, P., Lightbody, J., Sato, G., Levine, L., & Sweet, W. (1968). Differentiated rat glial cell strain in tissue culture. *Science (New York, N.Y.)*, *161*(3839), 370–1. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4873531

Beygi, S., Saadat, S., Jazayeri, S. B., & Rahimi-Movaghar, V. (2013). Epidemiology of pediatric primary malignant central nervous system tumors in Iran: a 10 year report of National Cancer Registry. *Cancer Epidemiology*, *37*(4), 396–401. doi:10.1016/j.canep.2013.03.002

Blattmann, H., Gebbers, J.-O., Bräuer-Krisch, E., Bravin, A., Le Duc, G., Burkard, W., … Laissue, J. a. (2005). Applications of synchrotron X-rays to radiotherapy. *Nuclear Instruments and Methods in Physics Research Section A: Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 17–22. doi:10.1016/j.nima.2005.03.060

Bouchet, A., Bidart, M., Miladi, I., Le Clec'h, C., Serduc, R., Coutton, C., … Le Duc, G. (2014). Characterization of the 9L gliosarcoma implanted in the Fischer rat: an orthotopic model for a grade IV brain tumor. *Tumour Biology : The Journal of the International Society for Oncodevelopmental Biology and Medicine*, *35*(7), 6221–33. doi:10.1007/s13277-014-1783-6

Bouchet, A., Lemasson, B., Christen, T., Potez, M., Rome, C., Coquery, N., … Serduc, R. (2013). Synchrotron microbeam radiation therapy induces hypoxia in intracerebral gliosarcoma but not in the normal brain. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, *108*(1), 143–8. doi:10.1016/j.radonc.2013.05.013

Bouchet, A., Lemasson, B., Le Duc, G., Maisin, C., Bräuer-Krisch, E., Siegbahn, E. A., … Serduc, R. (2010). Preferential effect of synchrotron microbeam radiation therapy on intracerebral 9L gliosarcoma vascular networks. *International Journal of Radiation Oncology, Biology, Physics*, *78*(5), 1503–12. doi:10.1016/j.ijrobp.2010.06.021

Bouchet, A., Sakakini, N., El Atifi, M., Le Clec'h, C., Brauer, E., Moisan, A., … Pelletier, L. (2013). Early gene expression analysis in 9L orthotopic tumor-bearing rats identifies immune modulation in molecular response to synchrotron microbeam radiation therapy. *PloS One*, *8*(12), e81874. doi:10.1371/journal.pone.0081874

Bouchet, A., Sakakini, N., El Atifi, M., Le Clec'h, C., Bräuer-Krisch, E., Rogalev, L., … Pelletier, L. (2014). Pelletier L. Identification of AREG and PLK1 pathway modulation as a potential key of the response of intracranial 9L tumor to microbeam radiation therapy. *International Journal of Cancer. Journal International Du Cancer*, *in print*.

Bräuer-Krisch, E., Serduc, R., Siegbahn, E. a, Le Duc, G., Prezado, Y., Bravin, A., … Laissue, J. A. (2010). Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. *Mutation Research*, *704*(1-3), 160–6. doi:10.1016/j.mrrev.2009.12.003

Charles, N. a., Holland, E. C., Gilbertson, R., Glass, R., & Kettenmann, H. (2012). The brain tumor microenvironment. *Glia*, *60*(3), 502–14. doi:10.1002/glia.21264

Cho, K.-T., Kim, D. G., Paek, S. H., Chung, H.-T., Jung, H. W., & Park, H. W. (2012). Concurrent treatment with BCNU and Gamma Knife radiosurgery in the rat malignant glioma model. *Journal of Neurological Surgery. Part A, Central European Neurosurgery*, *73*(3), 132–41. doi:10.1055/s-0032-1304216

Costes, S. V, Chiolo, I., Pluth, J. M., Barcellos-Hoff, M. H., & Jakob, B. (2010). Spatiotemporal characterization of ionizing radiation induced DNA damage foci and their relation to chromatin organization. *Mutation Research*, *704*(1-3), 78–87. doi:10.1016/j.mrrev.2009.12.006

Crosbie, J. C., Anderson, R. L., Rothkamm, K., Restall, C. M., Cann, L., Ruwanpura, S., … Rogers, P. a W. (2010). Tumor cell response to synchrotron microbeam radiation therapy differs markedly from cells

in normal tissues. *International Journal of Radiation Oncology, Biology, Physics*, *77*(3), 886–94. doi:10.1016/j.ijrobp.2010.01.035

Deverell, M. H., Bailey, N., & Whimster, W. F. (1989). Tissue Distortion in Three-Dimensional Reconstruction of Wax or Plastic Embedded Microscopic Structures. *Pathology - Research and Practice*, *185*(5), 598–601.

Dilmanian, F. a, Qu, Y., Liu, S., Cool, C. D., Gilbert, J., Hainfeld, J. F., … McDonald, J. W. (2005). X-ray microbeams: Tumor therapy and central nervous system research. *Nuclear Instruments & Methods in Physics Research. Section A, Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 30–37. doi:10.1016/j.nima.2005.03.062

Dilmanian, F. A., Button, T. M., Le Duc, G., Zhong, N., Peña, L. A., Smith, J. A. L., … Ren, B. (2002). Response of rat intracranial 9L gliosarcoma to microbeam radiation therapy. *Neuro-Oncology*, *4*(1), 26. doi:10.1016/j.ejrad.2008.04.055

Dilmanian, F. A., Morris, G. M., Le Duc, G., Huang, X., Ren, B., Bacarian, T., … Shivaprasad, H. L. (2001). Response of avian embryonic brain to spatially segmented x-ray microbeams. *Cellular and Molecular Biology (Noisy-Le-Grand, France)*, *47*(3), 485–93. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11441956

Dilmanian, F. A., Morris, G. M., Zhong, N., Bacarian, T., Hainfeld, J. F., Kalef-Ezra, J., … Rosen, E. M. (2003). Murine EMT-6 carcinoma: high therapeutic efficacy of microbeam radiation therapy. *Radiation Research*, *159*(5), 632–41. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12710874

Dilmanian, F. A., Qu, Y., Feinendegen, L. E., Peña, L. A., Bacarian, T., Henn, F. A., … McDonald, J. W. (2007). Tissue-sparing effect of x-ray microplanar beams particularly in the CNS: is a bystander effect involved? *Experimental Hematology*, *35*(4 Suppl 1), 69–77. doi:10.1016/j.exphem.2007.01.014

Dobrin, P. B. (1996). Effect of histologic preparation on the cross-sectional area of arterial rings. *The Journal of Surgical Research*, *61*(2), 413–5. doi:10.1006/jsre.1996.0138

Farrell, C. L., Stewart, P. a, & Del Maestro, R. F. (1987). A new glioma model in rat: the C6 spheroid implantation technique permeability and vascular characterization. *Journal of Neuro-Oncology*, *4*(4), 403–15. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/3572471

Fazzari, J., Mersov, A., Smith, R., Seymour, C., & Mothersill, C. (2012). Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect. *International Journal of Radiation Biology*, *88*(10), 786–90. doi:10.3109/09553002.2012.714519

Fernandez-Palomo, C., Bräuer-Krisch, E., Trippel, M., Schroll, C., Requardt, H., Bartzsch, S., … Schültke, E. (2013). DNA double strand breaks in the acute phase after synchrotron pencilbeam irradiation. *Journal of Instrumentation*, *8*(07), C07005–C07005. doi:10.1088/1748-0221/8/07/C07005

Fernandez-Palomo, C., Schültke, E., Smith, R., Bräuer-Krisch, E., Laissue, J., Schroll, C., … Mothersill, C. (2013). Bystander effects in tumor-free and tumor-bearing rat brains following irradiation by synchrotron X-rays. *International Journal of Radiation Biology*, *89*(6), 445–53. doi:10.3109/09553002.2013.766770

Gajewski, T. F., Schreiber, H., & Fu, Y.-X. (2013). Innate and adaptive immune cells in the tumor microenvironment. *Nature Immunology*, *14*(10), 1014–22. doi:10.1038/ni.2703

Greve, B., Bölling, T., Amler, S., Rössler, U., Gomolka, M., Mayer, C., … Schmezer, P. (2012). Evaluation of different biomarkers to predict individual radiosensitivity in an inter-laboratory comparison--lessons for future studies. *PloS One*, *7*(10), e47185. doi:10.1371/journal.pone.0047185

Iyer, R., & Lehnert, B. E. (2000). Factors underlying the cell growth-related bystander responses to ?? particles. *Cancer Research*, *60*(5), 1290–1298. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10728689

Jella, K. K., Rani, S., O'Driscoll, L., McClean, B., Byrne, H. J., & Lyng, F. M. (2014). Exosomes are involved in mediating radiation induced bystander signaling in human keratinocyte cells. *Radiation Research*, *181*(2), 138–45. doi:10.1667/RR13337.1

Kashino, G., Kondoh, T., Nariyama, N., Umetani, K., Ohigashi, T., Shinohara, K., … Ono, K. (2009). Induction of DNA double-strand breaks and cellular migration through bystander effects in cells irradiated with the slit-type microplanar beam of the spring-8 synchrotron. *International Journal of Radiation Oncology, Biology, Physics*, *74*(1), 229–36. doi:10.1016/j.ijrobp.2008.09.060

Laissue, J. a, Bartzsch, S., Blattmann, H., Bräuer-Krisch, E., Bravin, A., Dalléry, D., … Slatkin, D. N. (2013). Response of the rat spinal cord to X-ray microbeams. *Radiotherapy and Oncology : Journal of the European Society for Therapeutic Radiology and Oncology*, *106*(1), 106–11. doi:10.1016/j.radonc.2012.12.007

Laissue, J. A., Blattmann, H., Di Michiel, M., Slatkin, D. N., Lyubimova, N., Guzman, R., … Wagner, H.-P. (2001). The weanling piglet cerebellum: a surrogate for tolerance to MRT (microbeam radiation therapy) in pediatric neuro-oncology. *Penetrating Radiation Systems and Application*, *4508*, 65–73. doi:10.1117/12.450774

Laissue, J. A., Geiser, G., Spanne, P. O., Dilmanian, F. a, Gebbers, J. O., Geiser, M., … Slatkin, D. N. (1998). Neuropathology of ablation of rat gliosarcomas and contiguous brain tissues using a microplanar beam of synchrotron-wiggler-generated X rays. *International Journal of Cancer. Journal International Du Cancer*, *78*(5), 654–60. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med4&NEWS=N&AN=9808538

Laissue, J. A., Lyubimova, N., Wagner, H.-P., Archer, D. W., Slatkin, D. N., Di Michiel, M., … Blattmann, H. (1999). Microbeam radiation therapy. In H. B. Barber & H. Roehrig (Eds.), *SPIE's International Symposium on Optical Science, Engineering, and Instrumentation* (pp. 38–45). International Society for Optics and Photonics. doi:10.1117/12.368185

Le Duc, G., Corde, S., Elleaume, H., Estève, F., Charvet, A. M., Brochard, T., … Le Bas, J. F. (2000). Feasibility of synchrotron radiation computed tomography on rats bearing glioma after iodine or gadolinium injection. Jeune Equipe RSRM-UJF. *European Radiology*, *10*(9), 1487–92. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10997441

Le, M., McNeill, F. E., Seymour, C., Rainbow, A. J., & Mothersill, C. E. (2014). An observed effect of ultraviolet radiation emitted from β-irradiated HaCaT cells upon non-β-irradiated bystander cells. *Radiation Research*, (In Print).

Lyng, F. M., Maguire, P., McClean, B., Seymour, C., & Mothersill, C. (2006). The Involvement of Calcium and MAP Kinase Signaling Pathways in the Production of Radiation-Induced Bystander Effects. *Radiation Research*, *165*(4), 400–409. doi:10.1667/RR3527.1

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2000). Production of a signal by irradiated cells which leads to a response in unirradiated cells characteristic of initiation of apoptosis. *British Journal of Cancer*, *83*(9), 1223–30. doi:10.1054/bjoc.2000.1433

Lyng, F. M., Seymour, C. B., & Mothersill, C. (2001). Oxidative stress in cells exposed to low levels of ionizing radiation. *Biochemical Society Transactions*, *29*(2), 350. doi:10.1042/0300-5127:0290350

Lyng, F. M., Seymour, C., & 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*(4), 365–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893237

Maeda, M., Kobayashi, K., Matsumoto, H., Usami, N., & Tomita, M. (2013). X-ray-induced bystander responses reduce spontaneous mutations in V79 cells. *Journal of Radiation Research*, *54*(6), 1043–9. doi:10.1093/jrr/rrt068

Maeda, M., Tomita, M., Usami, N., & Kobayashi, K. (2010). Bystander cell death is modified by sites of energy deposition within cells irradiated with a synchrotron X-ray microbeam. *Radiation Research*, *174*(1), 37–45. doi:10.1667/RR2086.1

Martin, O. a, Redon, C. E., Nakamura, A. J., Dickey, J. S., Georgakilas, A. G., & Bonner, W. M. (2011). Systemic DNA damage related to cancer. *Cancer Research*, *71*(10), 3437–41. doi:10.1158/0008-5472.CAN-10-4579

Miura, M., Blattmann, H., Bräuer-Krisch, E., Bravin, A., Hanson, A. L., Nawrocky, M. M., … Laissue, J. A. (2006). Radiosurgical palliation of aggressive murine SCCVII squamous cell carcinomas using synchrotron-generated X-ray microbeams. *The British Journal of Radiology*, *79*(937), 71–5. doi:10.1259/bjr/50464795

Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., … Seymour, C. (2014). Transmission of signals from rats receiving high doses of microbeam radiation to cage mates: an inter-mammal bystander effect. *Dose-Response : A Publication of International Hormesis Society*, *12*(1), 72–92. doi:10.2203/dose-response.13-011.Mothersill

Mothersill, C., & Seymour, C. (1997). Medium from irradiated human epithelial cells but not human fibroblasts reduces the clonogenic survival of unirradiated cells. *International Journal of Radiation Biology*, *71*(4), 421–7. doi:10.1080/095530097144030

Mothersill, C., & Seymour, C. B. (2004). Radiation-induced bystander effects--implications for cancer. *Nature Reviews. Cancer*, *4*(2), 158–164. doi:10.1038/nrc1277

Murphy, J. E. J., Nugent, S., Seymour, C., & Mothersill, C. (2005). Mitochondrial DNA point mutations and a novel deletion induced by direct low-LET radiation and by medium from irradiated cells. *Mutation Research*, *585*(1-2), 127–36. doi:10.1016/j.mrgentox.2005.04.011

Mutou-Yoshihara, Y., Funayama, T., Yokota, Y., & Kobayashi, Y. (2012). Involvement of bystander effect in suppression of the cytokine production induced by heavy-ion broad beams. *International Journal of Radiation Biology*, *88*(3), 258–66. doi:10.3109/09553002.2012.636138

Nagano, N., Sasaki, H., Aoyagi, M., & Hirakawa, K. (1993). Invasion of experimental rat brain tumor: early morphological changes following microinjection of C6 glioma cells. *Acta Neuropathologica*, *86*(2), 117–25. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/8213067

Nagasawa, H., & Little, J. B. (1992). Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Research*, *52*(22), 6394–6396. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1423287

Narayanan, P. K., Goodwin, E. H., & Lehnert, B. E. (1997). Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Research*, *57*(18), 3963–71. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9307280

Narayanan, P. K., LaRue, K. E., Goodwin, E. H., & Lehnert, B. E. (1999). Alpha particles induce the production of interleukin-8 by human cells. *Radiation Research*, *152*(1), 57–63. doi:10.2307/3580049

Persano, L., Rampazzo, E., Basso, G., & Viola, G. (2013). Glioblastoma cancer stem cells: role of the microenvironment and therapeutic targeting. *Biochemical Pharmacology*, *85*(5), 612–22. doi:10.1016/j.bcp.2012.10.001

Prise, K. M., & O'Sullivan, J. M. (2009). Radiation-induced bystander signalling in cancer therapy. *Nature Reviews. Cancer*, *9*(5), 351–60. doi:10.1038/nrc2603

Priyadarshika, R. C. U., Crosbie, J. C., Kumar, B., & Rogers, P. a W. (2011). Biodosimetric quantification of short-term synchrotron microbeam versus broad-beam radiation damage to mouse skin using a dermatopathological scoring system. *The British Journal of Radiology*, *84*(1005), 833–42. doi:10.1259/bjr/58503354

Redon, C. E., Dickey, J. S., Nakamura, A. J., Kareva, I. G., Naf, D., Nowsheen, S., … Sedelnikova, O. a. (2010). Tumors induce complex DNA damage in distant proliferative tissues in vivo. *Proceedings of the National Academy of Sciences of the United States of America*, *107*(42), 17992–7. doi:10.1073/pnas.1008260107

Ricard, D., Idbaih, A., Ducray, F., Lahutte, M., Hoang-Xuan, K., & Delattre, J.-Y. (2012). Primary brain tumours in adults. *Lancet*, *379*(9830), 1984–96. doi:10.1016/S0140-6736(11)61346-9

Rogakou, E. P., Pilch, D. R., Orr, a. H., Ivanova, V. S., & Bonner, W. M. (1998). DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *The Journal of Biological Chemistry*, *273*(10), 5858–68. doi:10.1074/jbc.273.10.5858

Rothkamm, K., Crosbie, J. C., Daley, F., Bourne, S., Barber, P. R., Vojnovic, B., … Rogers, P. a W. (2012). In situ biological dose mapping estimates the radiation burden delivered to "spared" tissue between synchrotron X-ray microbeam radiotherapy tracks. *PloS One*, *7*(1), e29853. doi:10.1371/journal.pone.0029853

Russell, J. S., & Brown, J. M. (2013). The irradiated tumor microenvironment: role of tumor-associated macrophages in vascular recovery. *Frontiers in Physiology*, *4*(July), 157. doi:10.3389/fphys.2013.00157

Safdie, F., Brandhorst, S., Wei, M., Wang, W., Lee, C., Hwang, S., … Longo, V. D. (2012). Fasting enhances the response of glioma to chemo- and radiotherapy. *PloS One*, *7*(9), e44603. doi:10.1371/journal.pone.0044603

Saroya, R., Smith, R., Seymour, C., & Mothersill, C. (2009). Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism. *Dose-Response : A Publication of International Hormesis Society*, *8*(3), 317–30. doi:10.2203/dose-response.09-043.Saroya

Schültke, E., Juurlink, B. H. J., Ataelmannan, K., Laissue, J., Blattmann, H., Bräuer-Krisch, E., … Fourney, D. (2008). Memory and survival after microbeam radiation therapy. *European Journal of Radiology*, *68*(3 SUPPL.), 142–146. doi:10.1016/j.ejrad.2008.04.051

Sedelnikova, O. a, Nakamura, A., Kovalchuk, O., Koturbash, I., Mitchell, S. a, Marino, S. a, … Bonner, W. M. (2007). DNA double-strand breaks form in bystander cells after microbeam irradiation of three-dimensional human tissue models. *Cancer Research*, *67*(9), 4295–302. doi:10.1158/0008-5472.CAN-06-4442

Serduc, R., Berruyer, G., Brochard, T., Renier, M., & Nemoz, C. (2010). In vivo pink-beam imaging and fast alignment procedure for rat brain lesion microbeam radiation therapy. *Journal of Synchrotron Radiation*, *17*(3), 325–31. doi:10.1107/S0909049510006667

Serduc, R., Bouchet, A., Bräuer-Krisch, E., Laissue, J. A., Spiga, J., Sarun, S., … Le Duc, G. (2009). Synchrotron microbeam radiation therapy for rat brain tumor palliation-influence of the microbeam width at constant valley dose. *Physics in Medicine and Biology*, *54*(21), 6711–24. doi:10.1088/0031-9155/54/21/017

Shao, C., Stewart, V., Folkard, M., Michael, B. D., & Prise, K. M. (2003). Nitric Oxide-Mediated Signaling in the Bystander Response of Individually Targeted Glioma Cells. *Cancer Research*, *63*(23), 8437–8442.

Sheehan, J., Ionescu, A., Pouratian, N., Hamilton, D. K., Schlesinger, D., Oskouian, R. J., & Sansur, C. (2008). Use of trans sodium crocetinate for sensitizing glioblastoma multiforme to radiation: laboratory investigation. *Journal of Neurosurgery*, *108*(5), 972–8. doi:10.3171/JNS/2008/108/5/0972

Slatkin, D. N., Spanne, P., Dilmanian, F. a, Gebbers, J. O., & Laissue, J. A. (1995). Subacute neuropathological effects of microplanar beams of x-rays from a synchrotron wiggler. *Proceedings of*

the National Academy of Sciences of the United States of America, *92*(19), 8783–8787. doi:10.1073/pnas.92.19.8783

Slatkin, D. N., Spanne, P., Dilmanian, F. A., & Sandborg, M. (1992). Microbeam radiation therapy. *Medical Physics*, *19*(6), 1395–1400. doi:10.1118/1.596771

Smith, R. W., Wang, J., Schültke, E., Seymour, C. B., Bräuer-Krisch, E., Laissue, J. A., … Mothersill, C. E. (2013). Proteomic changes in the rat brain induced by homogenous irradiation and by the bystander effect resulting from high energy synchrotron X-ray microbeams. *International Journal of Radiation Biology*, *89*(2), 118–27. doi:10.3109/09553002.2013.732252

Spiga, J., Siegbahn, E. a., Bräuer-Krisch, E., Randaccio, P., & Bravin, A. (2007). The GEANT4 toolkit for microdosimetry calculations: Application to microbeam radiation therapy (MRT). *Medical Physics*, *34*(11), 4322. doi:10.1118/1.2794170

Sprung, C. N., Cholewa, M., Usami, N., Kobayashi, K., & Crosbie, J. C. (2011). DNA damage and repair kinetics after microbeam radiation therapy emulation in living cells using monoenergetic synchrotron X-ray microbeams. *Journal of Synchrotron Radiation*, *18*(Pt 4), 630–6. doi:10.1107/S0909049511011836

Tofilon, P. J., & Fike, J. R. (2000). The radioresponse of the central nervous system: a dynamic process. *Radiation Research*, *153*(4), 357–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10798963

Tomita, M., Kobayashi, K., & Maeda, M. (2012). Microbeam studies of soft X-ray induced bystander cell killing using microbeam X-ray cell irradiation system at CRIEPI. *Journal of Radiation Research*, *53*(3), 482–8. doi:10.1269/jrr.11055

Tomita, M., Maeda, M., Maezawa, H., Usami, N., & Kobayashi, K. (2010). Bystander cell killing in normal human fibroblasts is induced by synchrotron X-ray microbeams. *Radiation Research*, *173*(3), 380–5. doi:10.1667/RR1995.1

World Health Organization. (2008). *World cancer report*. (P. Boyle & B. Levin, Eds.). Lyon. Retrieved from http://www.cabdirect.org/abstracts/20103010665.html

Zhong, N., Morris, G. M., Bacarian, T., Rosen, E. M., & Avraham Dilmanian, F. (2003). Response of Rat Skin to High-Dose Unidirectional X-Ray Microbeams: A Histological Study. *Radiation Research*, *160*(2), 133–142. doi:10.1667/3033

Zhou, H., Ivanov, V. N., Lien, Y.-C., Davidson, M., & Hei, T. K. (2008). Mitochondrial function and nuclear factor-kappaB-mediated signaling in radiation-induced bystander effects. *Cancer Research*, *68*(7), 2233–40. doi:10.1158/0008-5472.CAN-07-5278

Chapter 7

7 DNA DOUBLE STRAND BREAKS IN THE ACUTE PHASE AFTER SYNCHROTRON PENCILBEAM IRRADIATION

C. Fernandez-Palomo, E. Bräuer-Krisch, M. Trippel, C. Schroll, H. Requardt, S. Bartzsch, G. Nikkhah, E. Schültke.

This research project was design by Schültke and the first author of this thesis. The irradiations were performed by the first author, Schültke, Bräuer-Krisch, Trippel and Schroll. Tissue collection, processing and data recollection were performed by the first author. Data analysis was performed by the first author, Bartzsch, Requardt and Schültke. The manuscript was written by the first author, Schültke and Bartzsch. Nikkhah provided financial support and access to his laboratories.

The manuscript is published in the Journal of Instrumentation.

Published on July 16, 2013.

Volume 08; 07, doi:10.1088/1748-0221/8/07/C07005

7.1 ABSTRACT

Introduction: At the biomedical beamline of the European Synchrotron Radiation Facility (ESRF), we have established a method to study pencilbeam irradiation in-vivo in small animal models. The pencilbeam irradiation technique is based on the principle of microbeam irradiation, a concept of spatially fractionated high-dose irradiation. Using γH2AX as marker, we followed the development of DNA double strand breaks (DSBs) over 48 hrs after whole brain irradiation with the pencilbeam technique.

Method: Almost square pencilbeams with an individual size of 51 x 50 μm were produced with an MSC collimator using a step and shoot approach, while the animals were moved vertically through the beam. The center-to-center distance (ctc) was 400 μm, with a peak-to-valley dose ratio (PVDR) of about 400. Five groups of healthy adult mice received peak irradiation doses of either 330 Gy or 2,460 Gy and valley doses of 0.82 Gy and 6.15 Gy, respectively. Animals were sacrificed at 2, 12 and 48 hrs after irradiation.

Results: DNA DSBs are observed in the path of the pencilbeam. The size of the area affected undergoes changes within the first 48 hours after irradiation.

Conclusions: The extent of DNA damage caused by pencilbeam irradiation, as assessed by H2AX antibody staining, is dose- dependent.

KEYWORDS: Animal model, brain, DNA double strand breaks, microbeam irradiation, pencilbeam irradiation, synchrotron

7.2 INTRODUCTION

Pencilbeam irradiation is a new concept of spatially fractionated irradiation, based on the principle of microbeam radiation therapy (MRT). Based on the dose-volume principle, the spatial fractionation allows the application of X-ray doses in one single irradiation session. The synchrotron doses are higher by almost two orders of magnitude than X-ray doses usually applied in a single session with broad beam irradiation in the hospital environment. This should be an advantage in the therapy of highly radioresistant tumours. The technique requires a high-flux of photons and therefore the use of a synchrotrton is still a prerequisite.

It has been shown in studies with a classic MRT approach that the overall structure of the healthy tissue between the paths of the microbeams stays intact [1] and that the function of healthy tissue in the path of the beam is greatly preserved, possibly taking over part of the function of the tissue destroyed by the microbeams [2], [3]. The classic MRT approach delivers synchrotron radiation by essentially sectioning the tissue into multiple parallel slices following a radiadisurgical-like manner. On the contrary, the three-dimensional distribution of the pencilbeam irradiation avoids sectioning the tissue into slices while, at the same time, reducing the volume of the irradiated tissue. Theoretically, this should allow better brain tissue tolerance and as a consequence make pencilbeam irradiation suitable for whole brain radiotherapy (WBRT).

Having previously demonstrated the feasibility of pencilbeam irradiation with the technical setup available at the biomedical beamline ID 17 of the ESRF [4], we have now designed a first experiment to study the consequences of pencilbeam irradiation on DNA integrity.

7.3 MATERIALS AND METHODS

7.3.1 Animal model and ethics statement

The animals were housed and cared for in a temperature-regulated animal facility exposed to a 12-hr light/dark cycle. All experiments were performed in accordance with the guidelines of the French and German Councils on Animal Care; with permissions of the institutional committees for animal care specifically obtained at the Freiburg University Medical Center and at the ESRF.

A total of 18 healthy adult male mice (NMRI nu/nu, Charles River Germany) were used for the experiments. Of these, 15 animals were submitted to pencil beam irradiation of the entire brain, with a lateral beam propagation from right to left. Three animals served as non-irradiated controls. Of the irradiated animals, 9 were irradiated with a lower peak dose (330 Gy) and 6 animals were irradiated with a higher peak dose (2,460 Gy). The irradiation experiments were conducted with the animals under general anaesthesia following an intraperitoneal injection of a ketamine and xylazine cocktail (ketamine 1 mg / 10 g, xylazine 0.1 mg / 10g). Animals were sacrificed at 2, 12 and 48 hrs after

irradiation (n=3/group), the brains were removed and preserved in 10% phosphate-buffered formalin.

7.3.2 Monte Carlo Calculations

The general approach to Monte Carlo simulations for pencilbeam irradiation has been described previously [4]. The calculations were carried out in the Geant4 tool set (Version 4.9.3 p02) using an adjoint simulation technique. A cube of water with 16 cm side length was defined as target for modelling process. The reference depth was 3 mm, corresponding to the assumed center of the implanted tumors. Energy was scored in 5 x 5 μm² voxels with a height of 4 mm. At the reference depth of 3 mm the height was reduced to 0.5 mm in an additional simulation, since the dose dependency at shallow penetration depth is steep. At this depth, Monte Carlo simulations were compared to film dosimetry. The measured peak-to-valley dose ratio (PVDR) was about 400, somewhat lower than the calculated PVDR of 530, which is most likely due to the imperfection of the model used for the measurements [4]. For safety reasons, we will continue working with the lower, measured values.

7.3.3 Pencilbeam irradiation

The irradiation experiments were conducted at ID 17, the biomedical beamline at the ESRF. The original synchrotron beam is first modified by a

wiggler source, resulting in pink beam for use at the biomedical beamline. Square pencilbeams were produced using a step and shoot approach with the insertion of a multislit collimator (MSC) [5]. White synchrotron radiation X-ray beam, issued from a wiggler source, was filtered through 1 mm copper and 1.5 mm aluminum, the energy spectrum extending from about 50 keV to well above 350 keV with a maximum at 83 keV [6]. The animals were placed prone on the goniometer, with the top of the skull horizontal and their teeth hooked into a small fixed holding device, and moved in vertical direction through the beam. The whole brain was irradiated in unidirectional mode, using a stop-and shoot approach during this vertical movement. Almost square pencilbeams of 50 x 51μm were produced, the width being determined by the collimator and the hight by the beam slits (the horizontal beam divergence responsible for the 50 becoming 51 μm. With the right lateral side of the skull positioned orthogonally to the direction of beam propagation, the right-to-left lateral irradiation created a grid of square pencil beams (Figure 7.1) in sagittal and an array of 50 μm wide parallel stripes visible in the axial tissue sections. The animals were irradiated with a center-to-center distance (ctc) of 400 μm and received peak irradiation doses of either 330 Gy or 2460 Gy. The irradiation pattern and the irradiation dose were recorded on Gafchromic film [7]. The irradiation times varied, depending on the ctc and total irradiation dose, between 0.035 seconds for 330 Gy and 0,206 seconds for 2,460 Gy. Those irradiation parameters had been chosen based on the results from our earlier feasibility study (REF). The irradiation doses chosen for this study are

slightly below the lowest and slightly above the highest doses tested in the previous experiments.

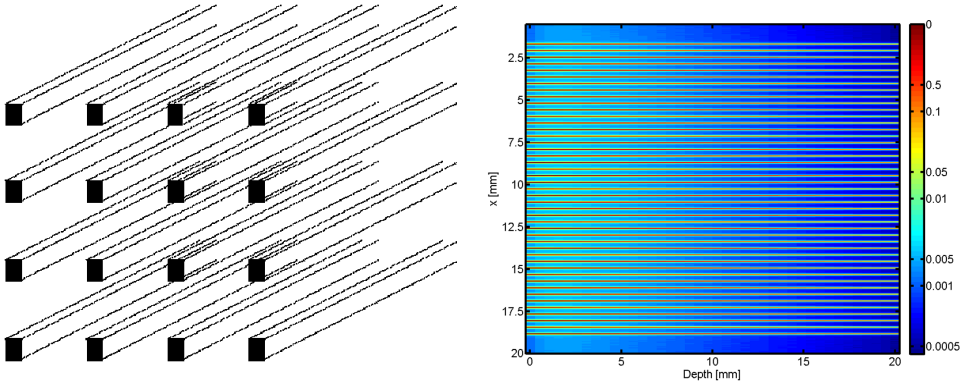

Figure 7.1 Schematic of the lateral pencilbeam profile (A) and modelling of depth dose depending on tissue depth using Monte Carlo calculations (B)

7.3.4 Histology and immunofluorescence

The brains were sectioned either sagittally along the midline or axially through the olfactory bulbs, placed in standard histology cassettes and stored in 10% phosphate-buffered formalin for 7 days. They were then embedded in paraffin and 3 μm thick sagittal sections were mounted on microscope slides. Histology sections were stained for Haematoxylin and Eosin (H&E) according to a standard protocol. Immunochemistry sections were stained for fluorescence imaging with yH2AX antibody (Abcam, Cambridge, UK) and DAPI (Sigma). The sections were first deparaffinised and rehydrated using a series of alcohol and

xylene washes. Vapour-based heat epitope retrieval was conducted by placing the slices in a pH 6 solution of citric acid at a temperature of 95ºC for 40 minutes. Blocking of the host's proteins was performed using 5% Goat Serum over an hour. Immediately after, the yH2AX antibody was applied using a 1:100 dilution for incubation overnight at 4ºC. On the next day, the slides were rinsed 3 times with PBS and fluorochrome-conjugated secondary antibody Alexa-488 (1:200 dilution) was applied over an hour at room temperature together with DAPI to counterstain the DNA. Fluorescence microscopy was conducted in the Laboratory of Molecular Neurosurgery at the Freiburg Neurocenter, using an Olympus AX 70 fluorescence microscope.

7.4 RESULTS

The only unexpected death occurred in the ctc 400 μm high dose irradiation group. One animal irradiated with 2,460 Gy was found death in his cage 5 hrs after irradiation. It was the only death occuring in this high dose irradiation group that included 14 animals, nine of them with brain tumours. However, we might have been touching the upper permissible dose limit for whole brain irradiation with a ctc of 400 μm.

The pattern seen in the sections stained with γH2AX clearly reproduced the irradiation patterns recorded on Gafchromic film (Figure 7.2). The kinetics of the double strand breaks (DSBs) over time can be observed as a change of the spot diameter stained by the H2AX stain on sagittal brain sections. The number

of DSBs per beam spot seem to slightly increase between 2 to 12 hours and then to decrease at 48 hours after irradiation, suggesting that either DNA repair mechanisms are taking place or that the damaged cells have died and been removed by macrophages within the latter time period.

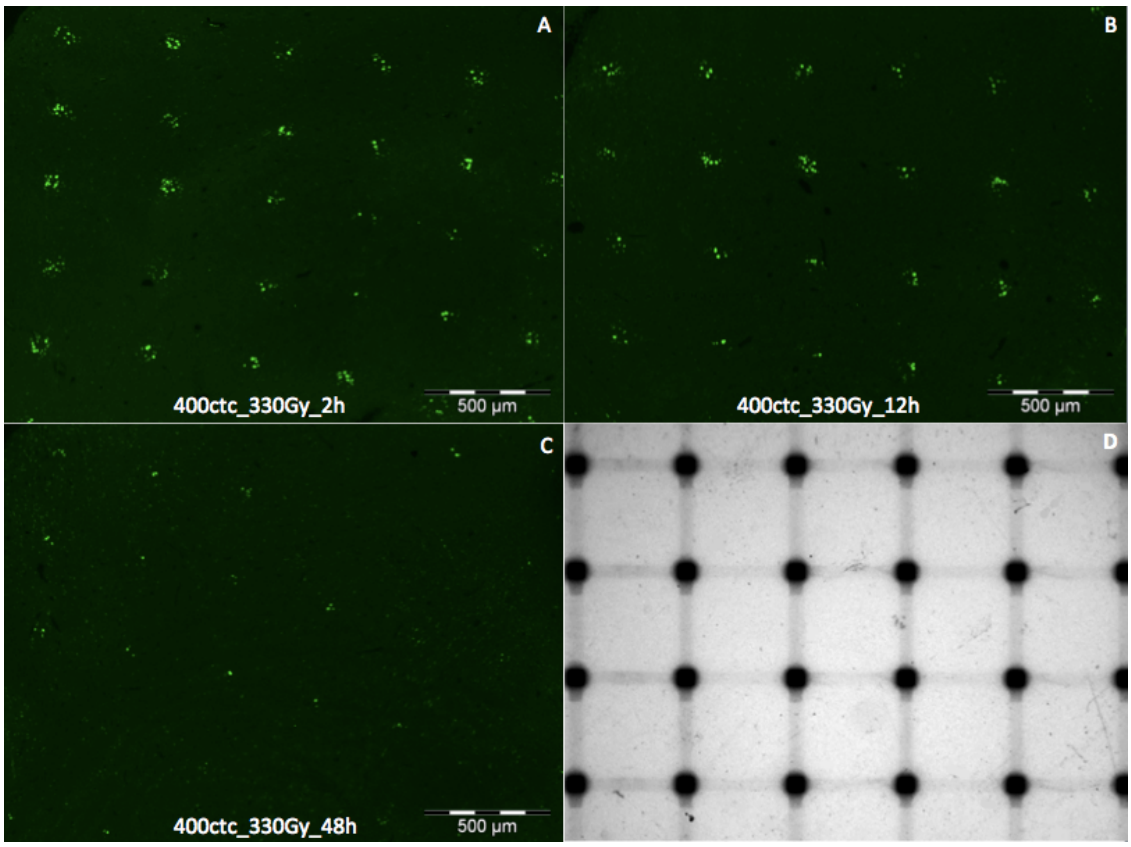

Figure 7.2 High magnification images, sagittal sections of mouse cortex stained with γH2AX antibody at 2, 12 and 48 hrs after irradiation wit 330 Gy peak dose (A-C) and the microscopy image of a corresponding Gafchromic film (D).

The pattern of cells with DNA DSBs (green) in the immunofluorescence microscopy image clearly reproduce the pattern recorded on the Gafchromic film.

The characteristic γH2AX patterns were present in and around the center of the pencilbeams at all three observation times, at 2, 12 and 48 hrs after irradiation. No DSBs were seen in the tissue corresponding to the centers of the dose valleys, not even after irradiation with 2,460 Gy. As expected, the area affected by DNA DSBs was larger after irradiation with 2,460 Gy than after irradiation with 330 Gy. There was an increase in the size of the area affected by DNA DSBs around the paths of the pencilbeams between 2 and 12 hrs after irradiation, followed by a decrease of the affected area between 12 and 48 hrs after irradiation (Figure 7.3). While at two hours after irradiation the γH2AX-positive areas appeared compact, at 12 hrs after irradiation we noticed that the activated region became more ring-shaped.

In the sections stained with the morphological standard stain H&E we found the characteristic dark pycnotic nuclei of cells destined for apoptosis in the area that corresponds to the center of the beams (Figure 7.4). This correlates with findings from classic microbeam radiation therapy, where the cells in the path of the beam become pycnotic and subsequently die.

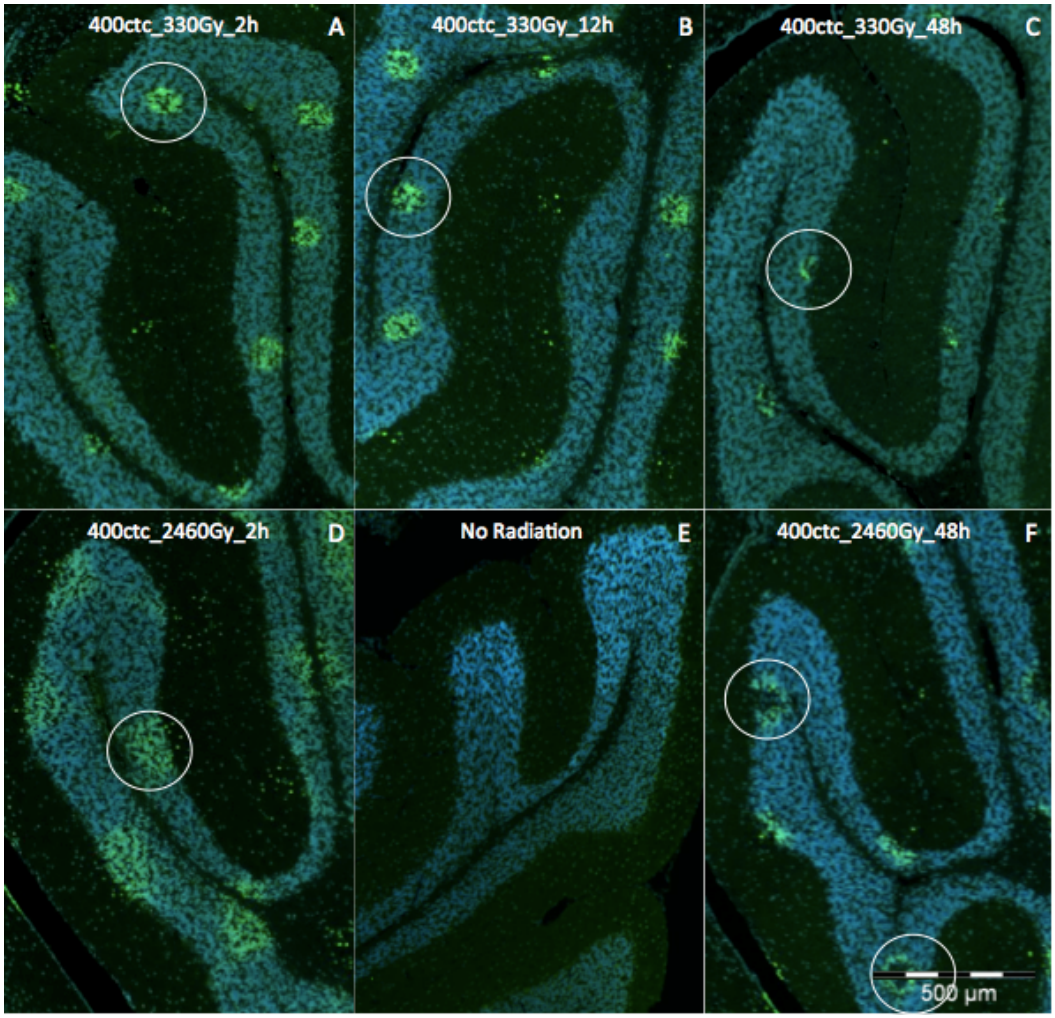

Figure 7.3 Low magnification images, γH2AX stain, sagittal sections through the cerebellum of adult mice sacrificed after irradiation with 330 Gy at 2 hrs (A), 12 hrs (B) and 48 hrs (C), and after irradiation with 2,460 Gy at 2 hrs (D), and 48 hrs (F). E: section through a non-irradiated cerebellum.

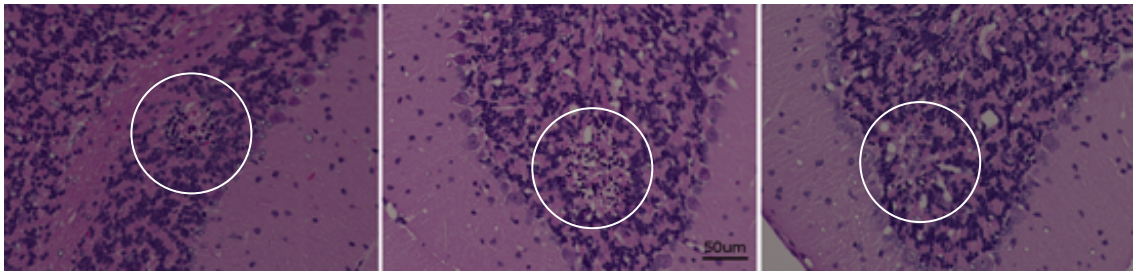

Figure 7.4 High magnification images, H&E stain of the cerebellum 2 (left), 12 (middle) and 48 hrs (right) after whole brain irradiation with 2,460 Gy. Dark pycnotic nuclei can be seen in the centers of the radiation-induced lesions, characteristic for cells destined to undergo apoptotic cell death. The diameter of the lesion increases over time.

7.5 DISCUSSION

Whole brain radiotherapy (WBRT) is often the last available therapeutic option for patients with multiple brain metastases. In the treatment of patients with highly malignant brain tumours, the WBRT approach has been abandoned for focal irradiation of the tumour. One of the reasons for this change of approach is the observation of changes in the white matter structure, frequently associated with cognitive and behavioural changes [8]. The therapeutic results, however, are very unsatisfactory. Patients with glioblastoma multiforme gain only a few months of survival with radiotherapy [9]. Besides radioresistance, the migration of tumour stem cells within the brain to areas outside the irradiated volume might be one of the reasons for this unsatisfactory outcome. Another reason might be that the repair capacity of tumour stem cells for damage caused by the iradiation doses applied in the hospital environment is suffciently high to allow them to survive.

Spatially fractionated radiotherapy allows the application of much higher irradiation doses and therefore might be a more successful approach in the treatment of those patients.

We have shown in our experiements that the extent of DNA damage caused by pencilbeam irradiation is dose-dependent, which is in agreement with results reported by Priyadarshika and colleagues for the classsic microbeam radiation approach [10]. The development of pycnotic nuclei in the centres of the beam path corresponds to the results of our earlier experiments, where at 6 months after irradiation the centres of the beam paths were almost devoid of living cells [4].

From the analysis of the γH2AX study we understand that the volume in which the DNA damage occurs is not limited to the path of the beam but becomes larger than the beam in a dose-dependent fashion. However, no γH2AX-positive cells were seen in the centres of the dose valleys. In our earlier experiments we have also shown that much of the tissue between the beams remained intact, which was also reflected in memory and motor function several months after irradiation [4]. This is in agreement with results reported from work with the classic microbeam radiation approach [1, 2, 3]. From work with the classic microbeam radiation approach we also know that with some treatment protocols, a significant increase of survival times and even cures have been achieved in small animal models despite the fact that only part of the tumour volume was in the path of the microbeams [11]-[14]. Thus, one of the next steps

will be to test in small animal models of malignant brain tumours whether pencilbeam irradiation is therapeutically effective and an increase of survival time can be achieved with the technique.

7.6 CONCLUSION

DNA DSB as assessed by γH2AX antibody occur after pencilbeam irradiation in the path of the beam and in parts of the dose valleys adjacent to the path of the beam. No DNA DSBs occur in the centers of the dose valleys at any time between 2 and 48 hrs after irradiation, even with doses as high as 2,460 Gy. The extent of the DNA damage is dose-dependent.

7.7 ACKNOWLEDGMENTS

We thank Ms. Charlène Caloud from the animal facility of ID 17 (ESRF) for her dedicated animal care at the ID 17 animal facility. Dr. Geraldine Le Duc (ESRF) and Dr. Rita Sanchez – Brandelik (Freiburg University) and their teams are thanked for logistic support with the animal reception and transportation across international borders. We thank Dr. Alberto Bravin (ID17) for generously providing in-house research time for this project. Professor Mothersill, the mentor of Cristian Fernandez at McMaster University, is thanked for providing travel support for this student exchange. Ms. Johanna Wessolleck (Laboratory of Molecular Neurosurgery) and the technicians of the Department of

Neuropathology in Freiburg are thanked for introducing Cristian Fernandez to immunofluorescence microscopy.

7.8 REFERENCES

[1] Laissue JA, Geiser G, Spanne PO, Dilmanian FA, Gebbers JO (1998) Neuropoathology of ablation of rat gliosarcomas and contiguous brain tissues using a microplanar beam of synchrotron-wiggler-generated x-rays. Int J Cancer 78: 654–660.

[2] Schültke E, Juurlink B, Ataelmannan K, Laissue J, Blattmann H, et al. (2008) Memory and Survival after Microbeam Radiation Therapy. Eur J Radiol 68S: S142–S146.

[3] Laissue JA, Blattmann H, Di Michiel M, Slatkin DN, Lyubimov N, et al. (2001) Weanling piglet cerebellum: a surrogate for tolerance to MRT (microbeam radiation therapy) in pediatric neuro-oncology. Proc SPIE 4508: 65-73.

[4] Schültke E, Trippel M, Bräuer-Krisch E, Renier M, Bartzsch S, Requardt H, Döbrössy MD, Nikkhah G (2012) Pencilbeam irradiation technique for whole brain radiotherapy: Technical and biological challenges in a small animal model. PLOS ONE, in print.

[5] Bräuer-Krisch E, Requardt H, Brochard T, Berruyer G, Renier M, et al. (2009) New technology enables high precision multislit collimators for microbeam radiation therapy, Rev Sci Instr 80: 074301.

[6] Bräuer-Krisch E, Serduc R, Siegbahn EA, Le Duc G, Prezado Y, et al. (2010) Effects of pulsed, spatially fractionated, microscopic synchrotron X-ray beams on normal and tumoral brain tissue. Mutat Res 704(1-3):160–166.

[7] ISP EBT Product Brief. International Speciality Products (ISP) Technologies (2007) http://online1.ispcorp.com/ lay-outs/GafChromic®/index.html.

[8] Mineo JF, Bordron A, Baroncini M, Ramirez C, Maurage CA, Blond S, Dam-Hieu P (2007) Prognosis factors of survival time in patients with glioblastoma multiforme: a multivariate analysis of 340 patients. Acta Neurochir (Wien): 149(3): 245-53.

[9] Armstrong CL, Shera DM, Lustig RA, Phillips PC (2012) Phase measurement of cognitive impairment specific to radiotherapy. Int J Oncol Biol Phys 83(3): e319-24.

[10] Priyadarshika RC, Crosbie JC, Kumar B, Rogers PA (2011) Biodosimetric quantification of short-term synchrotron microbeam versus broad-beam

radiation damage to mouse skin using a dermatopathological scoring system. Br J Radiol. 2011 Sep;84 (1005):833-42.

[11] Bouchet A, Lemasson B, Le Duc G, Maisin C, Bräuer-Krisch E, et al. (2010) Preferential effect of synchrotron microbeam radiation therapy on intracerebral 9L gliosarcoma vascular networks. Int J Radiat Oncol Biol Phys 78(5): 1503-12.

[12] Serduc R, Bouchet A, Bräuer-Krisch E, Laissue JA, Spiga J, et al. G (2009) Synchrotron microbeam radiation therapy for rat brain tumor palliation—influence of the microbeam width at constant valley dose. PMB 54: 6711-24.

[13] Regnard P, Duc GL, Bräuer-Krisch E, Troprès I, Siegbahn EA, et al. (2008) Irradiation of intracerebral 9L gliosarcoma by a single array of microplanar x-ray beams from a synchrotron: balance between curing and sparing. Phys Med Biol 53(4): 861-78.

[14] Dilmanian FA, Button TM, Le Duc G, Zhong N, Peña LA, et al. (2002) Response of rat intracranial 9L gliosarcoma to microbeam radiation therapy. Neuro-Oncology 4: 26-38.

Chapter 8

8 THESIS DISCUSSION WITH INTRODUCTION AND FUTURE WORK

8.1 INTRODUCTION

The research presented in this thesis sought to understand the role of bystander mechanisms – if any – in Microbeam Radiation Therapy (MRT) and Pencilbeam (PB) therapy. The purpose was to help understand relevance of bystander effects for these modalities, which are currently at the preclinical stage of testing for use in the treatment of glioma.

This thesis also sought to elucidate the dose response of the T98G, C6 and F98 glioma cell lines by understanding the relationship between the low-dose hyper-radiosensitivity (HRS) phenomenon– which is prevalent in these radioresistant lines – and bystander effects, which are also seen after low doses. The relevance of these effects for the MRT and PB modalities is because tissues in the valleys between the parallel beams are exposed to doses where HRS and bystander effects might be expected to dominate.

8.2 BYSTANDER EFFECTS AND HRS CAN COEXIST, AND THEY MIGHT BE INTER-RELATED

8.2.1 HRS and Bystander effects after Gamma Irradiation

The field of radiobiology has tried to elucidate the link between bystander effects and HRS for several years. Research involving more than ten cell lines revealed that cell populations displaying HRS did not show bystander effects (Mothersill, Seymour, & Joiner, 2002; Ryan et al., 2009). Concluding that HRS and bystander effects were mutually exclusive. Recent research however, has shown opposite results suggesting that bystander effects can also occur in cells displaying HRS (Heuskin, Wéra, Riquier, Michiels, & Lucas, 2013; Nuta & Darroudi, 2008). This contradictory data motivated the re-examination of the subject in this thesis, where we studied the response of the T98G glioma and epithelial HaCaT cell lines to ten different doses in the HRS range (0 to 1 Gy).

The data presented in Chapter two strongly suggests that HRS and bystander effects can co-exist, that HRS-positive cells can induce and exhibit bystander responses, and that the induction of Increased Radio-resistance (IRR) is associated with loss of BE. These bystander results were observed as decreases in cell survival or induction of cell-kill in the human T98G glioma. Moreover, the bystander signals from the T98G cells were able to decrease the survival of the human (HRS-negative) HaCaT cell line. These results are in agreement with published work by Nuta & Darroudi (2008), who suggested that

radiation-induced bystander factors may lead to cell inactivation after exposing BJ fibroblast to gamma and X-rays in the HRS dose range. Our results are also supported by Heuskin and colleagues who irradiated the HRS-positive A549 lung carcinoma using HRS doses and observed an enhanced cell-kill that could not solely be explained by the exposure to alpha particles (Heuskin et al., 2013). That led the authors to investigate the participation of bystander signals by blocking the gap junctions with Lindane, which abolished the enhanced HRS cell-kill.

Results in this thesis show that increases in calcium fluxes in the bystander cells – triggered by the conditioned medium – were inversely correlated with the bystander cell killing observed. These calcium observations are supported by other work from our laboratory (Fazzari, Mersov, Smith, Seymour, & Mothersill, 2012; Fernandez-Palomo et al., 2015; Saroya, Smith, Seymour, & Mothersill, 2009; Singh et al., 2011) and colleagues (F. M. Lyng, Maguire, McClean, Seymour, & Mothersill, 2006; Fiona M Lyng, Seymour, & Mothersill, 2002; Carmel Mothersill et al., 2005; Shao, Lyng, Folkard, & Prise, 2006). Demonstrating that transient calcium fluxes are associated with bystander effects.

Overall, the results presented in this section suggest that the HRS phenomenon can clearly coexist with bystander effects. Moreover, HRS and bystander effects show a direct correlation, which is corroborated by the decrease in their intensities as IRR takes place.

8.2.2 Bystander effects and HRS after Synchrotron Radiation

The animal experiments presented in this thesis involved the use of two tumour models, the C6 and the F98 rat gliomas. In an attempt to explore the radiobiological response of these tumour cell lines *in vitro*, we decided to follow the irradiation protocols established in Chapter two but exposing the cells to both gamma and Synchrotron radiation – Chapter three.

The results indicated that both cell lines displayed HRS, and that they induced opposite bystander effects at HRS doses after exposure to either gamma or Synchrotron radiation. Those findings were corroborated by measurements of calcium fluxes through the plasma membrane of the reporter cells, which revealed an inverse correlation with the bystander responses induced by both glioma cell lines. Nevertheless, the results presented in this section show that bystander effects and HRS can coexist.

It is not understood why the donor F98 cells induced bystander survival while donor C6 cells induced bystander cell killing. However, while the F98 results are in agreement with the *in vivo* data from Chapters four and five – where the presence of the F98 glioma in the irradiated Fisher rats and athymic nude mice blocked/counteracted the bystander effects – the C6 results are in agreement with published work from the author's Master degree (Fernandez-Palomo et al., 2013) showing that the presence of the C6 glioma in the irradiated Wistar rats induced stronger bystander effects. Although opposite *in vivo*

bystander responses could be associated with the different immunogenicity of both glioma cell lines (Barth & Kaur, 2009), it cannot explain our results *in vitro*. A more likely explanation could be attributed to the p53 statuses of these cell lines, which reveals a wild-type p53 for the C6 cells (Asai et al., 1994; Strigari et al., 2014; Yang et al., 2014) and a mutated p53 for F98 cells (Schlegel et al., 1999; Senatus et al., 2006). Mothersill et al. (Carmel Mothersill et al., 2011) demonstrated that while the p53 status of the human colon tumour 116 cells does not affect their ability to produce bystander signals, only the wild-type cell line responded to them. Similarly, the different p53 statuses of our C6 and F98 glioma could be behind of the opposite bystander effects observed at HRS doses.

In summary, the results from Chapters two and three, together with publish data (Heuskin et al., 2013; Nuta & Darroudi, 2008) highly suggest that HRS and bystander effects are closely inter-related, and that the primary cause of cell killing in the HRS region might be the bystander signalling mechanism.

8.3 INTER-ANIMAL COMMUNICATION OF BYSTANDER EFFECTS AFTER SYNCHROTRON RADIATION

Research by Surinov and Mothersill have reported that bystander signal communication can occur from irradiated to unexposed rats (Carmel Mothersill et al., 2014; B P Surinov, Isaeva, & Tokarev, 2001; B P Surinov, Karpova, Isaeva, & Kulish, 1998; B. P. Surinov, Isaeva, & Dukhova, 2005; B. P. Surinov, Isaeva, & Karpova, 1997) and fish (Mothersill, Smith, Hinton, Aizawa, & Seymour, 2009; Carmel Mothersill et al., 2006; Carmel Mothersill, Smith, Agnihotri, & Seymour, 2007; Saroya et al., 2009). To provide more evidence for this type of inter-animal transmission, we decided to study the influence a tumour during the signalling process – Chapter four.

These experiments were able to confirm that bystander effects can be communicated from Synchrotron MRT-irradiated Fisher rats to unirradiated rats. These results then support the preliminary data previously published by our group where transmission of bystander signals occurred from Synchrotron-irradiated Wistar rats to its unexposed cage mate companions (Carmel Mothersill et al., 2014). The results from this section are also in accordance with other data from our laboratory where inter-animal communication of radiation stress was observed in several species of fish (C Mothersill et al., 2009; Carmel Mothersill et al., 2006, 2007; Saroya et al., 2009), and it is also supported by data from Surinov and colleagues were bystander rats and mice showed decreases in

immune reactivity (B P Surinov et al., 2001, 1998; B. P. Surinov et al., 2005, 1997).

The mechanism by which the bystander signal travels from the irradiated animal to its cage mate companion is currently unknown. The most likely route is via excretion of molecules in urine, which are then absorbed into the cage mate animal. This is in accordance with work by Surinov suggesting that urine was the carrier of the signal (B P Surinov et al., 2001) and it is also in line with work from other researchers suggesting that the signal must be water soluble (Fernandez-Palomo et al., 2013; Saroya et al., 2009; Wang et al., 2011). A second explanation could be that the signals exit through the urine and become volatile, to then enter the cage mate animal through the respiratory system. This latter hypothesis is supported by published work by Surinov showing that volatiles from irradiated rats caused the cage mates to develop compromised immune responses (Abramova & Surinov; Mukherjee, Coates, Lorimore, & Wright, 2014; B P Surinov et al., 2001, 1998). However, further investigations are needed to establish the importance of volatiles. Perhaps, possible future experiments to evaluate the effect of the volatiles could follow Surinov's and place the bedding from the irradiated rats beneath the cage of unirradiated animals.

8.4 THE ROLE OF PVDR IN THE BYSTANDER RESPONSE

The experiments from Chapter five show evidence of the induction of significantly different bystander responses between MRT and PB, although only in the tumour bearing animals after 2 and 48 hours. Since the valley doses delivered by PB and MRT were the same (0.5 & 2.5 Gy), the only major differences were the peak doses – 22 & 110 Gy for MRT; 200 & 1000 Gy for PB – and the peak-to-valley dose ratios (PVDRs). PVDRs are reported to modulate the sparing of normal tissue (Dilmanian et al., 2007; Slatkin, Spanne, Dilmanian, & Sandborg, 1992) and an analysis of the experimental results revealed that the PVDR for MRT was 44, while the PVDR for PB was 400. Although speculative, these findings suggest that large PVDRs may be responsible for these different MRT and PB responses. However, further specifically designed investigations are needed to test this hypothesis.

8.5 γ-H2AX MARKER AS A TOOL FOR DNA DAMAGE IN-SITU AFTER EXPOSURE TO SYNCHROTRON RADIATION

When this PhD programme was being planned the published data showing the direct effects of Synchrotron radiation in the brain of rats was based on conventional histological analysis of the irradiated tissue (Dilmanian et al., 2005; Laissue et al., 1998; Laissue, Blattmann, Wagner, Grotzer, & Slatkin, 2007; Regnard et al., 2008). However cellular studies using immunocytochemical

methods, were showing that the use of the γ-H2AX antibody correlated well with expression of DNA damage in the cells exposed to MRT (Kashino et al., 2009; Usami, Maeda, Eguchi-Kasai, Maezawa, & Kobayashi, 2006). We wanted to take those investigations further and evaluate whether similar results could be obtained *in vivo*. To accomplish that, we irradiated the brain of rats with MRT (Chapter Six), and the brain of mice with PB (Chapter Seven).

8.5.1 γ-H2AX as marker of DNA damage after exposure of rats to MRT

By employing the γ-H2AX biomarker, we obtained visual confirmation that our physical microbeam parameters had been adequately delivered. Moreover, we were able to correlate the MRT doses with the number of immunopositive cells per unit area. Revealing that the overall number of cells showing DNA damage decrease overtime after exposure, which is indicative of the action of the DNA damage repair processes. These results are in agreement with studies developed by Kashino et al. (2009), which demonstrated that the number of foci in each irradiated C6 glioma cell was correlated with the MRT dose delivered. Our data are also supported by work from Crosbie et al. (2010) where normal skin and subcutaneous tumours showed a direct correlation between the intensity of γ-H2AX and the MRT dose delivered.

8.5.2 γ-H2AX as marker of DNA damage after exposure of mice to PB

While MRT was developed to treat primary brain tumours (Serduc et al., 2008; R. Serduc et al., 2009), PB was conceived to treat brain metastasis (Schültke et al., 2013). Moreover, research concerning MRT has been developed for over twenty years while PB is a recent concept developed by Schültke et al. (2013). We decided to use of the γ-H2AX biomarker to study for the first time the dynamics of DNA damage repair and dose deposition after PB. Our results indicated that the amount of DNA damage produced in the brain of mice was correlated with the amount of immunoreactive cells, which further supports the finding presented in this section.

8.6 A BRAIN TUMOUR MODIFIES THE NORMAL RESPONSE TO SYNCHROTRON RADIATION IN TWO MURINE MODELS.

As described earlier, this thesis project employed the F98 tumour during the in vivo investigations in Chapters four and five. The data from both chapters revealed that the presence of the F98 glioma – in the brain of the Fischer rats and the athymic mice – is associated with loss or suppression of the bystander cell killing in the reporter cell line exposed to the signals from brain explants. Similar effects were observed when these signals originated from the contralateral brain hemisphere, which had no implanted tumour. The F98 tumour also modulated the transmission of the bystander signal/effects from the

irradiated rats to the cage mate companions, which had no tumour in their brains. These data could suggest two competing mechanisms, which are modulated by the presence of F98 tumour; one that leads to the apparent suppression of the bystander effects (i.e. pro-survival signal) and another that could involve tumour- or associated normal tissue induced bystander signals. Either mechanism would then affect the host, which would consequently affect the reporter cells or other organism receiving the signals. However, it is not yet known whether the F98 tumour produces signals, which affect the unirradiated cage mate companions directly or if it attenuates the bystander signals before they depart from host to affect the cage mates.

Published data regarding Wistar rats harbouring the C6 glioma (Fernandez-Palomo et al., 2013) showed that the presence of the C6 tumour enhanced the radiation-induced bystander effect. This clearly contradicts the results observed in chapters four and five. However, a comparison between the cell lines revealed that while the F98 glioma is weakly immunogenic the C6 glioma is highly immunogenic (Barth & Kaur, 2009). This may suggest that the pro-survival signals released by the F98 tumour could be a consequence of having evaded the immune recognition; of having effectively suppressed the anti-tumour response of the host animal; or that the F98 tumour simply secretes pro-survival signals. Alternatively, since the F98 tumour displays mutant p53 and it is inoculated in the brain of a wild-type p53 animal, we might expect a different outcome to when both the tumour and the host animal are wild-type p53.

Considering that the F98 glioma was implanted one week before irradiation, it has to be considered that the signals may have been produced by the microenvironment in the rats or mice, as a response to presence of the tumour. Whether that is the real cause or not, this appears to be the first report showing that bystander effects – in healthy cage mates rats and in reporter cells exposed to conditioned media from tumour bearing mice – can be blocked by the presence of a rat glioma in the brain of the animal receiving radiation.

8.7 FINAL REMARKS AND FUTURE WORK

The goal of this thesis was to provide experimental data concerning radiation-induced bystander effects that could aid in the development of Synchrotron radiation as a novel brain radiotherapy treatment.

The key take-home messages of this thesis are four. First, bystander and abscopal effects can occur in Fisher rats after exposure to Synchrotron radiation and even in the immune-compromised nude mice. Second, bystander effects can be communicated to healthy unirradiated cage mate rats. Third, bystander effects may be highly relevant in the tissue valleys – between the peaks – especially in the PB modality where valley doses are very low and within the range of HRS and bystander effects. Fourth, the presence of a tumour modifies the bystander and abscopal responses. Finally, we suggest that future treatments should focus

on delivering valley doses within the range of 0.1 to 0.3 Gy to take advantage of enhanced cell killing produced by HRS and/or bystander effects.

8.8 REFERENCES

Abramova, M. R., & Surinov, B. P. Attractive and immunosuppressive properties of volatile secretions induced in mice separately and combine influence of ionizing radiation and cyclophosphamide. *Radiatsionnaia Biologiia, Radioecologiia / Rossiiskaia Akademiia Nauk*, *50*(1), 74–80. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/20297684

Asai, A., Miyagi, Y., Sugiyama, A., Gamanuma, M., Hong, S. H., Takamoto, S., … Kuchino, Y. (1994). Negative effects of wild-type p53 and s-Myc on cellular growth and tumorigenicity of glioma cells. Implication of the tumor suppressor genes for gene therapy. *Journal of Neuro-Oncology*, *19*(3), 259–68. doi:10.1007/BF01053280

Barth, R. F., & Kaur, B. (2009). Rat brain tumor models in experimental neuro-oncology: the C6, 9L, T9, RG2, F98, BT4C, RT-2 and CNS-1 gliomas. *Journal of Neuro-Oncology*, *94*(3), 299–312. doi:10.1007/s11060-009-9875-7

Crosbie, J. C., Anderson, R. L., Rothkamm, K., Restall, C. M., Cann, L., Ruwanpura, S., … Rogers, P. a W. (2010). Tumor cell response to synchrotron microbeam radiation therapy differs markedly from cells in normal tissues. *International Journal of Radiation Oncology, Biology, Physics*, *77*(3), 886–94. doi:10.1016/j.ijrobp.2010.01.035

Dilmanian, F. a, Qu, Y., Liu, S., Cool, C. D., Gilbert, J., Hainfeld, J. F., … McDonald, J. W. (2005). X-ray microbeams: Tumor therapy and central nervous system research. *Nuclear Instruments & Methods in Physics Research. Section A, Accelerators, Spectrometers, Detectors and Associated Equipment*, *548*(1-2), 30–37. doi:10.1016/j.nima.2005.03.062

Dilmanian, F. A., Qu, Y., Feinendegen, L. E., Peña, L. A., Bacarian, T., Henn, F. A., … McDonald, J. W. (2007). Tissue-sparing effect of x-ray microplanar beams particularly in the CNS: is a bystander effect involved? *Experimental Hematology*, *35*(4 Suppl 1), 69–77. doi:10.1016/j.exphem.2007.01.014

Fazzari, J., Mersov, A., Smith, R., Seymour, C., & Mothersill, C. (2012). Effect of 5-hydroxytryptamine (serotonin) receptor inhibitors on the radiation-induced bystander effect. *International Journal of Radiation Biology*, *88*(10), 786–90. doi:10.3109/09553002.2012.714519

Fernandez-Palomo, C., Bräuer-Krisch, E., Laissue, J., Vukmirovic, D., Blattmann, H., Seymour, C., … Mothersill, C. (2015). Use of synchrotron medical microbeam irradiation to investigate radiation-induced bystander and abscopal effects in vivo. *Physica Medica : PM : An International Journal Devoted to the Applications of Physics to Medicine and Biology : Official Journal of the Italian Association of Biomedical Physics (AIFB)*, 1–12. doi:10.1016/j.ejmp.2015.03.004

Fernandez-Palomo, C., Schültke, E., Smith, R., Bräuer-Krisch, E., Laissue, J., Schroll, C., … Mothersill, C. (2013). Bystander effects in tumor-free and tumor-bearing rat brains following irradiation by synchrotron X-rays. *International Journal of Radiation Biology*, *89*(6), 445–53. doi:10.3109/09553002.2013.766770

Heuskin, A.-C., Wéra, A.-C., Riquier, H., Michiels, C., & Lucas, S. (2013). Low-Dose Hypersensitivity and Bystander Effect are Not Mutually Exclusive in A549 Lung Carcinoma Cells after Irradiation with Charged Particles. *Radiation Research*, *180*(5), 491–8. doi:10.1667/RR13358.1

Kashino, G., Kondoh, T., Nariyama, N., Umetani, K., Ohigashi, T., Shinohara, K., … Ono, K. (2009). Induction of DNA double-strand breaks and cellular migration through bystander effects in cells irradiated with the slit-type microplanar beam of the spring-8 synchrotron. *International Journal of Radiation Oncology, Biology, Physics*, *74*(1), 229–36. doi:10.1016/j.ijrobp.2008.09.060

Laissue, J. A., Blattmann, H., Wagner, H. P., Grotzer, M. A., & Slatkin, D. N. (2007). Prospects for microbeam radiation therapy of brain tumours in children to reduce neurological sequelae. *Developmental Medicine and Child Neurology*, *49*(8), 577–81. doi:10.1111/j.1469-8749.2007.00577.x

Laissue, J. A., Geiser, G., Spanne, P. O., Dilmanian, F. a, Gebbers, J. O., Geiser, M., … Slatkin, D. N. (1998). Neuropathology of ablation of rat gliosarcomas and contiguous brain tissues using a microplanar beam of synchrotron-wiggler-generated X rays. *International Journal of Cancer. Journal International Du Cancer*, *78*(5), 654–60. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med4&NEWS=N&AN=9808538

Lyng, F. M., Maguire, P., McClean, B., Seymour, C., & Mothersill, C. (2006). The Involvement of Calcium and MAP Kinase Signaling Pathways in the Production of Radiation-Induced Bystander Effects. *Radiation Research*, *165*(4), 400–409. doi:10.1667/RR3527.1

Lyng, F. M., Seymour, C., & 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*(4), 365–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11893237

Mothersill, C., Bristow, R. G., Harding, S. M., Smith, R. W., Mersov, A., & Seymour, C. B. (2011). A role for p53 in the response of bystander cells to receipt of medium borne signals from irradiated cells. *International Journal of Radiation Biology*, *87*(11), 1120–5. doi:10.3109/09553002.2011.610866

Mothersill, C., Bucking, C., Smith, R., Agnihotri, N., Oneill, a, Kilemade, M., & Seymour, C. (2006). Communication of radiation-induced stress or bystander signals between fish in vivo. *Environmental Science & Technology*, *40*(21), 6859–64. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17144322

Mothersill, C., Fernandez-Palomo, C., Fazzari, J., Smith, R., Schültke, E., Bräuer-Krisch, E., … Seymour, C. (2014). Transmission of signals from rats receiving high doses of microbeam radiation to cage mates: an inter-mammal bystander effect. *Dose-Response : A Publication of International Hormesis Society*, *12*(1), 72–92. doi:10.2203/dose-response.13-011.Mothersill

Mothersill, C., Lyng, F., Seymour, C., Maguire, P., Lorimore, S., & Wright, E. (2005). Genetic factors influencing bystander signaling in murine bladder epithelium after low-dose irradiation in vivo. *Radiation Research*, *163*(4), 391–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15799694

Mothersill, C., Seymour, C. B., & Joiner, M. C. (2002). Relationship between radiation-induced low-dose hypersensitivity and the bystander effect. *Radiation Research*, *157*(5), 526–32. doi:10.1667/0033-7587(2002)157[0526:RBRILD]2.0.CO;2

Mothersill, C., Smith, R. W., Agnihotri, N., & Seymour, C. B. (2007). Characterization of a radiation-induced stress response communicated in vivo between zebrafish. *Environmental Science & Technology*, *41*(9), 3382–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17539553

Mothersill, C., Smith, R. W., Hinton, T. G., Aizawa, K., & Seymour, C. B. (2009). Communication of radiation-induced signals in vivo between DNA repair deficient and proficient medaka (Oryzias latipes). *Environmental Science & Technology*, *43*(9), 3335–42. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19534155

Mukherjee, D., Coates, P. J., Lorimore, S. a, & Wright, E. G. (2014). Responses to ionizing radiation mediated by inflammatory mechanisms. *The Journal of Pathology*, *232*(3), 289–99. doi:10.1002/path.4299

Nuta, O., & Darroudi, F. (2008). The impact of the bystander effect on the low-dose hypersensitivity phenomenon. *Radiation and Environmental Biophysics*, *47*(2), 265–74. doi:10.1007/s00411-007-0145-9

Regnard, P., Le Duc, G., Bräuer-Krisch, E., Troprès, I., Siegbahn, E. A., Kusak, A., … Bravin, A. (2008). Irradiation of intracerebral 9L gliosarcoma by a single array of microplanar x-ray beams from a synchrotron: balance between curing and sparing. *Physics in Medicine and Biology*, *53*(4), 861–78. doi:10.1088/0031-9155/53/4/003

Ryan, L. a, Seymour, C. B., Joiner, M. C., & Mothersill, C. E. (2009). Radiation-induced adaptive response is not seen in cell lines showing a bystander effect but is seen in lines showing HRS/IRR response. *International Journal of Radiation Biology*, *85*(1), 87–95. doi:10.1080/09553000802635062

Saroya, R., Smith, R., Seymour, C., & Mothersill, C. (2009). Injection of resperpine into zebrafish, prevents fish to fish communication of radiation-induced bystander signals: confirmation in vivo of a role for serotonin in the mechanism. *Dose-Response : A Publication of International Hormesis Society*, *8*(3), 317–30. doi:10.2203/dose-response.09-043.Saroya

Schlegel, J., Piontek, G., Kersting, M., Schuermann, M., Kappler, R., Scherthan, H., … Mennel, H. (1999). The p16/Cdkn2a/Ink4a gene is frequently deleted in nitrosourea-induced rat glial tumors. *Pathobiology : Journal of Immunopathology, Molecular and Cellular Biology*, *67*(4), 202–6. doi:28073

Schültke, E., Trippel, M., Bräuer-Krisch, E., Renier, M., Bartzsch, S., Requardt, H., … Nikkhah, G. (2013). Pencilbeam irradiation technique for whole brain radiotherapy: technical and biological challenges in a small animal model. *PloS One*, *8*(1), e54960. doi:10.1371/journal.pone.0054960

Senatus, P. B., Li, Y., Mandigo, C., Nichols, G., Moise, G., Mao, Y., … Fine, R. L. (2006). Restoration of p53 function for selective Fas-mediated apoptosis in human and rat glioma cells in vitro and in vivo by a p53 COOH-terminal peptide. *Molecular Cancer Therapeutics*, *5*(1), 20–8. doi:10.1158/1535-7163.MCT-05-0181

Serduc, R., Bouchet, A., Bräuer-Krisch, E., Laissue, J. A., Spiga, J., Sarun, S., … Le Duc, G. (2009). Synchrotron microbeam radiation therapy for rat brain tumor palliation-influence of the microbeam width at constant valley dose. *Physics in Medicine and Biology*, *54*(21), 6711–24. doi:10.1088/0031-9155/54/21/017

Serduc, R. R., Christen, T., Laissue, J. A., Farion, R. R., Bouchet, A., Sanden, B. van der, … Rémy, C. (2008). Brain tumor vessel response to synchrotron microbeam radiation therapy: a short-term in vivo study. *Physics in Medicine and Biology*, *53*(13), 3609–22. doi:10.1088/0031-9155/53/13/015

Shao, C., Lyng, F. M., Folkard, M., & Prise, K. M. (2006). Calcium fluxes modulate the radiation-induced bystander responses in targeted glioma and fibroblast cells. *Radiation Research*, *166*(3), 479–487. doi:10.1667/RR3600.1

Singh, H., Saroya, R., Smith, R., Mantha, R., Guindon, L., Mitchel, R. E. J., … Mothersill, C. (2011). Radiation induced bystander effects in mice given low doses of radiation in vivo. *Dose-Response : A Publication of International Hormesis Society*, *9*(2), 225–42. doi:10.2203/dose-response.09-062.Singh

Slatkin, D. N., Spanne, P., Dilmanian, F. A., & Sandborg, M. (1992). Microbeam radiation therapy. *Medical Physics*, *19*(6), 1395–1400. doi:10.1118/1.596771

Strigari, L., Mancuso, M., Ubertini, V., Soriani, A., Giardullo, P., Benassi, M., … Bossi, G. (2014). Abscopal effect of radiation therapy: Interplay between radiation dose and p53 status. *International Journal of Radiation Biology*, *90*(3), 248–55. doi:10.3109/09553002.2014.874608

Surinov, B. P., Isaeva, V. G., & Dukhova, N. N. (2005). Postradiation immunosuppressive and attractive volatile secretions: The “baystander effect” or allelopathy in groups of animals. *Doklady Biological Sciences*, *400*(1-6), 28–30. doi:10.1007/s10630-005-0034-9

Surinov, B. P., Isaeva, V. G., & Karpova, N. A. (1997). Post-radiation communicative induction of blood and immunity disorders. *Patologicheskaia Fiziologiia I Eksperimental’naia Terapiia*, (3), 7–10.

Surinov, B. P., Isaeva, V. G., & Tokarev, O. I. (2001). [Allelopathic activity of volatile secretions in irradiated animals]. *Radiatsionnaia Biologiia, Radioecologiia / Rossiĭskaia Akademiia Nauk*, *41*(6), 645–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11785304

Surinov, B. P., Karpova, N. A., Isaeva, V. G., & Kulish, I. S. (1998). [Communicative behavioral effects and disorders of immunity]. *Zhurnal Vyssheĭ Nervnoĭ Deiatelnosti Imeni I P Pavlova*, *48*(6), 1073–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9929917

Usami, N., Maeda, M., Eguchi-Kasai, K., Maezawa, H., & Kobayashi, K. (2006). Radiation-induced gamma-H2AX in mammalian cells irradiated with a synchrotron X-ray microbeam. *Radiation Protection Dosimetry*, *122*(1-4), 307–309. doi:10.1093/rpd/ncl434

Wang, C., Smith, R. W., Duhig, J., Prestwich, W. V, Byun, S. H., McNeill, F. E., … Mothersill, C. E. (2011). Neutrons do not produce a bystander effect in zebrafish irradiated in vivo. *International Journal of Radiation Biology*, *87*(9), 964–73. doi:10.3109/09553002.2011.584939

Yang, S.-H., Wang, S.-M., Syu, J.-P., Chen, Y., Wang, S.-D., Peng, Y.-S., … Kung, H.-N. (2014). Andrographolide induces apoptosis of C6 glioma cells via the ERK-p53-caspase 7-PARP pathway. *BioMed Research International*, *2014*, 312847. doi:10.1155/2014/312847