

RADIOBIOLOGY OF RAT BRAINS TO SYNCHROTRON MICROBEAM  
RADIATION

RADIOBIOLOGICAL RESPONSE OF HEALTHY AND TUMOUR-BEARING RAT  
BRAINS TO SYNCHROTRON MICROBEAM RADIATION

By

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# Abstract

Microbeam radiation therapy (MRT) is an experimental radiotherapy concept presently at the stage of pre-clinical evaluation. Although it has been primarily developed for the treatment of malignant brain tumours, it could be used in the therapy of other tumours in cases where surgery is not a suitable option. MRT uses high flux synchrotron x-rays delivered as an array of parallel microbeams in high doses of irradiation to tumours in fractions of seconds. The aims of this study were to 1) investigate the induction of bystander effects after normal and tumour-bearing rat brains were exposed to MRT and homogenous radiation; 2) validate a brain bystander proteome by detecting protein expression throughout immunohistochemistry; and 3) to investigate whether communication of bystander signals can be produced between animals.

Healthy and tumour-bearing Wistar rats were anaesthetised and exposed to 17.5, 35, 70 or 350 Gy of MRT or homogenous field of synchrotron radiation to the right brain hemisphere. After irradiation rats were allowed to recover in their own cages over 4 to 8 hours. To study the communication of bystander effects between animals, irradiated rats shared the same cage with non-irradiated rats over a period of 48 hours. After euthanasia of the animals, brains and bladders were dissected after 4, 8 or 48 hours, and samples for immunohistochemistry and bystander clonogenic assays were set up.

Clonogenic survival of the reporter HPVG cells showed that bystander effects occurred in both the non-irradiated hemisphere and bladder of normal and tumour-bearing rats, while the irradiated hemisphere showed the direct effects of radiation. Moreover, communication of bystander signals was confirmed in the non-irradiated rats using the same clonogenic reporter assay. Immunohistochemistry showed the expression of the antibodies studied but the results were not conclusive.

In conclusion, the results suggest that the MRT and homogenous radiation of unilateral normal and tumour-bearing rat brains produce bystander signals that affect the whole organism and that those signals also can be transmitted to non-irradiated animals. This represents a very important progress in the understanding of the mechanisms of transmission of bystander effects *in-vivo* and challenges the current concepts in health effects of radiation.

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# Chapter 1

## Introduction

### 1.1 Motivation

According to the World Health Organization (WHO) cancer is the leading cause of death in economically developed countries, and the second leading cause of death in developing countries <sup>1</sup>. In 2008, about 7.6 million cancer deaths were estimated to have occurred, with lung and breast cancer as the leading cause of death in male and female adults, respectively <sup>2</sup>. Brain tumours are the second leading cause of cancer-related deaths in both children under 20 years and males between 20 and 39 years <sup>3</sup>. The high incidence of cancer has led the scientific community to look for better treatment alternatives or to improve those currently available.

For more than 100 years ionizing radiation has been playing a key role in anticancer therapy due to its ability to kill tumour cells by inducing direct DNA damage <sup>4,5</sup>. Although radiation affects proteins, lipids, and cell structures, its effect on DNA double strand breaks (DSBs) is the determining factor for the desired cell death in radiotherapy <sup>6</sup>.

Different irradiation modalities have been developed in order to improve the delivery of the radiation dose to achieve tumour cell death. Teletherapy has become the most common form of radiotherapy, which directs an external radiation source toward the affected area of the body. Radiophysicists and doctors understood the need to focus the radiation onto the tumour whilst sparing the normal tissue, which brought the development of Confocal Radiotherapy (CFRT). This new treatment resulted in the inclusion of computed tomography for treatment planning, rotational delivery mechanisms, and the use of non-coplanar fields to reduce the amount of normal structures reached by radiation. Later on, Intensity-modulated radiation therapy was developed, which combines the delivery of non-uniform radiation field with variation in the radiation intensity (bixel by bixel) within the shaped field <sup>7</sup>. Although teletherapy has reached extraordinary technological advances, the delivery of high doses of radiation to tumours without damaging the skin was still only achievable by fractionating of the dose <sup>8</sup>.

Other types of treatment, such as Stereotactic Radiosurgery (SRS) and Spatially Fractionated Radiation (GRID Therapy) have, successfully reduced the number fractionations and overcome – in part – the extensive skin damage that occurs as a result of teletherapy by using a set of threedimensional coordinates to deliver highly precise small beams of ionizing radiation to tumours. However, SRS is efficient only in small neoplastic processes, making it unsuitable for larger sized



tumours. Despite this challenge, SRS poses a great alternative for brain cancer treatment<sup>9</sup>. Spatially fractionated radiation (GRID therapy) is another technique that allows for delivering high cumulative doses of radiation while overcoming the skin and subcutaneous tissue toxicity. GRID therapy was routinely used in the 1950s with orthovoltage radiation and it has been now developed in the megavoltage range for its use in palliative treatments<sup>9</sup>.

The Brookhaven National laboratory in Upton, New York took GRID therapy further, and with the use of synchrotron radiation, reduced the width of the beam's delivered from the centimeter to micrometer range. This reduction allowed the creation of Microbeam Radiation Therapy (MRT). MRT is currently an experimental form of radiosurgery developed for brain tumour treatment and it uses high confluence of synchrotron x-rays spatially fractionated into an array of parallel rectangular microbeams<sup>10,11</sup>. The unique spatial distribution of synchrotron microbeams allows MRT users to deliver extremely high doses of radiation in a fraction of a second. This treatment results in healthy tissue showing remarkable tolerance while tumours show high susceptibility<sup>12,13</sup>. MRT shows potential as a promising brain cancer treatment alternative because it can significantly increase survival time in animal models of malignant brain tumours<sup>12,14</sup>.

Although malignant brain tumours do not constitute the leading cause of cancer-related death, their infiltrative characteristics, aggressiveness and bad prognosis make them very hard to deal with. Furthermore, despite the notion that MRT is currently a promising radiation treatment against brain cancer in the pre-clinical stage, its human clinical applications are still hypothetical. Much more research is needed to evaluate safer uses and the potential benefits of this form of treatment.

## **1.2 Problem Description**

MRT shows great potential for treating brain cancer, but research has yet to learn many of its effects on humans. For it to be successfully used for clinical cancer treatment we need to increase our understanding of how synchrotron microbeams induce tissue damage and learn the extent of its effects in both normal and cancer tissue. Current research on MRT has been delving into these issues. Members of our team had already established in previous studies that MRT alone does not significantly affect the ability to form memory in treated rats<sup>15</sup>, and more work had been done by others which evidenced the superiority of MRT compared to conventional radiotherapy<sup>16-18</sup>. The current questions developing in research on the subject are concerned with how the latest knowledge in radiation biology helps us understand how synchrotron microbeams induce radiation damage.

For years the biological effects of radiotherapy were attributed only to the DNA damage caused by the energy deposition of ionizing radiation. However, this hypothesis was challenged by the confirmation that healthy cells show radiation-like responses when they are exposed to a medium from irradiated cells<sup>13,14,17,19-21</sup> or when they are located in the vicinity of irradiated cells<sup>22</sup>. This phenomenon is called radiation-induced bystander effects (RIBE).

If we remember the MRT configuration, radiation is delivered as an array of parallel microbeams instead of a broad beam; this means that a gap of non-irradiated tissue is present between all microbeams. Radiation-induced bystander effects in the vicinity of irradiated cells become very relevant at this point because they make it necessary to comprehend the extent of the bystander signal production when non-irradiated tissue gaps are present. Moreover, we need to understand what the implications of radiation-induced bystander effects for MRT are.

### **1.3 Proposed Solution**

To address the above problems researchers from McMaster University, the European Synchrotron Radiation Facility (ESRF), and the University of Freiburg completed work over the course of 2 years, between autumn 2009 and spring 2011. The experiments were conducted in these three different environments using rodents as an animal model, which were then exposed to different skin entry radiosurgical doses. Two different beam modalities were used to compare the induction of bystander responses, MRT and homogenous radiation. The confirmation of the presence of RIBE was made through Clonogenic HPV-G Reporter Bioassays.

During the first year, healthy rats were used to analyse the bystander effects after irradiating normal brain tissue. Clonogenic reporter bioassays were carried out to investigate the induction of bystander responses in the cerebral hemispheres and bladder as a distant organ. In addition, proteomic bystander results obtained from a previous pilot experiment were employed to perform immunohistochemical

analysis. Immunohistochemistry allowed studying both the cellular brain response and the distribution of the bystander proteome in the brain<sup>1</sup>.

During the second year, tumour-bearing rats were used to study the bystander responses. This time, clonogenic reporter bioassays were also carried out but to investigate the presence of bystander signals after irradiating a brain tumour<sup>2</sup>.

Additionally, a pilot experiment was performed to investigate whether the bystander signals can be transmitted from treated animals to non-irradiated animals<sup>3</sup>.

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<sup>1</sup> Refer to Chapter 4 for more details

<sup>2</sup> Refer to Chapter 5 for more details

<sup>3</sup> Refer to Chapter 6 for more details

## 1.4 Objectives

### 1.4.1 General Objective

Investigate the radiobiological effects of synchrotron microbeam radiation on both healthy and tumour-bearing rat brain.

### 1.4.2 Specific Objectives

Three experiments were developed to accomplish the general objective presented in this master thesis. Therefore, the specific objectives are divided as follows according to each research focus.

#### *I. Radiation-induced bystander effects in healthy rat brain after microbeam radiation therapy*

- a) Investigate the radiation-induced bystander effects on *non-irradiated* cerebral hemisphere after both MRT and homogenous radiation
- b) Investigate the radiation-induced bystander effects on *bladder* (as a distant organ) after both MRT and Homogenous radiation
- c) Investigate the cell response and distribution of bystander proteome in brain after MRT and homogenous radiation

**II. Radiation-induced bystander effects in tumour-bearing rat brain after synchrotron radiation**

- a) Investigate the radiation-induced bystander effects on the **non-irradiated** cerebral hemisphere after both MRT and homogenous radiation to the tumour
- b) Investigate the radiation-induced bystander effects on **bladder** (as distant organ) after both MRT and homogenous radiation to the tumour

**III. Communication of Radiation-Induced bystander signals in rats in vivo**

- a) Investigate the radiation-induced bystander effects in brain and bladder of **healthy, non-irradiated rats** after sharing the cage with **irradiated rats**

## 1.6 Outline

The present master thesis is divided into 7 chapters:

- Chapter 1 brings to the reader introductory material that will help to understand the motivation and the objectives of the project.
- Chapter 2 brings to the reader the background information needed to understand how the experiments were developed.
- Chapter 3 “Material and Methods” explains to the reader how the experiments were carried out, the techniques that were used, and the materials employed.
- Chapters 4, 5 and 6 show the correspondent results and discussions of the experiments 1, 2 and 3, which were developed to accomplish the general objective of this master project.
- Chapter 7 discuss the entire thesis including future work and conclusions.



## Chapter 2

# Background Information

## 2.1 Microbeam Radiation Therapy

### Historical Overview of MRT

The use of microbeams began during the mid-twenties when scientists from the Brookhaven National laboratory in the USA wanted to simulate the exposure of astronauts to heavy cosmic-particles using deuteron microbeams<sup>23-25</sup>. The microbeams were 25 $\mu$ m in diameter and produced tissue effects similar to the ionization track left by a single cosmic particle. Later on, in the 1980s, synchrotron-generated microbeams started to be used but only to attempt microtomography of the heads of mice. In this process, a 30 $\mu$ m pencil beam was used and 10 Gy were delivered to the skull. In order to improve the poor contrast of the images, they decided to increase the dose up to 200 Gy, keeping in mind work developed by Curtis<sup>26</sup>, which describes the high tolerance of normal tissue to elevated doses microbeam radiation. Interestingly, their results displayed no histological damage along the radiation track, which motivated them to gain a better understanding of the effect of microbeams in tissue. To accomplish this, they decided to switch from a pencil beam to microplanar beams of 50 $\mu$ m in

diameter with a distance of 50-200 $\mu$ m center-to-center<sup>27</sup>. Surprisingly, mice remained unaffected by these high doses, and no necrosis of tissue was observed<sup>28</sup>. This work is what initiated the use of synchrotron x-rays as radiosurgical alternative, and from it, Microbeam Radiation Therapy (MRT) was born.

### **Properties of the synchrotron microbeams**

Synchrotron-generated microbeams have great advantages when compared with particle microbeams. First, synchrotron radiation is characterized as having a very high fluence and an exceptionally low divergence, which allows us to spatially fractionate the beam. Second, it can be delivered using multichromatic or monochromatic beam-energies<sup>12</sup>. In MRT studies, the use of spatial fractionation is what allows for the delivery of high doses of radiation to the central nervous system with remarkably little damage to healthy non-targeted tissue. In order to achieve the spatial fractionation of the synchrotron x-rays a tungsten-multislit collimator is used, which is able to produce more than 100 identical quasi-parallel microbeams<sup>29,30</sup>. When it comes to the dose distribution in the tissue, the highest level of radiation is always found at the center of each microbeam and is called “peak dose”. Therefore, the lowest level of radiation is found in the space between the microbeams, and is called “valley dose”. Micro Monte Carlo simulations have

determined that the valley dose is in the range of 5 to 2.5 % of the of the peak dose<sup>14</sup>.

### **Pre-clinical studies of MRT**

MRT studies are led by the Synchrotron Radiation Facility (ESRF) and the Brookhaven National Laboratory (BNL), and have been focused on both investigating the sparing effects of microbeams and developing a technique that can be effective for brain-tumour treatment. In 1995, Slatkin<sup>28</sup> developed some very interesting findings in the BNL. He studied the tolerance of healthy tissue to different skin-entry doses of MRT. He delivered 312, 625, 1250, 2500, 5000 or even 10,000 grays to the brain of rats using either 20 or 37 $\mu$ m parallel microbeams. The results showed that the rats from the 10,000 Gy group developed brain tissue necrosis; the rats within the range of 1250 Gy to 5000 Gy showed loss of neuronal and astrocytic nuclei; and the rats between 312-625 Gy showed no adverse effects as result of the procedure. Moreover, Laissue et al.<sup>31,32</sup> studied the effects of MRT in the brain of piglets as a human paediatric model for developing brain. They irradiated the cerebellum of the piglets with between 66 and 263 Gy with a valley dose of around 5% of the peak dose and using 20-30 $\mu$ m of microbeams with a center-to-center distance of 210 $\mu$ m. The animals were allowed to live up to 465 days with no evidence of behavioural changes or pathology seen

with Magnetic Resonance Imaging. On the other hand, histology showed cell destruction along the path of the microbeam leaving the characteristic “stripes” of the MRT. Since microbeams only kill cells that are along the radiation path, the presence of tissue edema has been demonstrated to be lower than with conventional radiotherapy and radiosurgery<sup>12</sup>. Also Serduc et al.<sup>33</sup> reported that the amount of edema was very low despite the use of high doses of 312 Gy and 1,000 Gy, and the edema improved by day 7.

### **Tumour applications of MRT**

In addition to studying the effects of MRT on healthy tissue, researchers have been exploring its effects on animal models of cancer. Studies developed by Laissue et al.<sup>34</sup> involved the inoculation of 9L gliosarcoma in rats, which were irradiated after 14 days with unidirectional or intersecting microbeams. They array was of 25  $\mu\text{m}$  wide microbeams, spaced by 200  $\mu\text{m}$ , and delivering skin entrance doses of 625 Gy. The results showed that untreated controls had a median survival time of 21 days, while the median survival time observed in the 625 Gy bidirectional group increased to 47 days, which is translated to a 223% increase in life span. Following the same protocol Regnard et al<sup>35</sup> investigated the effects of decreasing the space between microbeams to 100 $\mu\text{m}$ . The median survival time of 9L-bearing rats increased from 40 to 67 days at 200 $\mu\text{m}$  and 100 $\mu\text{m}$  center-to-

center distance, respectively. However, it also increased abnormal clinical signs and weight patterns from 12 to 72%. In parallel, healthy brain tissue showed also an increase in histological lesions under the 100 $\mu$ m distance. Thus, the 200 $\mu$ m spacing was recommended for MRT as it better spares healthy tissue.

Moreover, Schülke et al.<sup>15</sup> studied survival-rates and memory in Wistar and Fisher rats that were implanted with C6 and F98 tumour cells respectively. The MRT was delivered 13 days after implantation using two orthogonal microbeams with a skin-entry dose of 350 Gy and an array configuration of 50 microbeams of 25 $\mu$ m width and spaced by 200 $\mu$ m. In both animal models the survival time was significantly increased compared to the untreated groups. Also MRT did not affect memory when it was compared to the untreated groups 1 month and 1 year after the time of irradiation.

When trying to explain why MRT increases tumour-eradication while allowing greater survival-rates of the different animal models, the answer seems to lie in the vascular response to synchrotron microbeam radiation<sup>36</sup>. Normally, MRT produces temporal disruption of the endothelium in healthy animal models. Moreover, the spatial fractionation of the dose reduces the edema in the brain and allows a rapid repair by the non-irradiated endothelial cells<sup>16</sup>. Tumour vasculature differs morphologically and functionally to normal blood vessels, showing high tortuosity, increased vessel diameter, high vessel permeability and abnormal

vessel contractibility<sup>14</sup>. This suggests that the abnormal tumour vasculature repairs the radiation damage less effectively than normal blood vessels and, therefore, it would explain why MRT is greatly effective for brain tumour treatment<sup>37</sup>.

## **2.2 Radiation-Induced Bystander Effects**

### **Introduction**

The cellular response to ionizing radiation is considered an active reaction against an agent that both threatens the integrity and alters the physiology of the irradiated cells. The response to this damage is indeed complex and can vary depending on the cell type, cell condition and even cell cycle state<sup>8</sup>. A key effect of the radiation stress in cells is the induction of double strand breaks (DSBs) of DNA. These lesions are considered one of the most important factors of gene toxicity and their correlation with cell death has been extensively documented. However, in the last years it has been investigated that ionizing radiation can significantly affect cells that have not received radiation but that were in contact or close to irradiated cells<sup>22,38,39</sup>. This phenomenon has been collectively named radiation-induced bystander effects (RIBE). The name of bystander effects was

taken and adapted from the field of experimental oncology. In 1993, in an attempt to use antivirals to treat tumours transfected with viral DNA, it was observed that apoptosis was also induced in cells that did not incorporate the gene<sup>40</sup>. A few years earlier, in 1992, Nagasawa and Little demonstrated that 30% of a cell population showed an increase in sister chromatid exchanges after exposing 1% of the population to alpha particles<sup>41</sup>. As a consequence, the effect of the unirradiated cells caused by the irradiation of a small fraction of the population was named RIBE, as an analogy to the effect observed in the gene therapy. The confirmation of the RIBE provided by Nagasawa and Little was supported by their statistical analysis that showed that the ratio of cells hit by a single alpha particle was smaller than the amount of cells showing chromosomal aberrations. Also, the use of particle microbeams for the study of RIBE allowed for the delivery of low radiation doses and greater accuracy while targeting individual structures within the cell<sup>42</sup>. Moreover, bystander effects were seen after using alpha particles to target exclusively the nucleus or mitochondria<sup>43-45</sup>. The damage observed in the cells includes sister chromatid exchanges, micronuclei formation, mutations, deletions, apoptosis, and genetic instability<sup>46</sup>

## **Mechanism of the Bystander Effect**

Although the specific mechanisms of RIBE are still unknown, researchers hypothesize that two main processes are involved: gap-junction intercellular communications (GJIC) and extracellular soluble factors. Several studies have reviewed that GJIC is necessary for the induction of bystander effects<sup>47,48</sup>. Initially, Azzam et al.<sup>49</sup> compared human fibroblasts cultures that were abundant and deficient in gap-junction communications. The results showed that RIBE were produced by low doses of alpha particles to only the first group. In addition, the blocking of gap-junction using different agents such as lindane abolished the bystander effects<sup>49,50</sup>. Moreover, it was observed that low doses of alpha particles induced the expression of connexin43, with an increase in its transcription and synthesis within different cell types<sup>51</sup>.

Wide ranges of studies are also showing evidence that extracellular soluble factors are involved in the induction of RIBE and that a direct cellular contact is not required for the signal transmission. One experimental model involves the irradiation of cells and the posterior harvesting and transferring of the media to unirradiated cells<sup>22,52</sup>. Another model uses irradiated cells cultured in the same medium with unirradiated cells, but they are not in direct contact<sup>53,54</sup>.



### **Factors causing the bystander effects**

Although the factor(s) causing RIBE have yet to be identified, some extracellular mediators and intracellular pathways have been recognized, including molecules, receptors, and second messengers that participate in a large number of processes involved in cellular stress. Within the group of extracellular mediators involved in RIBE we can identify the tumour necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>54</sup>, transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)<sup>54</sup>, interleukin-8 (IL8)<sup>55</sup>, reactive oxygen species (ROS)<sup>56</sup>, reactive nitrogen species (RNS)<sup>57</sup> and serotonin<sup>58</sup>. In addition, three enzymes which participate in the bystander effects have been identified: the nitric oxide synthase (NOS), the prostaglandin G/H synthase 2 or also called Cyclooxygenase-2 (COX2), and the NADPH oxidase<sup>6,59,60</sup>. When it comes to intracellular pathways we know that the extracellular mediators IL8, TGF- $\beta$ 1 and TNF- $\alpha$  interact with the membrane receptors. That activates both the mitogen-activated protein kinases (MAPKs) and the NF- $\kappa$ B transcription factor, which either individually or altogether stimulate the expression of COX2 and NOS2 in the nucleolus<sup>6,54</sup>. The ROS have been associated with the presence of NAD(P)H oxidase, which seems to be a key contributor to the production super oxide  $O_2^-$  from oxygen<sup>59</sup>. ROS can also activate the expression of apoptotic and cell cycle regulatory molecules like p53, p21<sup>Waf1</sup>, p34, and MDM2<sup>48,60,61</sup>. Additionally ROS and NOS can induce  $Ca^{2+}$  fluxes in unirradiated cells<sup>62</sup>. Moreover, blocking calcium channels can modify the bystander effect response<sup>63,64</sup>. Mitochondria

have also been associated with the induction of RIBE. In fact, radiation can induce loss of mitochondrial membrane potential<sup>65</sup>. Additionally, it has been demonstrated that radiation can affect both mitochondrial DNA<sup>66</sup> and function<sup>67</sup>. Other studies demonstrate that bystander fibroblast cells show a higher activation of protein kinase C compared to irradiated fibroblasts and controls<sup>68</sup>. Moreover, blocking the expression of protein kinase C significantly decreased the formation of micronuclei as result of RIBE when irradiation was given with alpha particles and gamma rays<sup>69</sup>.

### **Radiation-Induced Bystander Effects *in vivo***

Since their discovery RIBE have been considered either beneficial or harmful to the cells, and it raises the question about their consequences in an organism<sup>70</sup>. The majority of studies have demonstrated the presence of bystander effects mainly *in vitro* when culturing cells or tissue<sup>46,48,71,72</sup>. However, there is increasing evidence that indicates the existence of RIBE in a whole organism *in vivo*<sup>73</sup>. Pant et al. and Goh<sup>74,75</sup> showed that blood plasma from irradiated people either therapeutically or after an accident contains factor(s) that are able to induce chromosomal aberrations in normal leukocytes. Those clastogenic factors can persist in plasma for years, as it has been seen in the blood of Chernobyl survivor where the intensity of the clastogenic response seems to be related with the dose

received<sup>76,77</sup>. In addition, it is believed that ROS are also involved because the clastogenic activity can be inhibited by superoxide dismutase<sup>76</sup>.

Studies in rodents have demonstrated that irradiations of part of rat organs such as the liver<sup>78</sup> or lung<sup>79</sup> can produce cytotoxic effects in non-irradiated normal tissue. When radiation was delivered to one lung, the contralateral-shielded organ also showed damage<sup>80</sup>. Moreover, the damage was attenuated when radical scavengers were used. Additionally, interleukin and inflammatory cytokines like TNF- $\alpha$  and TGF- $\beta$  were found<sup>81</sup>. Furthermore, when mice were partially irradiated using a lead shield to protect their heads a marked increase of medulloblastoma was observed in mice that were shielded compared with the ones that received whole body irradiation<sup>82</sup>. Moreover, Singh et al. gave single and serial low doses to mice and observed that the media harvested from bladder explants was able to induce clonogenic death<sup>83</sup>.

Mothersill et al. have conducted extensive work in fish, mainly rainbow trout, medaka, and zebra fish. She reported that rainbow trout irradiated with 0.5 Gy total body dose can release factors into the water. Those factors seem to signal unirradiated fish and cause the induction of bystander effects, which are expressed as cell death in a reporter HPV-G cell system<sup>84</sup>. When repeating this experiment with Zebra fish they also observed bystander responses which do not seem to be retained in water for long periods of time<sup>85</sup>. In contrast, when irradiating rainbow

trout in their early life stages the production of signals was persistent during the animals' life span <sup>86</sup>.

The immune system seems to play an important role in the induction of RIBE *in vivo* <sup>87</sup>. When comparing normal and p53-null mice it was seen that macrophage activation and neutrophil infiltration were not direct effects of radiation. In fact, they were a consequence of the detection and clearance of the apoptotic cells induced by radiation. Moreover, the macrophages remain active for months, which suggests a sustained pro-inflammatory activity in the progeny of the irradiated cells <sup>88</sup>.

## Chapter 3

# Materials and Methods

### 3.1 Animal Model

Normal Wistar rats were used as 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 guidelines. Since the goal of this Masters project is to show the effects of MRT on healthy and tumour-bearing rats, experiments 1 and 3 were conducted with healthy rats whereas experiment 2 used tumour-bearing rats (Table1).

In preparation for the irradiations, rats were deeply anesthetized using 3% isoflurane in 2L/min compressed air and maintained with a intraperitoneal injection of a Ketamine-Xylazine cocktail (Ketamine : Xylazine = 1: 0.625; Ket 1000 and Paxman from Virback France).

**Table 1 - Design of the Research Work**

Nomination	Experimental Goal	Number of Rats per experiment	Results and discussion
Experiment 1	Study the RIBE in healthy rat brain after MRT	87	Chapter 4
Experiment 2	Study the RIBE in tumour-bearing rat brain after MRT	76	Chapter 5
Experiment 3	Study the communication of RIBE in rats in vivo	29	Chapter 6

Each nomination is related to its experimental objective, number of animals used, and the correspondent chapter where results and discussions can be found.

**Table 2 - Irradiation group schedule for Experiment 1 (Healthy Rats)**

Group	Number of Rats		Modality	Dose	Dissection
	IHC	Explant			
A	2		MRT	350 Gy	4 hrs
B	2		MRT	350 Gy	8 hrs
C	2	5	MRT	35 Gy	4 hrs
D	2	5	MRT	35 Gy	8 hrs
E		5	MRT	70 Gy	4 hrs
F		5	MRT	70 Gy	8 hrs
G		5	MRT	17.5 Gy	4 hrs
H		5	MRT	17.5 Gy	8 hrs
I	2		Homogenous	350 Gy	4 hrs
J	2		Homogenous	350 Gy	8 hrs
K	2	5	Homogenous	35 Gy	4 hrs
L	2	5	Homogenous	35 Gy	8 hrs
M		5	Homogenous	70 Gy	4 hrs
N		5	Homogenous	70 Gy	8 hrs
O		5	Homogenous	17.5 Gy	4 hrs
P		5	Homogenous	17.5 Gy	8 hrs
X1		1	Whole body Irradiation	1 mGy	Immediate
X2		1	Whole body Irradiation	2 mGy	Immediate
Sham		4	Rats anaesthetized, but not Irradiated		8 hrs
Controls		5	Rats never left the cage		

Experiment 1 was set up to investigate the radiation-induced bystander effects on normal rat brains. Synchrotron radiation was administered in a single session to the right cerebral hemisphere. For homogenous radiation, a broad beam with a size of 10mm width and 14mm height was used. MRT had the same size of the irradiation field but an array composition of 25 $\mu$ m thick and 14mm high rectangular microbeams, with 200 $\mu$ m of distance between the centres of two beams.

### 3.2 Tumour Cell Line

C6 glioma cells were used as malignant tumour model because they are morphologically similar to glioblastoma multiforme when injected into the brain of rats<sup>89</sup>. C6 Gliomas are a rapidly proliferating rat cell line that was originally produced in random-bred of Wistar-Furth rats by exposing them to N-nitrosomethylurea<sup>90</sup>. After injection into the brain, intracranial growth of C6 gliomas results in the formation of malignant solid tumours, which are delineated by a rim of reactive astrocytes but the tumour itself does not express glial fibrillary acidic protein (GFAP)<sup>91</sup>. Additionally C6 cells show high association for endothelial cells, which is characterized by their growth around blood vessels<sup>91</sup>.

Several C6 cultures were grown in T75 flasks to assure enough availability of the cells during the inoculations. Dulbecco's Modified Eagle Medium (GIBCO) was used as growth culture medium supplemented with 10% FBS (Gibco) and 5mL Penicillin-Streptomycin (Gibco). Cell line was maintained in a 37°C, 5% CO<sub>2</sub> and 95% humidity incubator. In preparation for implantation a 90% confluent flask was selected. Cells were gently washed with 10 mL of DPBS in order to regulate pH and eliminate remaining grown media. To detach cells, trypsin was replaced by 20 ml of Hank's Balanced Salt Solution (HBSS) in order to maintain high cell viability at the moment of the implantation. The flask was then placed in

the incubator until cells were floating freely – after approximately 10 min. Immediately 20mL of growth medium was added to neutralize HBSS and cell suspension was placed in a 45mL conical Falcon tube. Cells were then centrifuged at 1000 rpm during 4 min. The pellet was re-suspended in 1mL of fresh growth medium and cells were counted using an haemocytometer. Aliquots were prepared aiming a cell concentration of 100,000 cells per 10uL. As soon as aliquots were ready, they were placed on ice and transported to the surgical lab for animal implantation.

### **3.3 Tumour Inoculation:**

Upon arrival of the cell suspension, animals were anesthetized using 4% isoflurane and placed on a stereotactic frame. General anaesthesia was maintained at 2 – 2.5% isoflurane in 2L/min compressed air. The heads of the rats were shaved and disinfected with 70% alcohol. A vertical straight incision of 2 to 2.5 cm was made on the skin following the sagittal plane. A small hole was then drilled in the skull over the right hemisphere. The coordinates were 3mm to the right from the middle line and 3 mm posterior from the coronal suture. Tumour cells were next inoculated into the brain using a 27G needle connected to a pre-loaded Hamilton syringe mounted on the vertical arm of a stereotactic frame. For the inoculation the needle was carefully lowered through the hole until



touching the cortical surface. Then it was further lowered 3mm into the brain tissue and 10 $\mu$ L of medium containing 100,000 cells were introduced within a 4 minute period. The needle was then allowed to rest undisturbed for another minute in order to allow distribution of the new content into the brain and to avoid its escape to the surface when retrieving the needle. The incision was then closed and the rats were allowed to recover. Animals were housed for 8 days until the tumour developed. In preparation for the irradiations animals were assigned randomly to one of the treatment, sham or control groups of the Experiment 2 (Table 3).

**Table 3 Irradiation group schedule for Experiment 2 (Tumour-bearing rats)**

Group	Number of rats		Modality	Dose	Dissection
	IHC	Explant			
A	1	4	MRT	350 Gy	4 hrs
B	1	4	MRT	350 Gy	8 hrs
C	1	4	MRT	70 Gy	4 hrs
D	1	4	MRT	70 Gy	8 hrs
E	1	4	MRT	35 Gy	4 hrs
F	1	4	MRT	35 Gy	8 hrs
G	1	4	Homogenous	350 Gy	4 hrs
H	1	4	Homogenous	350 Gy	8 hrs
I	1	4	Homogenous	70 Gy	4 hrs
J	1	4	Homogenous	70 Gy	8 hrs
K	1	4	Homogenous	35 Gy	4 hrs
L	1	4	Homogenous	35 Gy	8 hrs
NO-IR	1	4	No-irradiation, Tumour only		
Reverse		2	Left irradiation, Tumour in right hemisphere		35 Gy 8 hrs
No-TU		5	Right irradiation, No Tumour		35 Gy 8 hrs
Controls	2	4	Rats never left the cage (No Tumour)		

Experiment 2 corresponds to the investigation of Radiation Induced-bystander effects on tumour-bearing rat brains after microbeam radiation therapy. Besides those in the control group, all rats contained tumours unless otherwise stated. Rats were irradiated to the right cerebral hemisphere using MRT and Homogenous beams. Alternative a pilot experiment was performed where rats containing tumour received irradiation on the healthy cerebral hemisphere. This was performed in order to analyse the extent of variation of the bystander signal released by the tumour when it is not irradiated.

**Table 4 - Irradiation group schedule for Experiment 3 (Cage Mates)**

Group	Irradiated Rats	Cage Mates (non-irradiated)	Modality	Dose	Dissection
A	4	4	MRT	350 Gy	48 hrs
B	4	4	MRT	35 Gy	48 hrs
C	2	2	Homogenous	350 Gy	48 hrs
D	2	2	Homogenous	35 Gy	48 hrs
Controls	5	5	Rats never left the cage		

Experiment 3 corresponds to the Communication of Radiation-induced bystander signal in rats in vivo. Only normal rats were used to perform this experiment. Rats were irradiated to the right cerebral hemisphere using MRT and Homogenous radiation. The purpose of this experiment is to analyse whether bystander signals can be transmitted from irradiated rats to their unexposed cage mates.

## **3.2 Irradiations**

Animals were transported from the Animal Facility to the biomedical beam line ID17, which takes less than 5 minutes. Each rat was then individually placed on the goniometer and the corresponding radiation dose for its treatment group was applied exclusively to the right cerebral hemisphere by setting the beam 2mm towards the right from the midline, unless otherwise stated. The left non-irradiated cerebral hemispheres served as fields of study for bystander effects. Details of the irradiation modalities were as follows:

### **3.2.1 MRT:**

Animals were exposed in a single treatment session following the schedule presented for the Experiments 1, 2 and 3 (on page 24 and 28) at 17.5, 35, 70, or 350 Gy skin-entry doses. Although multi-directional treatment is more successful in increasing survival, the geometry of the unidirectional beam works better for understanding RIBE. The above is supported by the fact that we wanted to understand whether the gaps of normal tissue present between the microbeams increases the induction of bystander effects. Therefore, an array of 10mm wide and 14mm high monochromatic anteroposterior beam was delivered. The MRT beam array was composed of 50 quasi-parallel rectangular microbeams, which were

25µm thick with 200µm centre-to-centre distance. Additionally, the synchrotron was set to deliver a multi-chromatic synchrotron beam with a dose rate of 16,000 Gy/sec.

### **3.2.2 Homogenous Radiation:**

In order to differentiate whether the bystander response produced by the spatially fractionated microbeams differs from the response to a broad beam, a uniform radiation dose was delivered to another group of rats, with an equivalent dose to the dose delivered with the corresponding MRT protocols.

As well as MRT, homogenous radiation was administered in one single treatment session following the schedule presented for the Experiments 1, 2 and 3 (on page 24, 28) at the same skin entry doses. The direction to the target and energy of the homogenous beam was the same as for MRT.

### **3.2.3 Scatter:**

In order to rule out that bystander responses of unirradiated tissue are produced by scatter radiation, two animals were selected as scatter

controls (X1 and X2, Experiment 1). To accomplish this, a PTW ion chamber semiflex was used to measure the dose received at the bladder after 350Gy Homogenous and 350Gy MRT. The doses obtained were 30.6mGy for the homogenous configuration and 5.8 mGy for MRT.

Next, an X-r-ray generator was adapted with different additional filters to obtain an adequate dose rate, in order to deliver as well again 30.6mGy and 5.8mGy to the whole rats respectively.

HD and MD Gafchromic Films (Nuclear Associates, NY, USA) were used to verify all irradiation doses and modalities applied.

After irradiation, rats were sent back to the ESRF animal facility for recovery in their correspondent treatment groups and were killed after 4, 8 or 48 hrs. depending on the experimental schedule.

### **3.3 Untreated and Sham Radiation Controls**

Untreated controls stayed always in the ESRF animal facility and never left the cage. They received neither radiation nor anaesthesia and thus constituted our absolute controls. Animals were killed at the end of the experiments. Sham radiation controls, on the contrary, did leave the cage, received anaesthesia, were transported to the biomedical beam line, and were placed on the goniometer. However, they did not receive radiation. This was performed in order to take into account any possible animal stress coming from being handled that could affect our results. Sham rats were then placed in the same cage and transported back to the animal facility for immediate dissection and sampling.

### **3.4 Dissections and Sampling**

Animals were transported with no exception to the ESRF animal facility after irradiation. Once their recovery time had passed rats were deeply anaesthetised, beheaded, and brain and bladder were obtained in the surgical laboratory.

#### **3.4.1 Dissections and Sampling for Explant Culture and Proteomics**

The rats' brains were quickly and carefully extracted from the skull using sterile instruments. Dissection of the brain was performed in a

biosafety cabinet. Two pieces of brain tissue (5mm x 5mm x 3mm) were taken from both the right and the left cerebral hemispheres, which were placed in a 5ml sterile tube containing 1mL of RPMI 1640 (Gibco) growth medium, supplemented with 10% FBS, 5mL of Penicillin-Streptomycin (Gibco), 5mL of L-glutamine (Gibco), 0.5 ug/ml of Hydrocortisone (Sigma-Aldrich), and 12.5 mL of 1M HEPES buffer solution (Gibco); and immediately transported on ice to the tissue culture laboratory. The remaining brain tissue was frozen with liquid nitrogen and stored at -80°C for further proteomic studies. To retrieve the bladder, an abdominal midline incision was made using a sterile technique. The bladder was then also placed in a 5ml tube containing 1ml of complete growth medium and immediately processed as tissue explant.

### **3.4.2 Dissection and Fixation for Immunohistochemistry**

In order to perform immunohistochemical studies in Experiments 1 and 2, an entire rat brain was required. For this purpose, rats were irradiated according to the schedule previously presented in tables 2 and 3. Dissection was carried out under the same conditions as above. After retrieving the brains from the skull, they were individually fixed with 10% phosphate-buffered Formalin over 4 hours. Next, the brains were dissected

horizontally into one upper and one lower part in order to allow formalin to fix the brain's internal structures. The upper and lower sections were individually placed in pre-labeled histological cassettes, and re-immersed in fresh 10% formalin. The tissue samples were kept in these conditions and transported to the University of Freiburg for paraffin embedding.

### **3.5 Explant Tissue Culture and Medium Harvesting**

Explant tissue culture was performed in the biosafety level 2 laboratory of the ESRF animal Facility. Brain tissue blocks and bladder were cut in 3 equal-size pieces of approximately  $2\text{mm}^3$  in a biosafety cabinet. Next, each piece was immediately plated as single explants in the centre of a  $25\text{cm}^2$  growth area 40 mL volume flask, containing 2mL of previously described growth medium. Flasks were then left undisturbed in a  $27^\circ\text{C}$ , 5%  $\text{CO}_2$  and 95% humidity incubator.

Growth medium from each of the three explant pieces was harvested 48 hours later by pouring it off into a sterile plastic container. This was then filtered through a  $0.22\mu\text{m}$  filter (Acrodisc Syringe Filter with HT Tuffryn Membrane, Pall Life Sciences) to ensure that cells were not present in the transferred medium, and placed in a 7mL tube. Conditioned growth medium was kept in  $4^\circ\text{C}$  until all medium was collected and then transported to McMaster University for clonogenic reporter bioassays.



### 3.6 Clonogenic Reporter Cell Line

The HPV-G cells are epithelial cells derived originally from human foreskin primary culture and immortalized through transfections of complete Human Papillomavirus 16 genes (HPV16-Genes)<sup>92</sup>. The HPV16 genes that directly participate in the immortalization of the epithelial cells are E6 and E7<sup>93</sup>. Although, E6 protein inactivates p53 pathways<sup>94</sup>, and activates the host telomerase<sup>95</sup> it only increases lifespan of the cell when it is present by itself. Therefore, the presence of E7, which inactivates the retinoblastoma Rb/p16 pathway<sup>96</sup> is also required to altogether give immortality to the cell. In fact, the full-length of E6/E7 oncogenes are sufficient for a successful cell transformation<sup>93</sup>.

HPV-G cells were obtained as a gift by Professor J. DiPaolo, NIH, Bethesda, MD, and have been use in a wide range of experiments due to their reliable and stable response to bystander signals<sup>22,52,85,97</sup>. Moreover, they show a reduction of around 40% in colony survival over a wide range exposure conditions and doses<sup>98,99</sup>

### **3.7 Culturing HPV-G Cells**

The HPV-G cells were cultured in T75 flasks with RPMI 1640 supplemented as above. Once a confluence of 90-95% was reached cells were detached using 1:1 solution of 0.25 % Trypsin/EDTA (10x) (Gibco) and Dulbecco's Phosphate Solution (1x) (Gibco). Cells were then placed in the incubator during 8 to 10 minutes until detachment was completed. Once cells were completely detached, 10ml of growth medium was used to neutralize the trypsin, and this solution was gently pipetted in order to produce a single cell suspension. The concentration of cells was determined by using a Coulter Counter (Beckman Coulter), and the cell line was subcultured plating around of 1,000,000 cells in a new T75 flasks. All procedures were carried out in a biosafety Cabinet Class II in Dr. Mothersill level 2 laboratory at McMaster University.

### **3.8 Clonogenic HPV-G Reporter Bioassay**

Upon arrival at McMaster University, in order to assess the effects of irradiated and non-irradiated rat tissue explants, the conditioned medium needed to be transferred into 25cm<sup>2</sup> flasks containing the HPV-G reporter cells. Reporter flasks were seeded with 500 HPV-G cells, which were 90-95% confluent, and that had received a medium change 24 hour before were selected.

Proceeding with the medium transfer technique, the already filtered explant conditioned medium obtained at the ESRF was placed into the reporter cell flasks, from which the growth medium had been previously removed. Cells were then placed in a 37°C, 5% CO<sub>2</sub> incubator to allow for colony formation during 10-12 days. Plating efficiency controls were also prepared.

### 3.9 Colonies Count and Bystander Activity

Reporter flasks needed to be stained before cells grow over the neighbouring colonies. This was accomplished by periodically checking the HPV-G colony growth under a light inverted microscope. Once colonies reached the expected size, flasks were removed from the incubator, and 2mL of 1:4 Carbol Fuchsin solution was used to stain cells.

Colonies were counted using a 50 cells threshold and the percentage survival fraction was calculated using the plating efficiency of the reporter cells.

**Equation 1 Plating Efficiency**

$$\text{Plating Efficiency (PE)} = \frac{\# \text{ of Colonies}}{\# \text{ of Cells seeded}} \times 100$$

**Equation 2 Survival Fraction**

$$\textit{Survival Fraction} = \frac{\textit{PE of Treated Cells}}{\textit{PE of Control Cells}} \times 100$$

### **3.10 Statistical Analysis**

Data are presented as standard deviation of the mean for the specific n value of each experiment. Significance was determined using the paired t-test. In all cases p values < 0.05 were selected as significant.

### **3.12 Immunohistochemistry**

Immunohistochemistry procedures were carried out entirely in the Neuropathology Department of the University of Freiburg Hospital, Germany. Distribution of the bystander proteome through the brain has hitherto not been discussed in immunohistochemical research. Because of this, we decided to first look into the normal irradiated rat brains instead of those inoculated with brain tumours as a trial measure. This trial would allow us to evaluate the results and make improvements to the research process without wasting valuable tissue material from inoculated rats. Although only cassettes from Experiment 1 were

selected for immunohistochemistry, all tissue samples were embedded in paraffin to allow their conservation over time.

### **3.12.1 Antibody Selection**

Antibodies were selected following the results obtained from a previous proteomics pilot experiment at the ESRF. This project was the first joint work developed by McMaster University, the European Synchrotron Radiation Facility, and the University of Freiburg. In that opportunity, Dr. Richard Smith from McMaster University identified the brain bystander protein expression after irradiating normal Wistar rats to 35Gy and 350Gy doses of MRT (Table 5). Dr. Smith kindly allowed the use of his results to look for both the protein functions and the availability of antibodies in the market.

Originally, immunofluorescence (IF) was the technique we wanted to use. Unfortunately, antibodies against our bystander proteins were poorly developed for IF compared to for immunohistochemistry in paraffin embedded tissue (IHC-P). Thus, it was decided to work only with antibodies for IHC-P in order to standardize our results.

Seven antibodies were found from the total of eight, Aconitate hydratase, mitochondrial (ACO2) (LifeSpan Biosciences, BIOZOL, Germany), Triosephosphate isomerase (TPI1) (LifeSpan Biosciences, BIOZOL, Germany), Heat shock cognate 71kDa protein (Hsc70) (Abnova, USA), Tubulin alpha-1A chain (TUBA1) (LifeSpan Biosciences, BIOZOL, Germany), Prohibitin (PHB) (LifeSpan Biosciences, BIOZOL, Germany), Glial Fibrillary Acidic (GFAP) (Dako, Germany), and NADH dehydrogenase (ubiquinone) flavoprotein 1 mitochondrial (NDUFV1) (ProteinTech Group, USA).

**Table 5 - Bystander Proteome Changes in Rat Brain after MRT**

Right Hemisphere (Irradiated)		Left Cerebral Hemisphere (non-Irradiated)	
Dihydropyridinyl dehydrogenase, mitochondrial (DLD)	↓	Tubulin alpha-1A chain (Tuba1a)	↓
Aconitate hydratase, mitochondrial (ACO2)	↑	Prohibitin (Phb)	↓
Triosephosphate isomerase (TPI1)	↑	Glial fibrillary acidic (GFAP)	↑
Heat shock cognate 71kDa protein (Hspa8)	↑	NADH dehydrogenase (ubiquinone) flavoprotein 1 mitochondrial	↑
		Heat shock cognate 71kDa protein (Hspa8)	↑

Bystander proteome changes in the right and left hemisphere of wistar rat brains after right hemisphere irradiation with 35 or 350 MRT. ↑ and ↓ indicate increase and decrease in protein expression compared to controls. Results were kindly shared by Dr. Richard Smith

### **3.12.2 Paraffin Embedding**

All fixed samples from experiments 1 and 2 were embedded using an automatic tissue processor (Leica ASP300). For the embedding process the longest automated cycle was chosen in order to allow better formalin removal. Cassettes were processed on incremental groups of 20 units per run, during a 58 hours 24 minutes cycle. The total fixation time was accounted to be between 3 to 15 days depending on the order that the tissue samples were processed.

### **3.12.3 Sectioning**

Sectioning was performed only in the upper sections of brains from experiment 1. Cassettes were placed on the microtome and 4 $\mu$ m sections were obtained at room temperature. Thirty slides were prepared per brain sample, in which 3 sections were mounted. To ensure that a representative area of the brain was being explored a 100 $\mu$ m distance was set between sections of each slide. Then, the slides were placed into a 35°C incubator over night to complete mounting process. Once completed, slides were conserved in 4°C until immunohistochemistry was performed.

#### **3.12.4 Antibody Optimization**

Primary antibodies were optimized in order to find both the optimal dilution and the appropriate epitope retrieval method, which allows a correct saturation of the tissue by the antibody. Positive controls were used at all times, which were provided by the Neuropathology Department of the Freiburg University Medical Centre. In order to detect the optimal primary antibody dilution a series of titrations were performed following the manufacturer data sheet recommendations. To retrieve epitopes back from binding with formalin, proteinase K and a vapour based heat system were used.

#### **3.12.5 Immunostaining**

Sections were first deparaffinised and rehydrated using a series of alcohol and xylene washes. Then, a cooking vapour system was used to induce heat epitope retrieval. The retrieval was conducted by placing the slices in a pH 6 solution of citric acid at a temperature of 95°C over 20 minutes. Next, blocking of tissue-endogenous peroxidases was performed using 3% Oxygen Peroxide (J.T.Baker) over 10 minutes. This was followed by blocking the host's proteins using 10% Fetal Calf Serum over an hour. Immediately after, incubation with the first antibody was



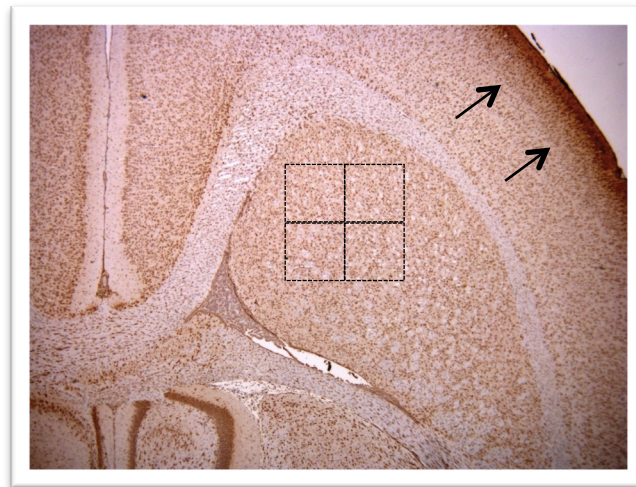
performed at room temperature over another hour. Incubation with the second antibody was then carried out also at room temperature for 30 minutes. Next, Avidin-peroxidase (Thermo Scientific) was applied over 20 minutes followed by Diaminobenzidine (Sigma) over 10 minutes. To finish the process, slides were counterstained with Haematoxylin, and dehydrated with alcohol and xylene washes.

### **3.12.6 Image Analysis**

Slides were analysed under the microscope Olympus Bx41 and images were acquired under a microscope Leica DMRA. Slide analysis consisted on screening the tissue samples in order to identify the affectivity of the immunohistological stain by analysing the positive and negative controls of each antibody and the integrity of the samples. For the image acquisition, it was established to detect the intensity of the dye in a specific area of the tissue sample using the Image-Pro software. Originally we decided to perform the detections across the brain cerebral cortex because that was the area explored during the proteomic studies. However, several staining artefacts observed along the cortex after the IHC did not allow its use as part of the research (Figure 1). Instead, the left caudate

nucleus was used as the target for detection of the dye because of both its close cellular architecture and functional correlation to the brain cortex<sup>100</sup>.

To develop a standard technique for our experiments, the 8 hours dissection time treatments were chosen for the software analysis. Then, the left caudate nucleus was identified under the microscope and 4 images were acquired (Figure 1). Next, each image was loaded into the Image-Pro software, which was used to first perform a digital filtration of the blue colour, which represents the haematoxylin counter stain; and second to detect the intensity of the remaining colour in the image, which represented the immunohistochemical stain for the specific antibodies. Once the intensity values were obtained, they were organized by antibody and posterior data analysis was performed.



**Figure 1** – Squares show the four areas of the caudate nucleus that were acquired in order to perform the image analysis. Arrows show the staining artefact of the cerebral cortex. (Antibody employed was Hspa8)

## Chapter 4

# Radiation-Induced Bystander Effects in Healthy Rat Brain After Microbeam Radiation Therapy

## 4.1 Results

### 4.1.1 Clonogenic survival of reporter cells grown in explant-conditioned medium from the irradiated right cerebral hemisphere

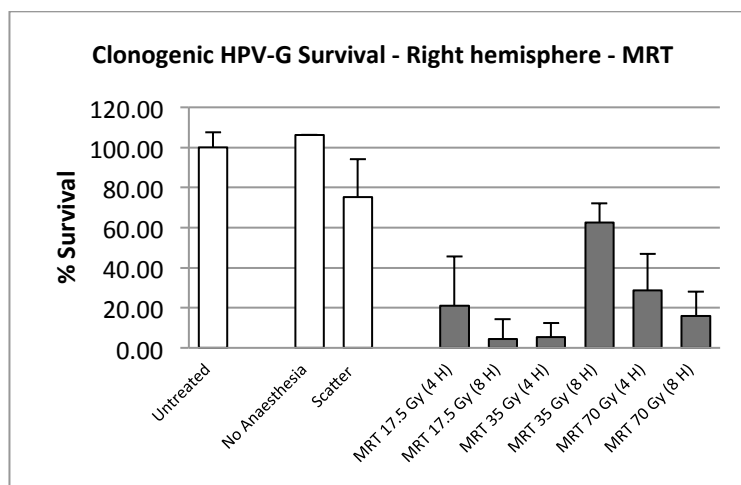
#### Clonogenic Survival After MRT

Figure 2 shows a significant reduction in the percentage of survival of HPV-G cells that received medium from irradiated right hemisphere tissue explants. Less than 10% of survival was observed after 17.5 Gy and 35 Gy of MRT doses were delivered and dissection was performed 8 and 4 hours afterwards, respectively. Close to a 20% of survival was seen after 17.5 Gy, 70 Gy, and 70 Gy of MRT were given to the rats and dissections were carried out 4, 4 and 8 hours after exposure, respectively. 60% of reporter survival was observed

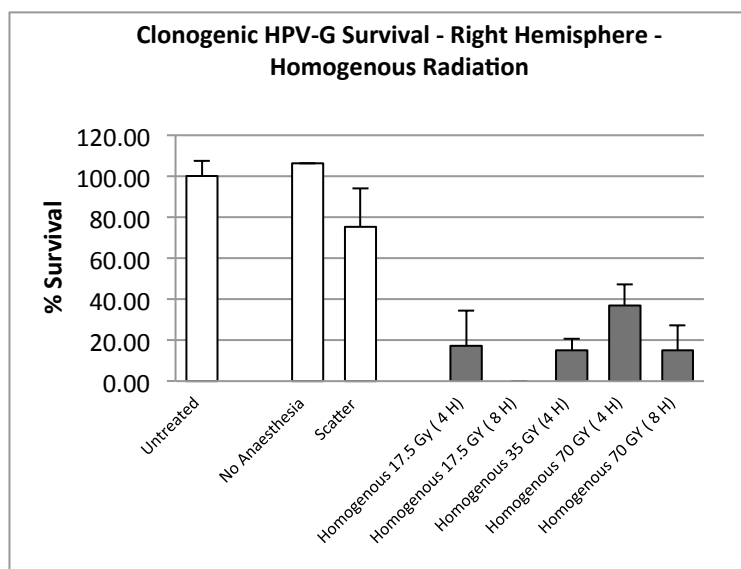
after 35 Gy MRT with a dissection time of 8 hours. Scatter controls showed a survival that is close to 70% compared to the untreated group.

### **Clonogenic Survival After Homogenous Radiation**

HPV-G clonogenic survival showed a significant reduction after being grown in explant-conditioned medium from the irradiated right cerebral hemisphere (Figure 3). No evidence of survival was observed in flasks that received medium from explants taken from cerebral hemispheres treated with 17.5 Gy of homogenous radiation and dissected in the following 8 hours. Less than 20% of survival was observed in the 17.5 Gy, 35 Gy and 70 Gy treatment groups that received homogenous radiation and whose brains were dissected after 4, 4, and 8 hours respectively. 35% of HPV-G survival was observed after the 17.5 Gy of homogenous radiation was delivered to rats, which dissection was performed 4 hours later.



**Figure 2** - Reporter Survival of HPV-G cells that received medium from tissue explants extracted from the right hemisphere treated with MRT. Rats from the untreated group received anaesthesia but did not received radiation. Rats from the Scatter group received a whole body dose of 5.8 mGy. Rats from the 'no-anaesthesia' group did not receive radiation or anaesthesia (Error bars indicate mean standard deviation for n=5)



**Figure 3** - Reporter Survival of HPV-G cells, which received medium from tissue explants extracted from the right hemisphere after being exposed to homogenous radiation. Rats from the untreated group received anaesthesia but did not received radiation. Rats from the Scatter group received a whole body dose of 30.6 mGy. Rats from the 'no-anaesthesia' group did not receive radiation or anaesthesia (Error bars indicate mean standard deviation for n=5)

#### **4.1.2 Clonogenic survival of reporter cells grown in explant-conditioned medium from the non-irradiated left cerebral hemisphere**

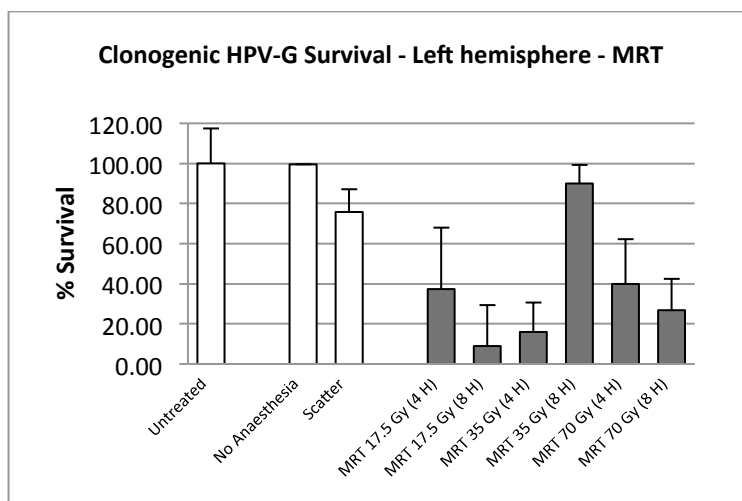
##### **Clonogenic Survival After MRT**

Figure 4 shows the response HPV-G reporter cells after being cultured with medium from rat tissue explants. Rats were exposed to different doses of MRT to the right cerebral hemisphere. However, the explants were taken from the non-irradiated left hemisphere. The results showed a decrease in the percentage of survival of all the treatments compared to the untreated group. A reporter cell survival lower than 20% was observed when the explant tissue was extracted after 8 and 4 hours of have applied 17.5 Gy and 35 Gy of MRT to the rats. Between 20 to 40% of clonogenic survival was seen after 17.5 Gy, 70 Gy and 70 Gy doses of MRT when dissection times were 4, 4, and 8 hours respectively. 90% of clonogenic survival was observed when dissection took place 8 hours after of have delivered 35 Gy of MRT. Scatter controls showed a survival that is close to 80% compared to the untreated group.

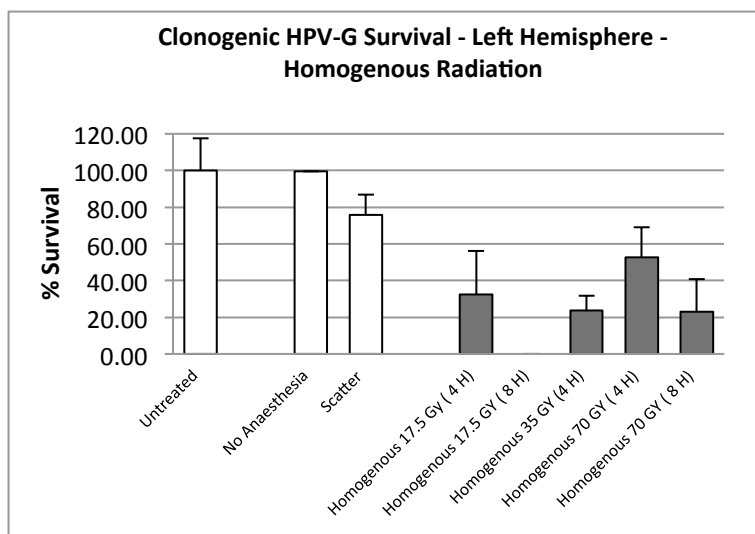
### **Clonogenic Survival After Homogenous Radiation**

Clonogenic of reporters showed a significant reduction of survival after being grown in explant-conditioned medium (Figure 5). Explants were obtained from the left cerebral hemisphere of rats that originally received homogenous radiation to their right hemisphere. No evidence of survival was seen after exposing reporter cells to medium resultant of 17.5 Gy of homogenous radiation when dissection was performed 8 hour after. Between 20 to 30% of clonogenic survival was observed when 17.5 Gy, 35 Gy, or 70 Gy of homogenous radiation was delivered to the rats and they were then killed at 4, 4, and 8 hours after exposure, respectively. Around 50% of survival was observed after a homogenous dose of 70 Gy was delivered and the dissection was made 4 hours later.





**Figure 4** - Reporter Survival of HPV-G cells, which received medium from non-irradiated left hemisphere after MRT was delivered to the contralateral right cerebral hemisphere. Rats from the untreated group received anaesthesia but did not receive radiation. Rats from the Scatter group received a whole body dose of 5.8 mGy. Rats from the no-anaesthesia group did not receive radiation or anaesthesia (Error bars indicate mean standard deviation for n=5)



**Figure 5** - Reporter Survival of HPV-G cells, which received medium from non-irradiated left hemisphere after homogenous radiation was delivered to the contralateral right cerebral hemisphere. Rats from the untreated group received anaesthesia but did not receive radiation. Rats from the Scatter group received a whole body dose of 5.8 mGy. Rats from the no-anaesthesia group did not receive radiation or anaesthesia (Error bars indicate mean standard deviation for n=5)

### **4.1.3 Clonogenic survival of reporter cells exposed to medium from bladder tissue explants after MRT or Homogenous radiation was delivered to the right cerebral hemisphere**

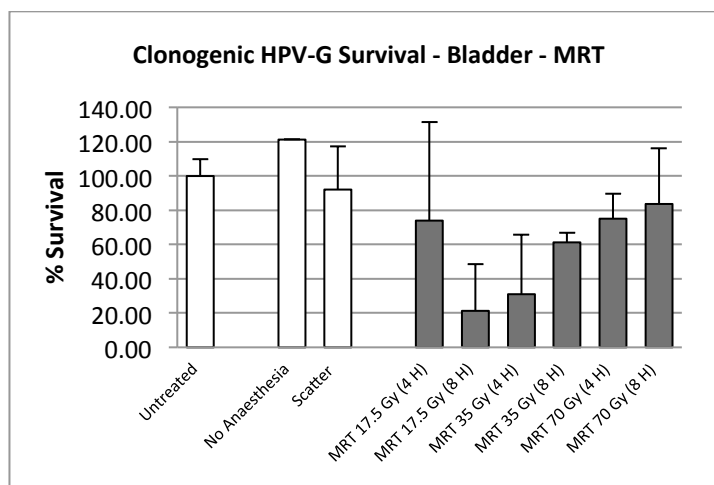
#### **Clonogenic Survival After MRT**

Figure 6 shows the response of the reporter cells when they were cultured in medium transferred from bladder explants, which originally belonged to rats that received MRT to the right cerebral hemisphere. Close to 20% of survival was observed in the 17.5 Gy and 35 Gy MRT treatment groups whose brains were respectively dissected 8 and 4 hours after exposure. Between 50-70% of HPV-G survival was observed in the last 4 MRT treatment groups. Scatter controls showed around of 80% of reporter survival compared to the untreated group.

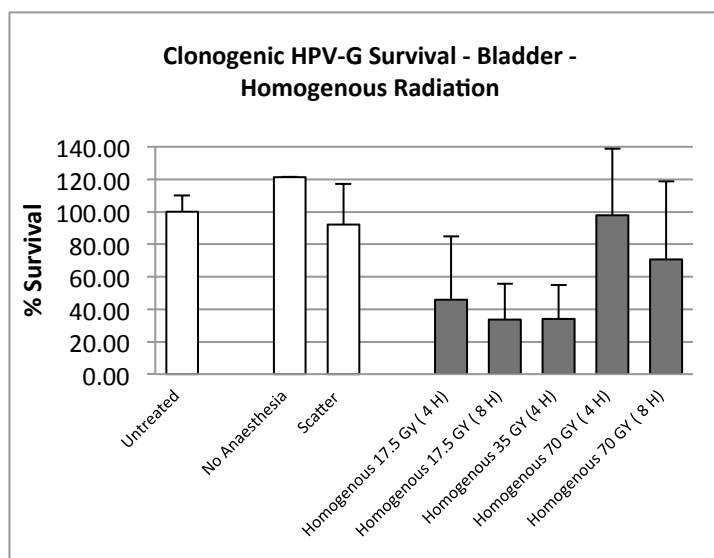
#### **Clonogenic Survival After Homogenous Radiation**

Clonogenic survival of reporter cells shows that bladder tissue conditioned medium caused a decrease in the number of colonies of both the treatment and control groups (Figure 7). Between 30 to 40% of HPV-G survival was observed in 3 homogenous radiation treatment groups, 17.5 Gy with 4 and 8 hours dissection times, and 35 Gy with 4 hours dissection time. 60% of survival was observed only when rats from the 70 Gy treatment group were dissected 8 hours after exposure.

80% of survival was seen when the conditioned medium came from rats exposed to 70 Gy, whose dissection time were 4 hours. Close to 80% of survival was observed in the handling and scatter controls groups.



**Figure 6** – Reporter Survival of HPV-G cells, which received medium from bladder after MRT was delivered to the right cerebral hemisphere. Rats from the untreated group received anaesthesia but did not received radiation. Rats from the Scatter group received a whole body dose of 5.8 mGy. Rats from the ‘no-anaesthesia’ group did not receive radiation or anaesthesia (Error bars indicate mean standard deviation for n=5)



**Figure 7** - Reporter Survival of HPV-G cells, which received medium from bladder after homogenous radiation was delivered to the right cerebral hemisphere. Rats from the untreated group received anaesthesia but did not received radiation. Rats from the Scatter group received a whole body dose of 30.6 mGy. Rats from the ‘no-anaesthesia’ group did not receive radiation or anaesthesia (Error bars indicate mean standard deviation for n=5)

**Table 6 – Statistical Analysis**

Treatment	Right Cerebral Hemisphere				Left Cerebral Hemisphere				Bladder			
	Mean SF	STANDV	t-test	Significant (p<0.05)	Mean SF	STANDV	t-test	Significant (p<0.05)	Mean SF	STANDV	t-test	Significant (p<0.05)
Untreated	44.2	±7.8	-	-	45.2	±3.3	-	-	87.4	±8.7	-	-
No Anesthetic	44.0	-	-	-	48.0	-	-	-	106.0	-	-	-
Scatter	33.5	±4.9	0.069747	No	34.0	±8.5	0.019690	Yes	80.5	±21.9	0.269345	No
MRT 17.5 Gy (4 H)	16.5	±13.5	0.002960	Yes	9.5	±11.2	0.000117	Yes	64.5	±50.4	0.171978	No
MRT 17.5 Gy (8 H)	4.0	±8.9	0.000032	Yes	2.0	±4.5	0.000000	Yes	18.6	±24.0	0.000156	Yes
MRT 35 Gy (4 H)	7.0	±6.6	0.000019	Yes	2.4	±3.2	0.000000	Yes	27.2	±30.2	0.001331	Yes
MRT 35 Gy (8 H)	39.8	±4.0	0.146480	No	28.2	±4.4	0.000066	Yes	53.6	±4.7	0.000030	Yes
MRT 70 Gy (4 H)	17.6	±9.9	0.000733	Yes	13.0	±8.1	0.000018	Yes	65.8	±12.6	0.006655	Yes
MRT 70 Gy (8 H)	11.8	±7.0	0.186698	No	7.2	±5.4	0.000000	Yes	73.2	±28.5	0.158611	No
Homogenous 17.5 Gy ( 4 H)	14.4	±10.4	0.000450	Yes	7.8	±7.8	0.000005	Yes	40.2	±34.0	0.008462	Yes
Homogenous 17.5 Gy ( 8 H)	0.0	±0.0	0.000001	Yes	0.0	±0.0	0.000000	Yes	29.2	±19.4	0.000142	Yes
Homogenous 35 Gy (4 H)	10.4	±3.6	0.000011	Yes	6.8	±2.5	0.000000	Yes	29.6	±18.4	0.000110	Yes
Homogenous 70 Gy ( 4 H)	23.2	±7.3	0.001144	Yes	16.6	±4.7	0.000002	Yes	85.6	±35.7	0.457728	No
Homogenous 70 Gy ( 8 H)	10.2	±7.9	0.000063	Yes	6.8	±5.5	0.000000	Yes	61.6	±42.0	0.107913	No

Statistical analysis of experiment 1, which shows how significantly different all treatment are compared to the untreated group. The study was performed using a paired t-test analysis.

**Table 7 – Statistical Analysis of all treatments compared to the Scatter group**

Treatment	Right Brain				Left Brain				Bladder			
	Mean SF	Standv	t-test	Significant (p<0.05)	Mean SF	Standv	t-test	Significant (p<0.05)	Mean SF	Standv	t-test	Significant (p<0.05)
<b>Scatter</b>	<b>34</b>	<b>±8.5</b>	<b>-</b>	<b>-</b>	<b>33.5</b>	<b>±4.9</b>	<b>-</b>	<b>-</b>	<b>80.5</b>	<b>±21.9</b>	<b>-</b>	<b>-</b>
Untreated	45.2	±3.3	0.0197	Yes	44.2	±7.8	0.0697	No	87.4	±8.7	0.2693	No
MRT 17.5 Gy (4 H)	9.5	±11.2	0.0276	Yes	16.5	±13.5	0.0876	No	64.5	±50.4	0.3512	No
MRT 17.5 Gy (8 H)	2	±4.5	0.0005	Yes	4	±8.9	0.0041	Yes	18.6	±24.0	0.0129	Yes
MRT 35 Gy (4 H)	2.4	±3.2	0.0003	Yes	7	±6.6	0.0020	Yes	27.2	±30.2	0.0386	Yes
MRT 35 Gy (8 H)	28.2	±4.4	0.1313	No	39.8	±4.0	0.0674	No	53.6	±4.7	0.0148	Yes
MRT 70 Gy (4 H)	13	±8.1	0.0140	Yes	17.6	±9.9	0.0455	Yes	65.8	±12.6	0.1458	No
MRT 70 Gy (8 H)	7.2	±5.4	0.0017	Yes	11.8	±7.0	0.0056	Yes	73.2	±28.5	0.3810	No
Homogenous 17.5 Gy (4 H)	7.8	±7.8	0.0054	Yes	14.4	±10.4	0.0315	Yes	40.2	±34.0	0.0961	No
Homogenous 17.5 GY (8 H)	0	±0.0	0.0001	Yes	0	±0.0	0.0000	Yes	29.2	±19.4	0.0139	Yes
Homogenous 35 GY (4 H)	6.8	±2.5	0.0004	Yes	10.4	±3.6	0.0005	Yes	29.6	±18.4	0.0123	Yes
Homogenous 70 GY (4 H)	16.6	±4.7	0.0073	Yes	23.2	±7.3	0.0676	No	85.6	±35.7	0.4312	No
Homogenous 70 GY (8 H)	6.8	±5.5	0.0017	Yes	10.2	±7.9	0.0064	Yes	61.6	±42.0	0.2931	No

Statistical analysis of all treatment groups compared to the Scatter controls. The study was performed using a paired t-test analysis

#### **4.1.4 Bystander Proteome**

As an introduction to the bystander proteome a compilation of the protein functions is presented in Table 8. Additionally, alternative names, correlation with cancer and additional data are presented in the same chart.

#### **4.1.5 Immunohistochemistry**

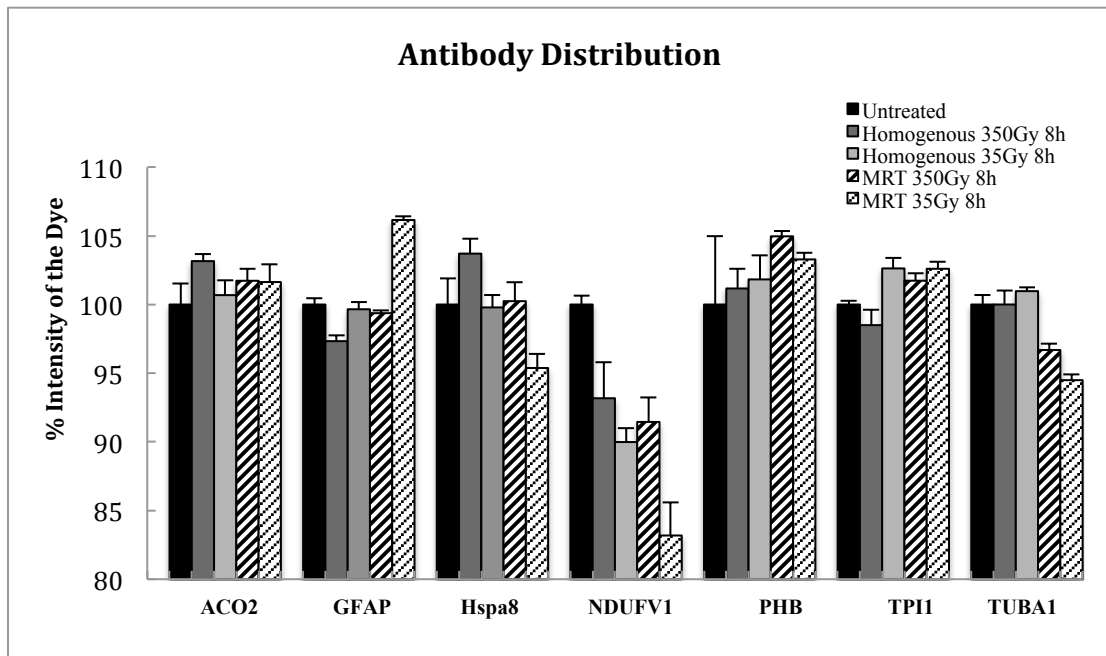
Figure 2 shows the protein distribution between the bystander left cerebral hemisphere and the untreated left cerebral hemisphere. The values represent the intensity of the immunohistochemistry dye after performing digital analysis using the Image-Pro software. Aconitate hydratase, mitochondrial (Aco2) showed an increase in its expression in all the treatment explored. Glial fibrillary acidic protein (GFAP) showed different levels of expression depending on the treatment. Heat shock cognate 71kDa protein (Hspa8) showed also different intensities of protein expression depending on the treatment. NADH dehydrogenase (ubiquinone) flavoprotein 1 mitochondrial (NDUFV1) showed a marked decrease in protein intensity compared to its control. Prohibitin (Phb) showed an increased percentage in dye intensity in all treatments and a similar trend was observed in Triosephosphate isomerase (Tpi1). On the contrary Tubulin alpha-1A chain (Tuba1a) showed a decrease in intensity.

**Table 8 - Brain Bystander Proteome, Functions and Description**

Bystander Protein	Alternative names	Relationship with Cancer in humans	Function and Cellular Component in Humans	Additional Data
Prohibitin (Phb)		Maybe a Tumour suppressor in humans	Prohibitin inhibits DNA synthesis. It has a role in regulating proliferation. As yet it is unclear if the protein or the mRNA exhibits this effect. May play a role in regulating mitochondrial respiration activity and aging.	Prohibitin is an evolutionarily conserved gene that is ubiquitously expressed. It is thought to be a negative regulator of cell proliferation and may be a tumour suppressor. Mutations in PHB have been linked to sporadic breast cancer.
			Can be found: membrane, nucleous, mitochondria	Prohibitin is expressed as two transcripts with varying lengths of 3' untranslated region. The longer transcript is present at higher levels in proliferating tissues and cells, suggesting that this longer 3' untranslated region may function as a trans-acting regulatory RNA.
Tubulin alpha-1A chain (Tuba1a)	Alpha-tubulin 1		The alpha and beta tubulins represent the major components of microtubules. There are multiple alpha and beta tubulin genes, which are highly conserved among species	
	Tubulin alpha-1 chain		This gene encodes alpha tubulin and is highly similar to mouse and rat Tuba1 gene. Northern blotting studies have shown that the gene expression is predominantly found in morphologically differentiated neurologic cells.	
Triosephosphate isomerase (Tpi1)	Triose-phosphate isomerase	when reduced it is considered as tumour suppressor -also known as a glioma marker	Can be found: Cytosol, nucleus, and soluble fraction	Tpi1 is essential for energy production because it is a glycolytic enzyme which participate in the glycolyte pathway, and since Cancer cells have an high rate of energy consumption (warburg effect), its reduction is considered to decrease tumour expression.
Heat shock cognate 71kDa protein (Hspa8)	Heat shock 70 kDa protein 8 (Hsc70, Hsc73)		This proteins binds to nascent polypeptides to faciitate correct folding. It also functions as an ATPase in the disassembly of clathrin-coated vesicles during transport membrane components through the cell. Cellular component: Extracellular vesicular exosome, melanosome, protein complex, ribonucleoprotein complex.	HSPA8 belongs to HSP70 family, which has two members: Heat-inducible proteins (Heat-shoke proteins, Hsp70) and Constitutively expressed proteins (heat-shock cognate proteins, Hsc70)
Serum Albumin (Alb)		Known as a glioma marker	Main protein of plasma, has a good binding capacity for water, Ca <sup>2+</sup> , K <sup>+</sup> , fatty acids, hormones, bilirubin and drugs. Its main function is the regulation of the colloidal osmotic pressure of blood. Major zinc transporter in plasma, typically binds about 80% of all plasma zinc.	
Aconitate hydratase, mitochondrial (Aco2)	Citrate hydro-lyase		The protein encoded by this gene belongs to the aconitase/IPM isomerase family. It is an enzyme that catalyzes the interconversion of citrate to isocitrate via cis-aconitate in the second step of the TCA cycle. This protein is encoded in the nucleus and functions in the mitochondrion. It was found to be one of the mitochondrial matrix proteins that are preferentially degraded by the serine protease, also known as Lon protease, after oxidative modification.	
NADH dehydrogenase (ubiquinone) flavoprotein 1 mitochondrial			Core subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I) that is believed to belong to the minimal assembly required for catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone	Defects in NDUFV1 are a cause of a disorder of the mitochondrial respiratory chain that causes a wide range of clinical disorders. Phenotypes include macrocephaly with progressive leukodystrophy, non-specific encephalopathy, cardiomyopathy, myopathy, liver disease, Leigh syndrome, Leber hereditary optic neuropathy, and some forms of Parkinson disease. It is also expressed in several tumours including glioma
Glial fibrillary acidic (GFAP)	GFAP	Known as a glioma and serum glioma marker	GFAP is expressed in the central nervous system in astrocyte cells. It is involved in many cellular functioning processes, such as cell structure and movement, cell communication, and the functioning of the blood brain barrier.	GFAP has been shown to play a role in mitosis by adjusting the filament network present in the cell. During mitosis, there is an increase in the amount of phosphorylated GFAP, and a movement of this modified protein to the cleavage furrow. Using knockout mice it can be seen a lack of intermediate filaments in the hippocampus and in the white matter of the spinal cord. Also the knocked-out mice undergo multiple degenerative processes including abnormal myelination, white matter structure deterioration, and alterations in the blood-brain barrier. These data suggest that GFAP is involved in maintenance of CNS myelin integrity.
			Glial fibrillary acidic protein (GFAP) is abundantly expressed in malignant gliomas	

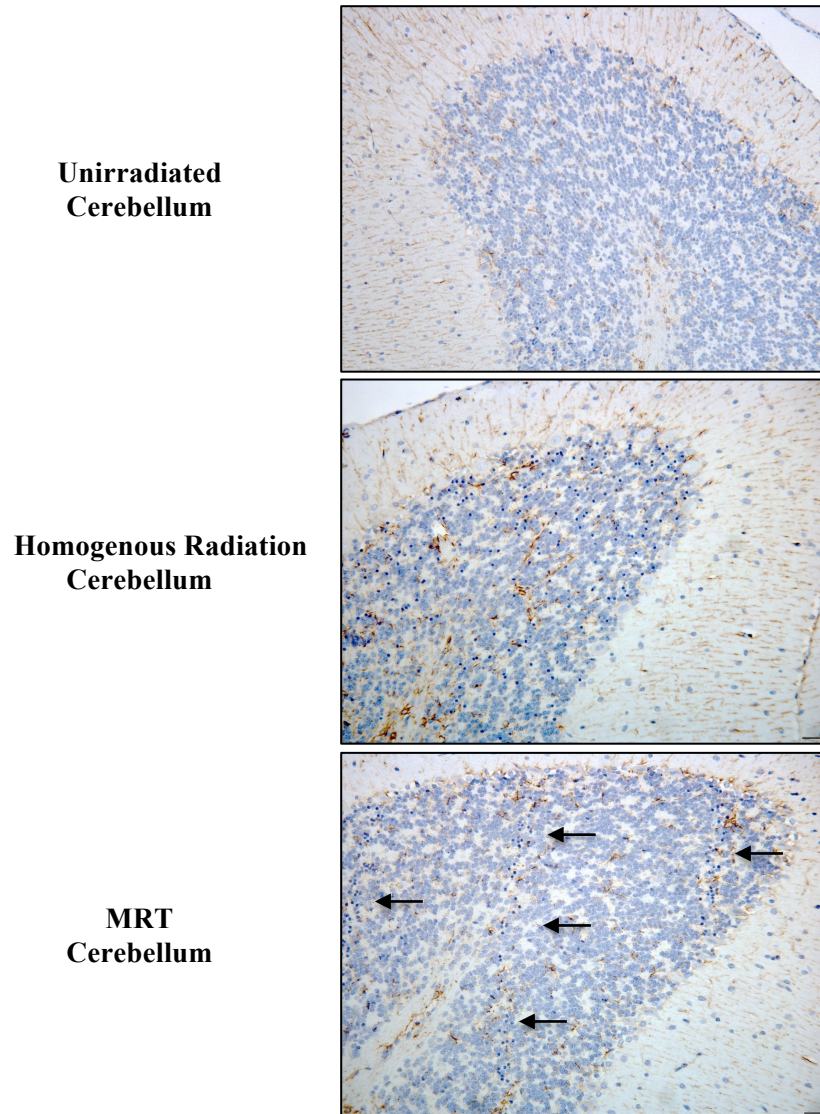
The chart shows the function of the bystander brain proteome, their alternatives names and additional cancer related.





**Figure 8** – Percentage of intensity of the 7 antibodies studied for correlation with bystander protein expression. Aconitate hydratase, mitochondrial (Aco2), Glial fibrillary acidic protein (GFAP), Heat shock cognate 71kDa protein (Hspa8), NADH dehydrogenase (ubiquinone) flavoprotein 1 mitochondrial (NDUFV1, Prohibitin (Phb), Triosephosphate isomerase (Tpi1) and Tubulin alpha-1A chain (Tuba1a) showed a decrease in intensity.

### Direct Effects of Synchrotron Radiation



**Figure 9** – Direct effect of synchrotron radiation in granular cell layer of the cerebellum. Radiation produced apoptotic/necrotic bodies that are observed as nuclear pyknosis. Images were obtained from the 350 Gy 8 hours dissection time homogenous and MRT treatment groups. Arrows indicate the track of the MRT.

## 4.2 Discussion

Radiation-induced bystander effects are radiation-like responses in cells that have not been directly irradiated<sup>39,72</sup>. Extensive work done *in-vitro* by Mothersill shows that RIBE are identified as a generalised stress response, which is expressed at the level of the tissue, organ or organism rather than at the level of individual cells<sup>70</sup>. Although all exposed cells may produce the signals, the response seems to require a quorum of cells in order to be manifested<sup>70</sup>. Following the above premise, our results show RIBE by observing a significant reduction in clonogenic survival after growing HPV-G cells in explant-conditioned media from the irradiated (right) hemisphere. In accordance with Mothersill, this is a consequence of the release of factors from the direct irradiated tissue. Those factors in the medium are detected by our reporter cells, which respond by undergoing apoptosis.

As it was explained in Chapters 1 and 2 the bystander effects were expected to occur in the “valley dose” region between the microbeams. However, no significant differences in clonogenic survival were observed after comparing homogenous radiation and MRT. In fact, both modalities seem to produce the same percentage of decrease in reporter cell survival (Figure 10) Additionally, bystander work *in-vivo* developed by Mothersill and O’Dowd<sup>71,101</sup>, showed that a whole body irradiation dose to mice and fish induced the production of bystander

signals that affected unirradiated animals. However, unlike Mothersill and O'Dowd our work showed that bystander effects can be observed after irradiating only one hemisphere of the rat brain instead. This suggests that the decrease in the clonogenic survival was produced first by messengers that were released from the irradiated hemisphere as consequence of the direct effect of radiation. Then, once that signal is distributed within the animal, it seems to target the contralateral brain hemisphere and bladder, which start to produce second messengers that affect the survival of our reporter cells when the clonogenic assay is performed. Furthermore, no matter the dose or radiation modality, the clonogenic survival of the bystander hemisphere and bladder show the same pattern as the direct irradiated groups (Figure 10). In terms of dose response, a slight decrease in survival at the low dose treatments can be observed, though it is not significant.

### **Scatter Controls**

Clonogenic survival of the scatter controls showed interesting results. First, by looking at the Figures 1-6 it is clear that the scatter controls demonstrated a decrease in survival compared to the untreated groups. However, as it is shown in Table 7 that decrease is only significant when comparing to the direct irradiated hemisphere. Second, the scatter clonogenic decrease was not deep enough to get close to the percentage of survival showed by the majority of treatment groups.

Moreover, the statistical analysis from Table 7 showed that the scatter clonogenic survival is significantly different from treatment groups. Finally, in terms of finding out whether anaesthesia was playing an important role in the induction of bystander effects, our results showed that there were not differences in clonogenic survival when comparing with the untreated group.

### **Immunohistochemistry**

The immunohistochemistry (IHC) staining showed a range of protein intensities in the bystander brain that don't show a strong correlation with the protein expression obtained by Dr. Smith. For example, some of the treatment groups showed staining intensities that are correlated with the bystander protein expression from the proteomic experiment. However, there are other antibodies that show the opposite response. For the antibodies ACO2, TPI1, and TUBA1, reaction matches that seen in the proteomics study, while NDUFV1 and PHB show opposite intensity levels compared to proteomics. In addition to that, the remaining antibodies, GFAP and Hsp8, showed a random stain intensity that cannot be significantly correlated to the original proteomic results.

A number of reasons are thought to be the cause of the inaccuracy in the immunohistochemical detection of the bystander protein distribution:

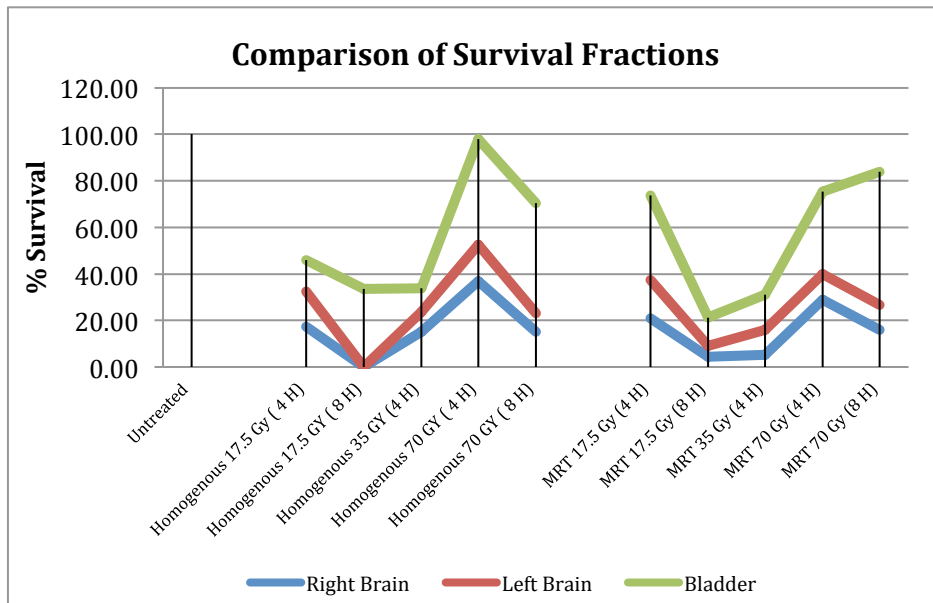
1. The immunohistochemical measurements were not made in the brain cortex. Originally, the brain cortex was the site from which tissue samples were extracted to perform the proteomic analysis. This may be a very important reason to think that the immunochemistry technique is in fact effective, but the differences in protein expression may be attributed to the variation in both cellular architecture and function of the caudate nucleus compared to the brain cortex.
2. It is well known that the use of formalin-fixed paraffin-embedded tissue generates masking of the epitopes <sup>102</sup>. This could be very relevant if we take in account that we are analyzing protein expression at very short times, 4 and 8 hours after irradiation. Therefore, the amount of protein will not be as large as in cancer detection for instance.
3. The use of IHC may not be the most appropriate technique to validate Dr. Smith bystander protein expression. IHC is sensitive enough to detect the small amount of protein changes but probably inefficient to enhance those changes and make them distinct from inherent background staining (Figure 11).

In order to improve the immunohistochemical findings the author suggests repeating the detection of the antibodies but selecting different areas within the brain. If this process is not conclusive, immunofluorescence (IF) would be an

excellent alternative, though not all the bystander proteins have their equivalent antibodies for IF.

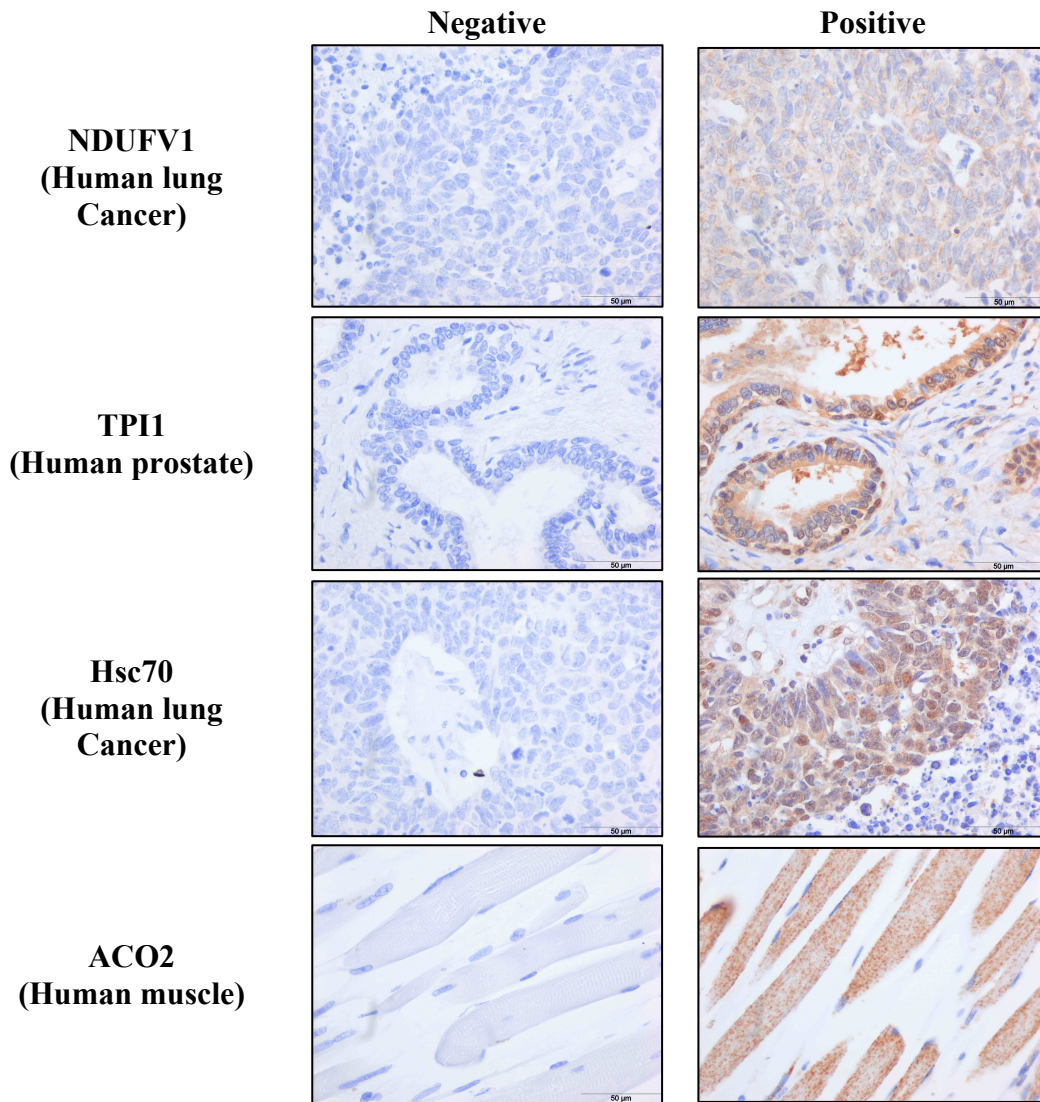
Positive and negative controls are included at the end of this discussion in order to discard any error that could have occurred during the IHC procedure, and that could have affect the level of stain in tissue (Figure 11). Images show an evident staining of all the positive groups demonstrating an adequate immunohistochemical performance. Additionally, when analyzing the negative controls, a reduced but important level of background color can be observed, which is especially evident in NDUFV1, TUBA1 and PHB.

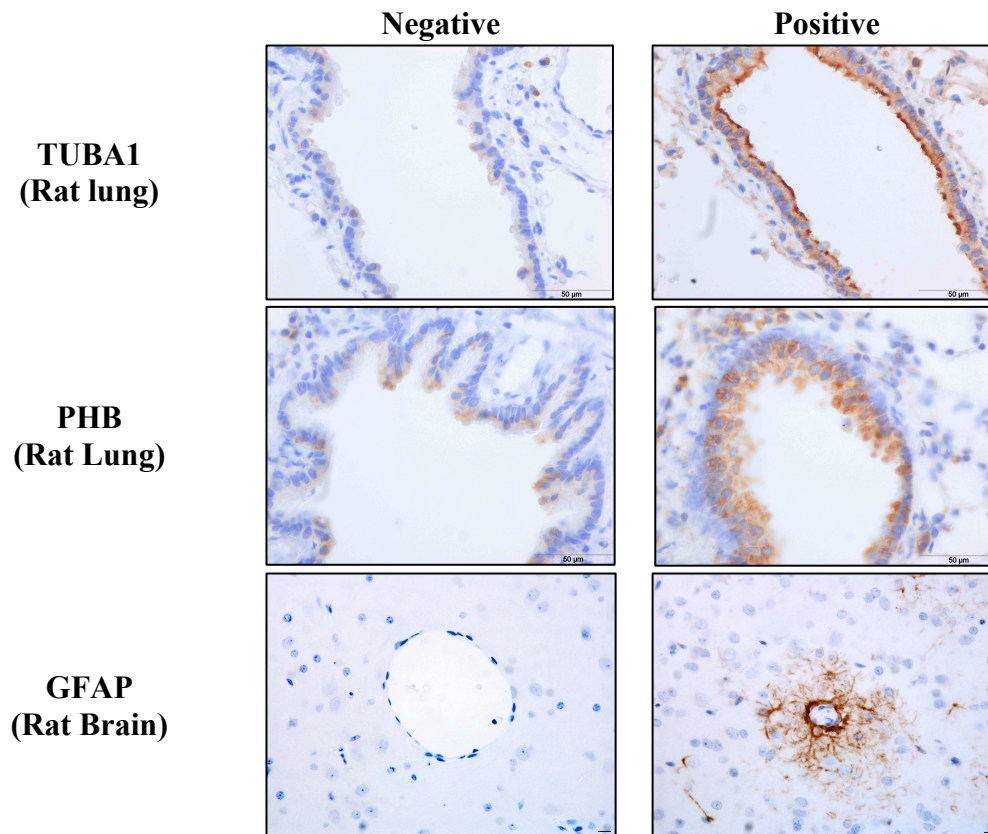
When looking at the direct effects of the synchrotron radiation in cerebellum a clear pattern of damage can be seen depending on the radiation modality (Figure 9). After homogenous radiation a uniform distribution of nuclear pyknosis can be observed. Whereas after MRT nuclear pyknosis can be seen only along the path of the microbeams.



**Figure 10** - Comparison of clonogenic reporter survival between MRT and homogenous radiation. Each treatment group includes the percentage of survival resulted from exposing HPV-G cells to the explant-conditioned medium from right brain hemisphere, left brain hemisphere and bladder. (Error bars indicate mean standard deviation for: untreated n=5)







**Figure 11** – Immunohistochemistry controls of the seven antibodies employed to investigate the bystander protein expression in rat brain. Images show the effectiveness of the assay in order to discard errors related with the technique.

## Chapter 5

# Radiation-Induced Bystander Effects in Tumour-Bearing Rat Brains After Microbeam Radiation Therapy

## 5.1 Results

### 5.1.1 Clonogenic survival of reporter cells grown in explant-conditioned medium from the irradiated right cerebral hemisphere of rats containing brain tumour.

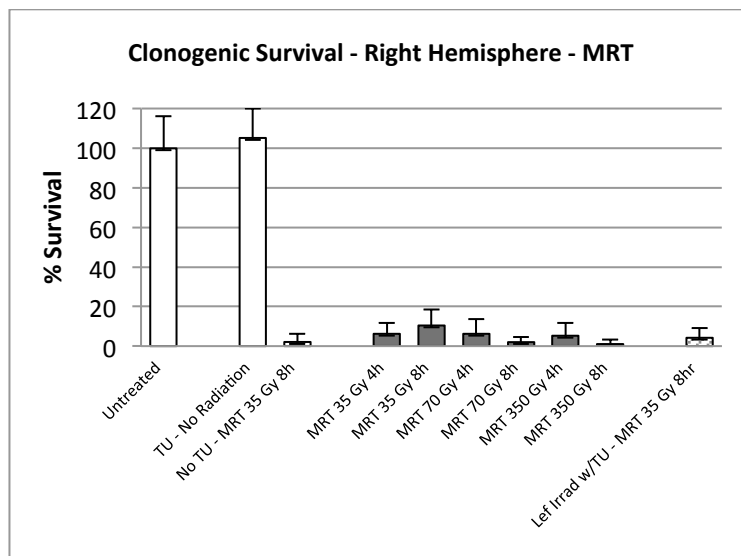
#### Clonogenic survival after MRT

A significant decrease in survival was observed after growing HPV-G cells in explant-conditioned medium from tumour-bearing rats (Figure 12). Tumour-bearing rats were exposed to different doses of MRT in their left cerebral hemisphere unless otherwise stated. Less than 10% of survival was observed in all the MRT treatment groups regardless of the dose delivered. The tumour-containing rats that were irradiated in the right cerebral hemisphere also showed less than 10% of reporter survival. Both untreated and tumour-bearing unirradiated groups showed a 100% rate of survival, whereas less than 5% of

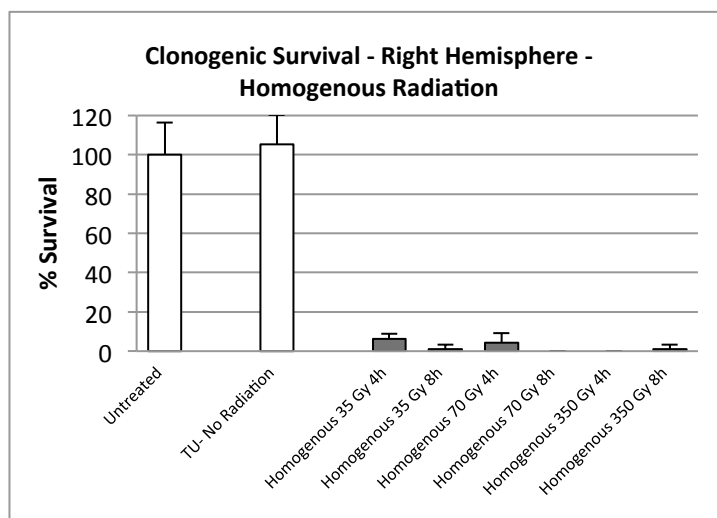
reporters in the 35Gy MRT group survived. The pilot experiment, which involved irradiation of the healthy left hemisphere with the tumour located in the contralateral side, showed less than 10% of reporter survival.

### **Clonogenic survival after homogenous radiation**

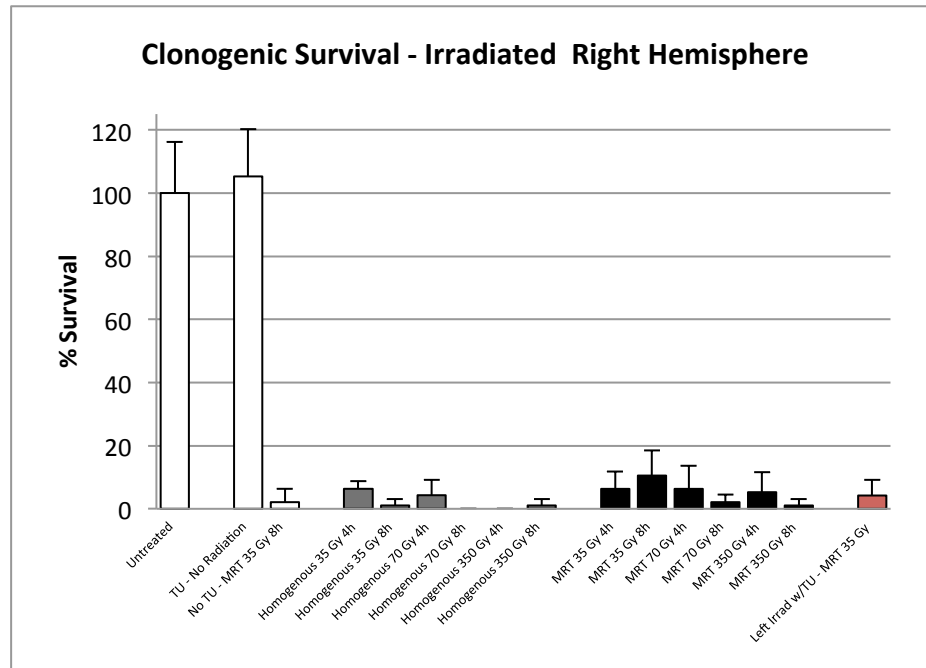
Figure 13 shows that explant-conditioned media generated a significant decrease in clonogenic reporter survival of the irradiated groups versus the controls. No presence of colonies were observed when dissections were performed 8 and 4 hours after irradiating rats with 70 and 350 Gy of homogenous radiation. Less than 10% of reporter cell survival was seen in the remaining radiation-treated groups, including those animals which had not been inoculated with tumour cells. Rats harbouring tumours but not exposed to radiation showed a clonogenic survival rate superior to 100%.



**Figure 12** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium. Explants were taken from the right hemisphere of the brain. Tumours were inoculated in the right hemisphere and MRT was also delivered to the right hemisphere. Rats from the untreated group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumours but did not receive radiation. Rats from the *No TU - MRT 35Gy* group were healthy rats, which only received MRT. Rats from the *Left Irrad w/TU-MRT 35Gy* group contained tumour but radiation was given to the left hemisphere. (Error bars indicate mean standard deviation for: Untreated n=4, “TU-No Radiation” n=4, No TU-MRT 35Gy n=5, tumour bearing rats n=4, “Left Irradiation w/TU-MRT” n=2).



**Figure 13** - Clonogenic survival of HPV-G cells grown in explant-conditioned media. Explants were taken from the right hemisphere of the brain, where tumours were inoculated. Homogenous radiation was given to the same right hemisphere. Rats from the untreated group received anaesthesia but did not received radiation. Rats from the *TU-No Radiation* group contained tumour but did not receive radiation. (Error bars indicate mean standard deviation for: Untreated n=4, tumour bearing rats n=4, “TU-No Radiation” n=4).



**Figure 14** - Comparison of reporter clonogenic survival between MRT and homogenous radiation. HPV-G cells were grown in explant-conditioned media. Explants were taken from the right hemisphere of the brain, where tumours were inoculated. MRT and Homogenous radiation was given to the same right hemisphere, unless otherwise stated. Rats from the *untreated* group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumour but did not receive radiation. Rats from the *No TU - MRT 35Gy* group were healthy rats, which only received MRT. Rats from the *Left Irrad w/TU-MRT 35Gy* group contained tumour but radiation was given to the left hemisphere. (Error bars indicate mean standard deviation for: Untreated n=4, tumour bearing rats n=4, “TU-No Radiation” n=4, “No TU-MRT 35Gy” n=5, “Left Irradiation w/TU-MRT” n=2).

### **5.1.2 Clonogenic survival of reporter cells grown in explant-conditioned medium from the non-irradiated left cerebral hemisphere of rat brains containing tumour.**

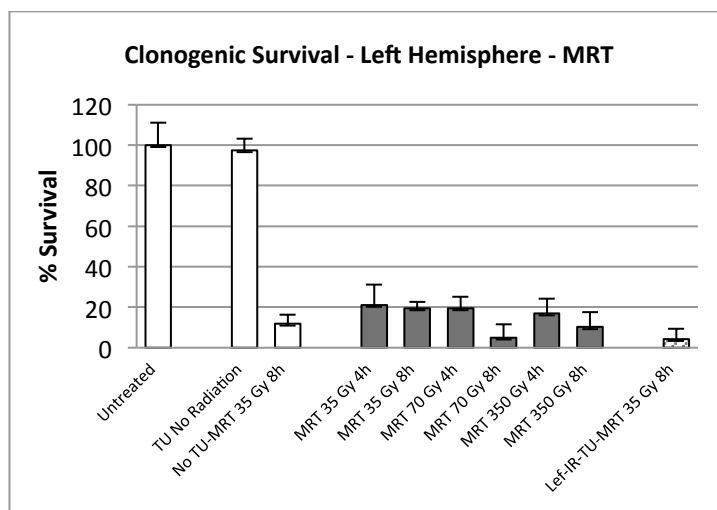
#### **Clonogenic survival after MRT**

Clonogenic survival showed a significant reduction after growing HPV-G cells in explant-conditioned medium (Figure 15). Explants were taken from the non-irradiated left hemisphere of the brain. Unless otherwise stated, MRT was delivered to the contralateral hemisphere, which was inoculated with tumour cells. Less than 10% of survival was observed in the 70 Gy MRT group, in which dissection was performed 4 hours after irradiation. Approximately 10% of survival was observed in both the “No TU–MRT 35Gy” and the “MRT 350Gy 8h” groups. The pilot experiment, which involved irradiation of the healthy left hemisphere while the tumour was in the contralateral side, showed less than 10% of reporter survival. An average of 20% survival was seen in the remaining 4 groups: MRT 35Gy 4h, MRT 35Gy 8h, MRT 70Gy 4h, and MRT 350Gy 4h.

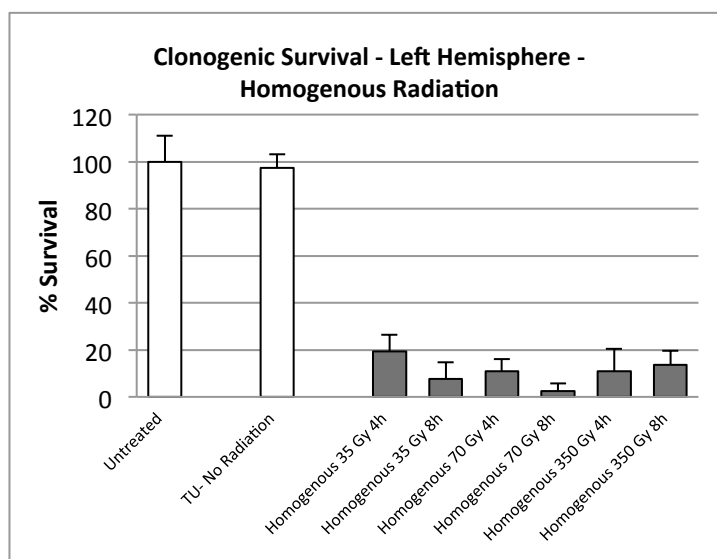
### **Clonogenic survival after homogenous radiation**

Clonogenic survival of HPV-G cells showed a significant decrease after being grown in explant-conditioned medium from the non-irradiated left cerebral hemisphere (Figure 16). Homogenous irradiation was given to the tumour inoculated in the contralateral right hemisphere. Less than 5% of survival was seen when reporters that received explant-conditioned medium from the group exposed to 70 Gy of homogenous radiation, on which dissections were performed 8 hours after irradiation. 20% of reporter survival was observed when HPV-G cells were grown in medium taken from tissue explants extracted 4 hours after irradiating rats to 35 Gy of homogenous radiation. The remaining 4 irradiated groups showed an average 10% of clonogenic survival. The tumour-inoculated group that did not receive radiation showed 100% of survival.

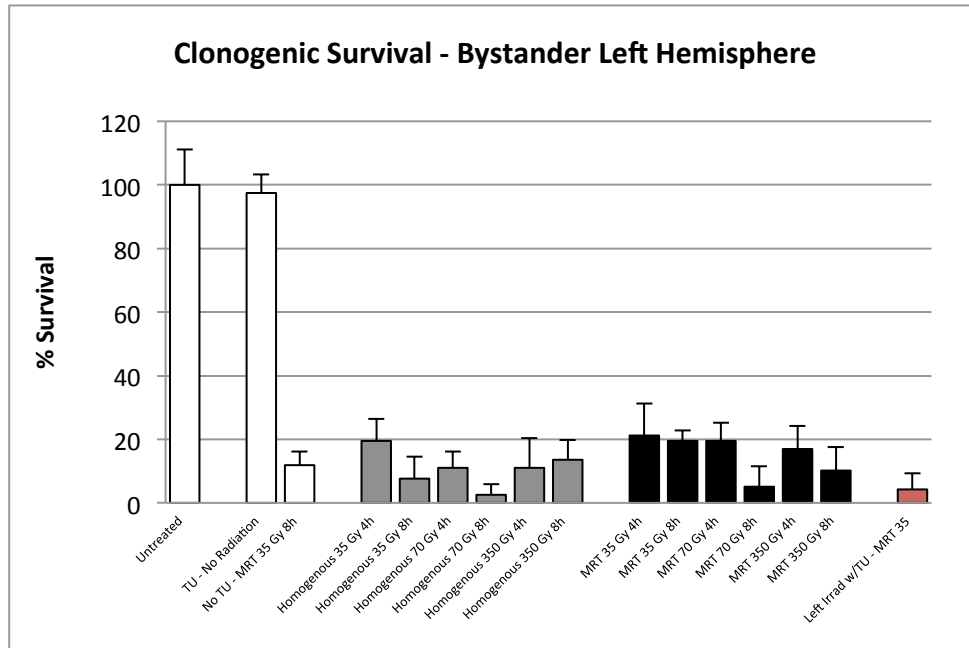




**Figure 15** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium. Explants were taken from the non-irradiated left hemisphere of the brain. Tumour was inoculated in the right hemisphere. MRT was given to the same right hemisphere. Rats from the *untreated* group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumour but did not receive radiation. Rats from the *No TU – MRT 35Gy* group were healthy rats, which only received MRT. Rats from the *Left Irrad w/TU-MRT 35Gy* group contained tumour but radiation was given to the left hemisphere. (Error bars indicate mean standard deviation for: Untreated n=4, “TU-No Radiation” n=4, No TU-MRT 35Gy n=5, tumour bearing rats n=4, “Left Irradiation w/TU–MRT” n=2).



**Figure 16** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium. Explants were taken from the left hemisphere of the brain, while tumour was inoculated into the contralateral side. Homogenous radiation was given to the same right hemisphere. Rats from the untreated group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumours but did not receive radiation. (Error bars indicate mean standard deviation for: Untreated n=4, “TU-No Radiation” n=4, tumour bearing rats n=4).



**Figure 17** - Comparison of reporter clonogenic survival between MRT and homogenous radiation. HPV-G cells were grown in explant-conditioned media. Explants were taken from the non-irradiated left hemisphere of the brain. Tumour was inoculated in the right hemisphere. MRT and Homogenous radiation was given to the same right hemisphere, unless otherwise stated. Rats from the *untreated* group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumour but did not receive radiation. Rats from the *No TU - MRT 35Gy* group were healthy rats, which only received MRT. Rats from the *Left Irrad w/TU-MRT 35Gy* group contained tumour but radiation was given to the left hemisphere. (Error bars indicate mean standard deviation for: Untreated n=4, “TU-No Radiation” n=4, “No TU-MRT 35Gy” n=5, tumour bearing rats n=4, “Left Irradiation w/TU-MRT” n=2).

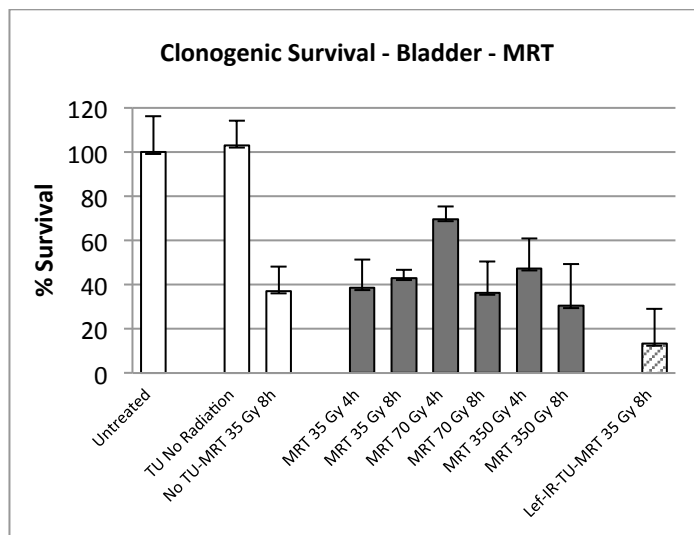
### **5.1.3 Clonogenic survival of reporter cells grown in explant-conditioned medium from bladder of rat brains containing tumour.**

#### **Clonogenic survival after MRT**

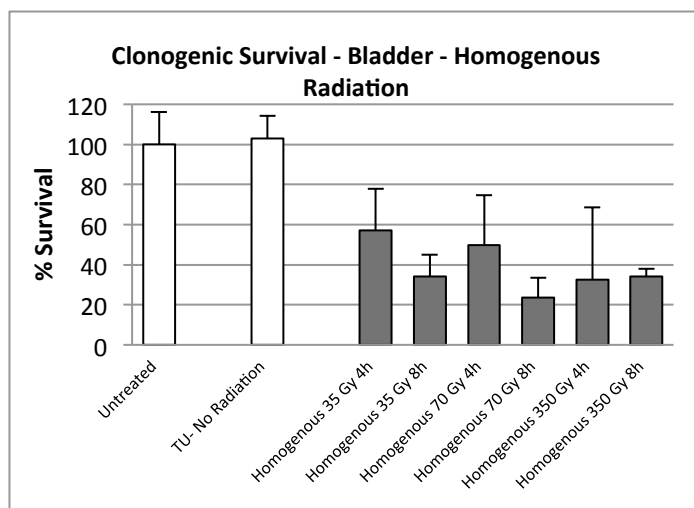
Explant-conditioned medium from bladders generated a decrease in clonogenic survival of reporter cells (Figure 18). Bladders belonged to rats that were both inoculated with tumour cells and exposed to MRT to the right cerebral hemisphere. The pilot experiment involving irradiation of the healthy left hemisphere with tumours in the contralateral side showed approximately 10% of reporter survival. Around 30% clonogenic survival was seen when explant-conditioned medium came from rats exposed to 350Gy of MRT whose brains were dissected 8 hours after irradiation. An average of 40% of survival was observed in 3 treatment groups, MRT 35Gy 4h, MRT 35Gy 8h, and MRT 70Gy 8h. Close to 30% of survival resulted from exposing reporter cells to explant-conditioned medium from MRT 350Gy 4h group. 70% of survival was observed when reporters were exposed to medium derived from explants dissected 4 hours after a 70 Gy MRT dose was delivered to rats.

### **Clonogenic survival after homogenous radiation**

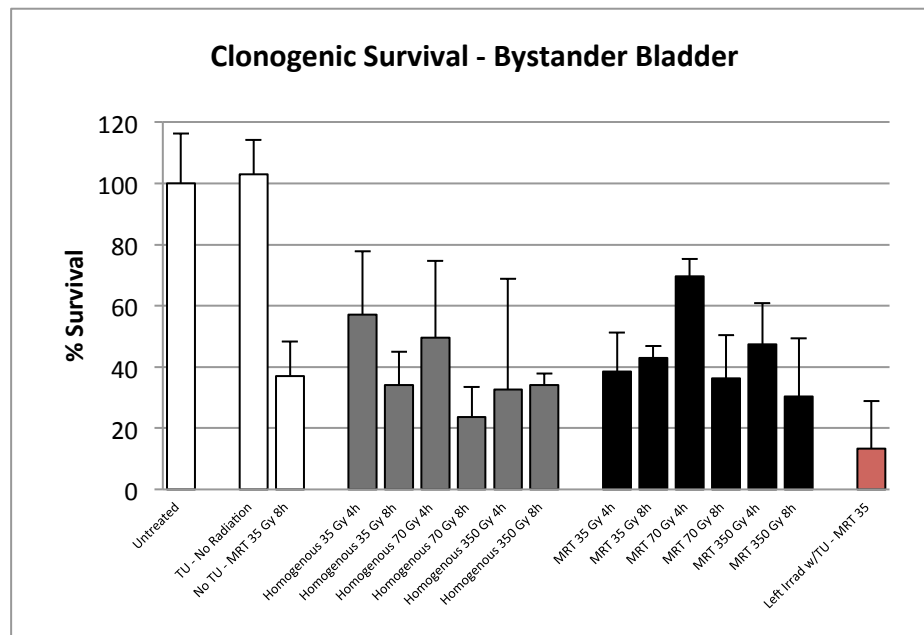
Figure 19 shows the response of reporter cells when they were cultured in a medium transferred from bladder explants, which originally belonged to rats that received MRT to the tumour-bearing right cerebral hemisphere. Close to 20% of survival was observed only when rats from the 70Gy treatment group were dissected 8 hours after exposure. Between 30 to 40% of HPV-G survival was observed in 3 homogenous radiation treatment groups: 35Gy with 8 hours of dissection time, and 35Gy with 4 and 8 hours dissection times. A rate of between 50 and 60% survival was observed when explants were extracted 4 hours after irradiating rats with 35Gy and 70Gy of homogenous radiation. Rats containing tumours that were not exposed to radiation showed 100% of survival.



**Figure 18** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium. Explants were taken from the bladder, which was extracted from rats that received MRT to the tumour-borne right cerebral hemisphere. Rats from the *untreated* group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumours but did not receive radiation. Rats from the *No TU – MRT 35Gy* group were healthy rats, which only received MRT. Rats from the *Left Irrad w/TU-MRT 35Gy* group contained tumour but radiation was given to the left hemisphere. (Error bars indicate mean standard deviation for: Untreated n=4, “TU-No Radiation” n=4, No TU-MRT 35Gy n=5, “Left Irradiation w/TU-MRT” n=2).



**Figure 19** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium. Explants were taken from bladder, which was extracted from rats that received homogenous radiation to the tumour-borne right cerebral hemisphere. Rats from the *untreated* group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumours but did not receive radiation. (Error bars indicate mean standard deviation for: Untreated n=4, “TU-No Radiation” n=4, tumour bearing rats n=4).



**Figure 20** - Comparison of reporter clonogenic survival between MRT and homogenous radiation. Explants were taken from the bladder, which was extracted from rats that received MRT and homogenous radiation to the tumour-borne right cerebral hemisphere, unless otherwise stated. Rats from the *untreated* group received anaesthesia but did not receive radiation. Rats from the *TU-No Radiation* group contained tumour but did not receive radiation. Rats from the *No TU - MRT 35Gy* group were healthy rats, which only received MRT. Rats from the *Left Irrad w/TU-MRT 35Gy* group contained tumour but radiation was given to the left hemisphere. (Error bars indicate mean standard deviation for: Untreated n=4, tumour bearing rats n=4, “TU-No Radiation” n=4, “No TU-MRT 35Gy” n=5, “Left Irradiation w/TU-MRT” n=2).

**Table 9** – Statistical Analysis of Experiment 2

Treatment	Right Cerebral Hemisphere				Left Cerebral Hemisphere				Bladder			
	Mean SF	Standv	t-test	Significant (p<0.05)	Mean SF	Standv	t-test	Significant (p<0.05)	Mean SF	Standv	t-test	Significant (p<0.05)
Untreated	23.75	±3.9	-	-	29.5	±3.3	-	-	33.75	±5.5	-	-
Tumour - No Radiation	25	±3.6	0.325452	No	28.75	±1.7	0.350775	No	34.75	±3.8	0.387218	No
No Tumour - MRT 35 Gy 8h	0.5	±1.0	0.000012	Yes	3.5	±1.3	0.000003	Yes	12.5	±3.8	0.000353	Yes
Homogenous 35 Gy 4h	1.5	±0.6	0.000014	Yes	5.75	±2.1	0.000009	Yes	19.25	±7.0	0.008632	Yes
Homogenous 35 Gy 8h	0.25	±0.5	0.000010	Yes	2.25	±2.1	0.000004	Yes	11.5	±3.7	0.000265	Yes
Homogenous 70 Gy 4h	1	±1.2	0.000014	Yes	3.25	±1.5	0.000003	Yes	16.75	±8.4	0.007426	Yes
Homogenous 70 Gy 8h	0	±0.0	0.000009	Yes	0.75	±1.0	0.000001	Yes	8	±3.3	0.000098	Yes
Homogenous 350 Gy 4h	0	±0.0	0.000009	Yes	3.25	±2.8	0.000009	Yes	11	±12.2	0.007234	Yes
Homogenous 350 Gy 8h	0.25	±0.5	0.000010	Yes	4	±1.8	0.000005	Yes	11.5	±1.3	0.000111	Yes
MRT 35 Gy 4h	1.5	±1.3	0.000017	Yes	6.25	±3.0	0.000023	Yes	13	±4.3	0.000511	Yes
MRT 35 Gy 8h	2.5	±1.9	0.000031	Yes	5.75	±1.0	0.000005	Yes	14.5	±1.3	0.000245	Yes
MRT 70 Gy 4h	1.5	±1.7	0.000022	Yes	5.75	±1.7	0.000007	Yes	23.5	±1.9	0.006259	Yes
MRT 70 Gy 8h	0.5	±0.6	0.000011	Yes	1.5	±1.9	0.000003	Yes	12.25	±4.8	0.000528	Yes
MRT 350 Gy 4h	1.25	±1.5	0.000018	Yes	5	±2.2	0.000008	Yes	16	±4.5	0.001257	Yes
MRT 350 Gy 8h	0.25	±0.5	0.000010	Yes	3	±2.2	0.000005	Yes	10.25	±6.4	0.000709	Yes
Left Irrad - Tumour - MRT 35 Gy 8hr	1	1.2	0.000014	Yes	1.25	1.5	0.000002	Yes	4.5	5.3	0.000127	Yes

Statistical analysis of experiment 2, which shows how significantly different all treatments were compared to the untreated group. The study was performed using a paired t-test analysis.

## 5.2 Discussion

The results from this chapter show that MRT and homogenous radiation delivered to an inoculated-hemispheric brain tumour produced the release of bystander signals, which can affect the unirradiated left cerebral hemisphere and bladder of the same animal. This was confirmed by observing a large decrease in clonogenic reporter cell survival among all of our treatment groups. In our experiments, tumour-bearing rats were irradiated following doses of 35 Gy, 70 Gy and 350 Gy of MRT and homogenous radiation. Rats were then kept alive for 4 and 8 hours and their brains and bladders were extracted and processed as tissue explants. Then explants were cultured over a 24 hours period and the medium was harvested, filtered, and transferred to flask containing HPV-G reporter. Results show that the non-irradiated control groups showed 100% of survival while all the irradiated groups showed a deep decrease in clonogenic survival, which sometime was completely absent.

### **Survival of reporter cells with growth medium from non-irradiated tumour-bearing rats**

In order to assess whether the presence of a tumour in the hemibrain of the animal could affect the survival of the reporters, a group of rats was inoculated



with tumours but left unirradiated. After 8 days of tumour maturation, host rats were killed, their brains and bladders extracted, and their correspondent tissue samples processed as explants for the clonogenic bystander assay. The results showed that the rate of survival remained similar between both the unirradiated tumour-bearing rats and the healthy untreated controls. These results suggest that the reduction in clonogenic survival seen after growing HPV-G cells in explant-conditioned medium from the irradiated rats is exclusively produced by RIBE and, therefore, not attributable to toxic factors that may have been produced by the inoculated C6 tumour cells.

### **Survival of irradiated tumour-bearing rats**

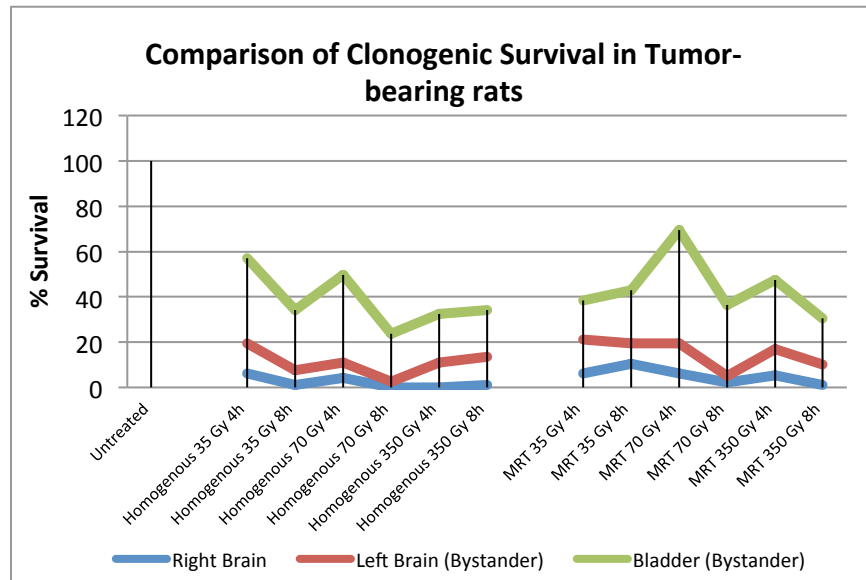
After exposing healthy and tumour-bearing rats to 350 Gy of MRT, no significant differences in survival fraction were observed between the two groups. These findings strongly suggest that the bystander factors may be released within equal amounts by the normal tissue and the inoculated C6 tumour cells. Moreover, a correlation in clonogenic survival, though not significant, was observed in the 35 Gy and 70 Gy homogenous groups depending on their dissection times. According to the above a trend of higher survival was observed when HPV-G cells received medium from explant extracted 4 hours after that homogenous irradiation was delivered compared to an evident lower survival when explants were extracted

after 8 hours (Figure 20). On the other hand, the 350 Gy homogenous groups showed a similar percentage of survival following both dissection times. These results suggest two possible explanations. First, the amount of bystander signals released is time-dependent and increases over time after a homogenous radiosurgical dose has been delivered to the cerebral hemisphere. Second, the production of the bystander factors may have a dose threshold around the 350 Gy of homogenous radiation, after which their release would be independent of time. It is interesting to notice that the same trend was observed in the MRT tumour-bearing rat groups, albeit not significantly. However, in this case, the pattern was seen in the rat groups that received the highest dose of radiation (70 Gy and 350 Gy), while the no difference in survival of reporter cells resulted from killing rats 4 and 8 hours after 35 Gy of MRT was delivered.

### **Contralateral Irradiation**

Extensive work in the last 40 years suggests that direct radiation to a human tumour can strongly affect other tumours in different locations of the body. This phenomenon has been named as abscopal effect<sup>6,103</sup>. In fact, work developed by Antoniades<sup>104</sup> shows that untreated lymph nodes exhibit regression after giving radiotherapy to bigger lymph nodes in subjects with non-Hodgkin's lymphoma. Moreover, work developed by Ohba<sup>105</sup>, shows that untreated

hepatocellular carcinoma displays a regression after bone metastasis radiotherapy. Furthermore, in a 2011 study, Okuma<sup>106</sup> describes a mass regression in untreated lung metastasis after radiotherapy. Non-irradiated tumours reduce their size because they are responding to bystander factors released by the irradiated tumour. This is very important because we are confronted by an unexpected post-radiation neoplastic behaviour that seems to be overall beneficial. However, these findings leave us with the question: how could irradiation of normal tissue affect the bystander response in unirradiated tumours? To assess this question, a pilot experiment was conducted in which rats were inoculated with tumours in the right cerebral hemisphere but received 35 Gy of MRT to the healthy (left) hemisphere. They were then housed and killed 8 hours after irradiation for the tissue extraction. The results showed that tumour-bearing rats whose healthy left hemisphere was irradiated responded with a lower clonogenic survival compared to the group that received direct irradiation to the tumour. Although the bystander signal production increased, it cannot be assumed that the response would be beneficial. In fact, it can only be said that the reporter HPV-G system had a stronger response to the factors.



**Figure 21** - Comparison of clonogenic reporter survival after exposing tumour-bearing rats to MRT and homogenous radiation. Each treatment group includes the percentage of survival resulted from exposing HPV-G cells to the explant-conditioned medium from right brain hemisphere, left brain hemisphere and bladder. (Error bars indicate mean standard deviation for: tumour bearing rats n=4, Untreated n=4)

## Chapter 6

# Communications of Radiation-Induced Bystander Signals in Rats in Vivo

## 6.1 Results

### 6.1.1 Comparison of clonogenic reporter survivals between non-irradiated rats who shared the cage with irradiated rats

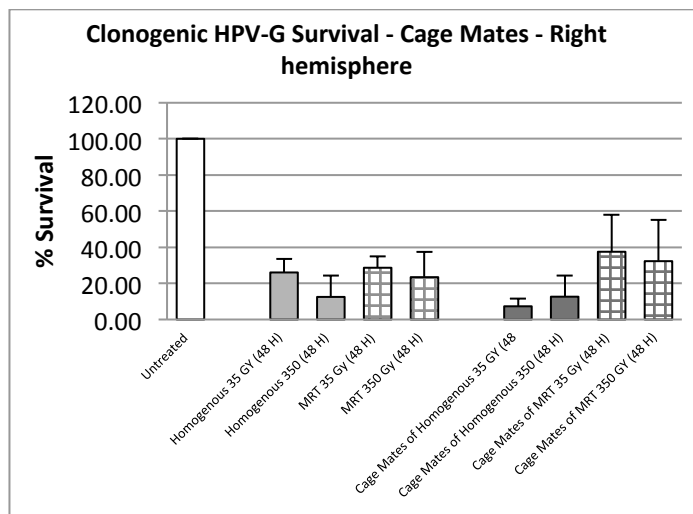
#### Clonogenic Survival after transferring medium from right cerebral hemisphere explants

Clonogenic survival of HPV-G cells showed a significant reduction when reporter cells were exposed to explant conditioned medium from the right cerebral hemisphere of both direct irradiated and cage-mate rats (Figure 22). A roughly 10% survival rate was observed amongst the irradiated rats when the conditioned medium was taken from the 350 Gy homogenous brain tissue explants, close to a 20% survival rate resulted from exposing reporter cells to conditioned medium from the 350 Gy MRT tissue explants, and close to a 30% survival rate was seen in the 35 Gy homogenous and MRT treatment groups. Concerning to the cage-mate rats, less than 20% of survival was observed in the

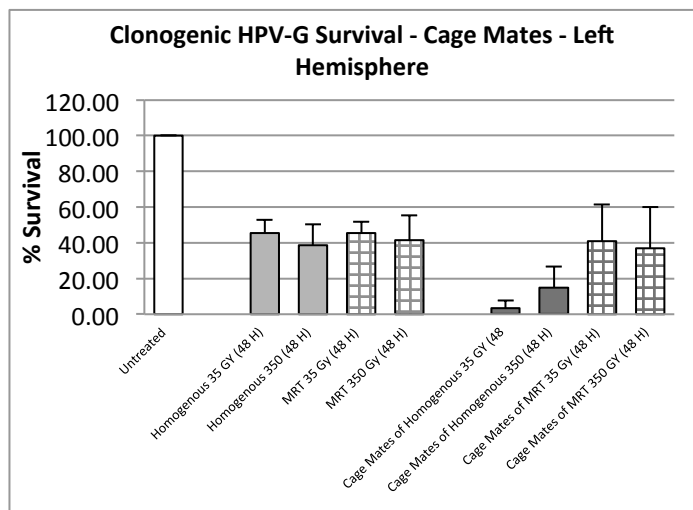
cage-mates of both 35 Gy and 350 Gy homogenous radiation groups, and between 30 to 40% of survival when medium was taken from the 35 Gy and 350 Gy MRT cage mate groups.

### **Clonogenic Survival after transferring medium from non-irradiated left cerebral hemisphere**

Clonogenic survival showed a significant reduction (Table 10) when HPV-G cells were grown in conditioned-explant medium transferred from the non-irradiated left cerebral hemisphere of both direct irradiated and cage-mate rats. The 4 direct irradiated groups showed an average of 40% of survival when medium was taken from explants extracted 48 hours after exposing rats to either MRT or homogenous radiation (Figure 23). The cage-mate survival response was less than 20% with a transferring medium that belonged to both the 35 Gy and 350 Gy homogenous groups, and around of 40% when the medium was taken from the MRT cage-mate groups.



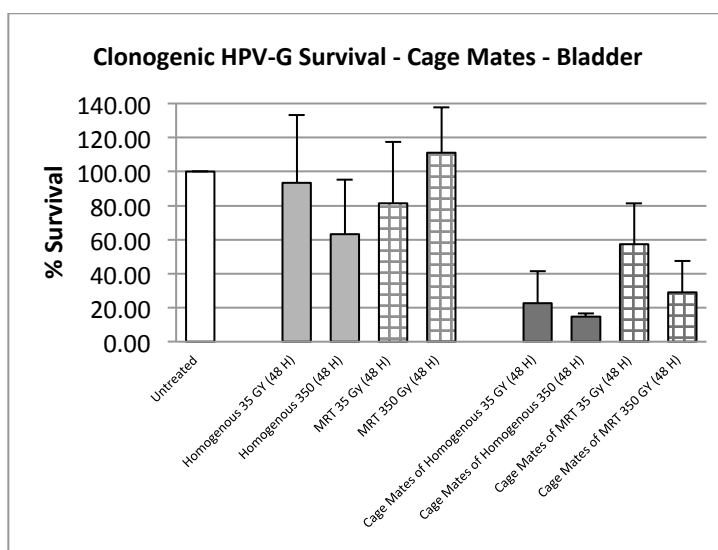
**Figure 22** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium taken from the right cerebral hemisphere of irradiated rats and their non-irradiated cage mates. Irradiated rats were exposed to either MRT or Homogenous Radiation in the left cerebral hemisphere. Non-irradiated rats were placed in the cage containing the irradiated rats during a 48 hours period and then all rats were killed and dissected. (Error bars indicate mean standard deviation for: untreated n=5; MRT and their cage mates n=4; homogenous and their cage mates n=2)



**Figure 23** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium taken from the left cerebral hemisphere of irradiated rats and their non-irradiated cage-mates. Irradiated rats were exposed to either MRT or Homogenous Radiation to the left cerebral hemisphere. Non-irradiated rats were placed in the cage containing the irradiated rats during a 48 hours period and then all rats were killed and dissected. (Error bars indicate mean standard deviation for: untreated n=5; MRT and their cage-mates n=4; homogenous and their cage mates n=2)

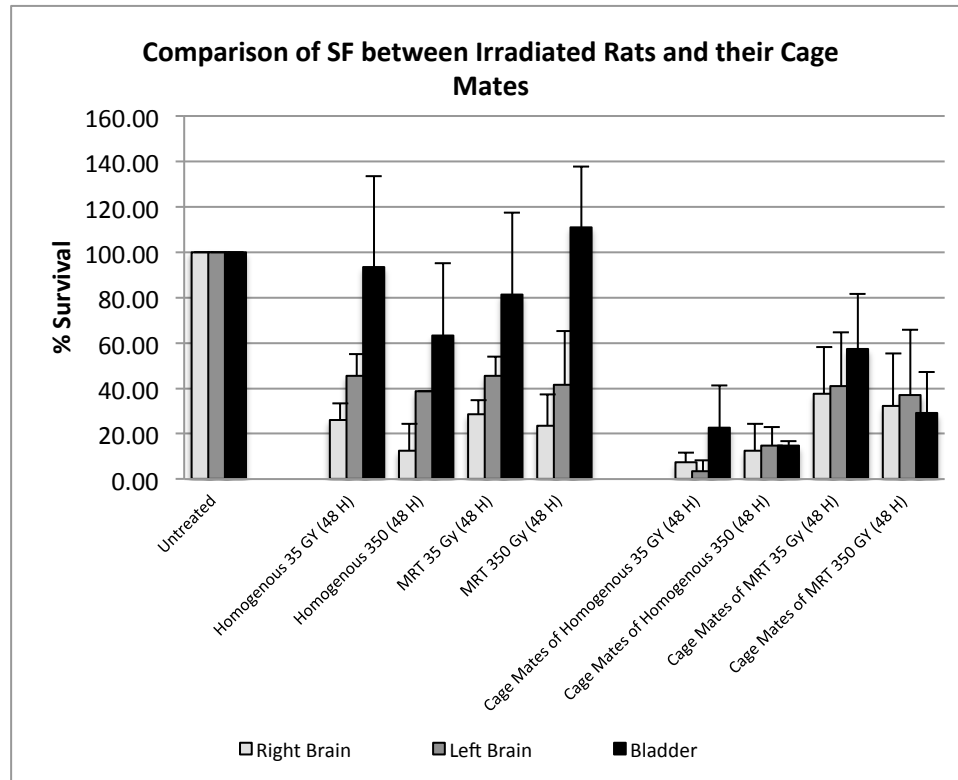
### Clonogenic Survival after transferring medium from bladder tissue explants

Reporters between the direct-irradiated and the cage-mate groups showed a varied reduction of clonogenic survival (Figure 24). Within the direct-irradiated groups, survival ranged from 60 to 110% with the 350Gy homogenous groups showing the lowest survival and the 350Gy MRT group showing the highest. Within the unirradiated cage-mates, an average of 20% survival was observed in both the 35Gy and the 350 Gy homogenous groups, and 30% and 60% survival was observed in the 350Gy and 35Gy MRT cage-mate groups.



**Figure 24** - Clonogenic survival of HPV-G cells grown in explant-conditioned medium taken from the bladder of irradiated rats and their non-irradiated cage-mates. Irradiated rats were exposed to either MRT or Homogenous Radiation to the left cerebral hemisphere. Non-irradiated rats were placed in the cage containing the irradiated rats during a 48 hour period and then all rats were killed and dissected. (Error bars indicate mean standard deviation for: untreated n=5; MRT and their cage mates n=4; homogenous and their cage mates n=2)





**Figure 25** - Comparison of reporter clonogenic survival between all the explant organs. Rats from the *untreated* group received anaesthesia but did not received radiation. Irradiated rats were exposed to either MRT or Homogenous Radiation in the left cerebral hemisphere. Non-irradiated rats were placed in the cage containing the irradiated rats during a 48 hours period and then all rats were killed and dissected. (Error bars indicate mean standard deviation for: untreated n=5; MRT and their cage-mates n=4; homogenous and their cage mates n=2)

**Table 10 – Statistical Analysis of direct irradiated treatment groups and their cage mates**

Treatment	Left Cerebral Hemisphere				Right Cerebral Hemisphere				Bladder			
	Mean SF	Standv	t-test	Significant (p<0.05)	Mean SF	Standv	t-test	Significant (p<0.05)	Mean SF	Standv	t-test	Significant (p<0.05)
Untreated	45.2	±3.3	-	-	44.2	±7.8	-	-	87.4	±8.7	-	-
Homogenous 35 Gy (48 H)	12.5	±3.5	0.000043	Yes	20.0	±4.2	0.005058	Yes	99.0	±42.4	0.264423	No
MRT 35 Gy (48 H)	13.8	±3.0	0.000001	Yes	20.0	±3.7	0.000377	Yes	86.3	±38.3	0.474583	No
Homogenous 350 (48 H)	6.0	±5.7	0.000036	Yes	17.0	±0.0	0.002706	Yes	67.0	±33.9	0.106025	No
MRT 350 Gy (48 H)	11.3	±6.7	0.000010	Yes	18.3	±10.4	0.001792	Yes	117.8	±28.4	0.027725	Yes
Cage Mates of MRT 35 Gy (48 H)	18.0	±9.9	0.000322	Yes	18.0	±10.5	0.001713	Yes	60.8	±25.7	0.031714	Yes
Cage Mates of MRT 350 Gy (48 H)	15.5	±11.0	0.000336	Yes	16.3	±12.7	0.002275	Yes	30.8	±19.4	0.000297	Yes
Cage Mates of Homogenous 35 Gy (48 H)	3.5	±2.1	0.000009	Yes	1.5	±2.1	0.000381	Yes	24.0	±19.8	0.000673	Yes
Cage Mates of Homogenous 350 (48 H)	6.0	±5.7	0.000036	Yes	6.5	±3.5	0.000725	Yes	15.5	±2.1	0.000054	Yes

Statistical analysis of experiment 3 showing how significantly different the treatments are compared to the untreated group. The study was performed using a paired t-test analysis.

## 6.2 Discussion

The results indicate that a single exposure of radiosurgical doses of MRT and homogenous radiation to one cerebral hemisphere generated the release of signals that altered the biological response in non-irradiated HPV-G cells. Moreover, these data confirm the communication of bystander factors from the irradiated rats to the brain and bladder of completely non-irradiated rats.

### **Radiation induced-bystander effect in-vivo**

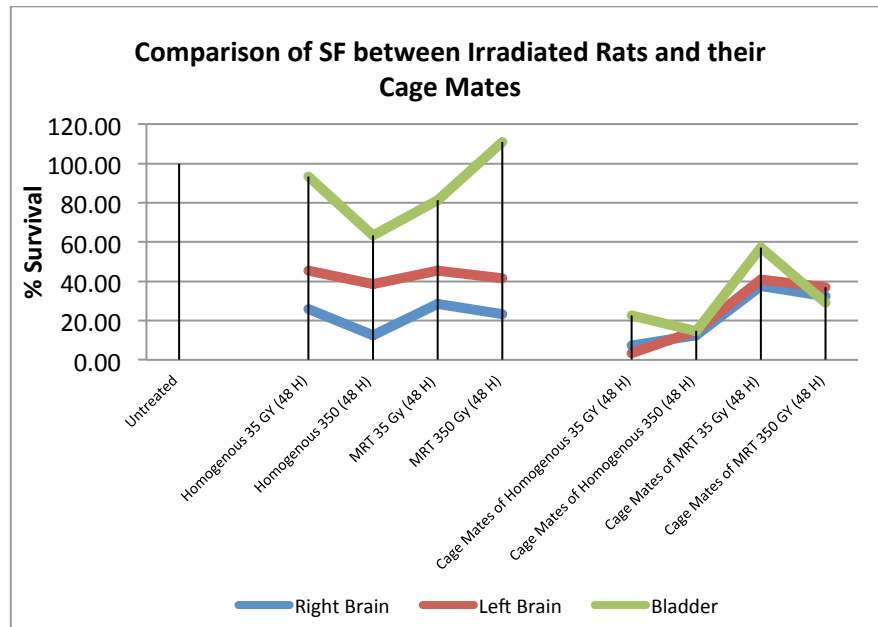
The results presented in this chapter show that bystander signals are produced *in-vivo* in rats after controlled radiosurgical doses of MRT and homogenous radiation were delivered to their right cerebral hemispheres. Confirmations of bystander responses were achieved by observing a decrease in clonogenic survival of HPV-G cells in all our treatment groups (Figures 22, 23, and 24). This is correlated by previous studies done by Mothersill<sup>52</sup> in which soluble factors presented in medium had the capacity to cause death in reporter recipient cells *in-vitro*. Moreover, first Mothersill<sup>71</sup> and then O'Dowd<sup>101</sup>, showed that bystander signals were produced *in-vivo* after irradiating mice and rainbow trout respectively. Our results clearly show that explant-conditioned media from the right and left cerebral hemispheres of direct irradiated rats significantly reduced the survival of reporters. A rate of less than 40% survival was observed

when explant-conditioned medium was harvested from the right cerebral hemisphere and of less than 50% survival when extracted from the left cerebral hemisphere. On the contrary, as shown in Table 10, the medium extracted from bladder explants produced significant results in only one treatment group. Although there are not significant differences between both radiation modalities, there seems to be a correlation between the dose and the clonogenic survival of the homogenous group. Evidently, as is shown in Figure 23 and Figure 24, 350 Gy of homogenous radiation produced a stronger reduction of survival than the 35 Gy group in both cerebral hemispheres and bladder. MRT groups, on the other hand, did not show that correlation. Furthermore, work performed by Mothersill<sup>86</sup> shows that whilst adult fish no longer produce bystander factors 6 hours after irradiation, fish irradiated in early life-stages continue producing signals during their entire life span. Our experiments showed a slightly different trend, in which adult rats conserved their capacity until at least 48 hours post-irradiation. The questions now involve whether rats have a prolonged capacity of signal-production, and whether their capacity could be attributed to all mammals—including humans.

### **Communications of bystander signals *in-vivo***

Non-irradiated rats placed in the same cage as irradiated rats over a 48-hour period showed a significant reduction in clonogenic reporter survival (Table 10). This trial experiment was performed in order to observe if the transmission of bystander signals could be possible within the same animal model. Previous studies done by Mothersill<sup>85,97</sup> showed that direct irradiated rainbow trout and zebrafish released signals in the surrounding water that affected non-irradiated fish. Moreover, work developed by Isaeva<sup>107</sup>, showed that irradiated mice induced immunosuppression in non-irradiated mice of various genotypes. Our results show that all cage-mate groups showed a significant decrease in clonogenic reporter survival. Furthermore, the cage-mate response seems to be related to the radiation modality but independent of the radiosurgical dose. In fact, as is shown in Figure 25, the cage-mates of the homogenous groups showed a higher decrease in survival compared to the cage-mates of the MRT group, suggesting that as we increase the irradiated tissue volume, we also increase the effect in cage-mate animals. These findings are particularly interesting because they make us wonder whether non-irradiated rats are more likely to detect these signals and therefore more sensitive to their effects, or whether once signals are detected, rats start their own bystander signal machinery that would enhance the final effect. A trend of survival can be easily observed in Figure 26 following the direct irradiated group, in which survival increases as we increase the distance

from the irradiated right hemisphere. On the contrary, the cage-mate clonogenic survival seems to respond equally in all organs but are dependent on dose and type of irradiation. These findings suggest that the production of the signal(s) in the irradiated rat is directly related to the distance between the organ involved in the bystander signal production and the radiation-target organ. Thus, once the factor(s) is/are expelled from the animal the systemic response in the non-irradiated rat may be a more complicated process, which could be related to the amount of signal intake. According to Mothersill, the signal(s) seem(s) to be both stable and soluble in water, which is confirmed in part by our results. Therefore, we could assume that the bystander factor(s) may be released through the urine. If we accept this assumption, the intake of the signal(s) by the non-irradiated rats may be as result of two mechanisms. First, the signals may be ingested through the gastrointestinal system as a result of rats grooming to each other as part of their social behavior; and second, the signal contained in the urine, as chemical compound, may be volatile, which would suggest that the intake of the factor(s) would be through the olfactory system. Consequently, these two mechanisms could either be independent or codependent and further studies are needed in order to confirm this hypothesis.



**Figure 26** - Comparison of clonogenic reporter survival between irradiated rats and their cage-mates. Each treatment group includes the percentage of survival resulted from exposing HPV-G cells to the medium from right brain hemisphere, left brain hemisphere and bladder. (Error bars indicate mean standard deviation for: untreated n=5; MRT and their cage mates n=4; homogenous and their cage mates n=2)

## Chapter 7

# Thesis Discussion, Future Work and Conclusion

The data in this thesis provides the first evidence that radiation-induced bystander effects are produced in Wistar rats *in-vivo* after delivering high radiosurgical doses of synchrotron radiation. In addition to that, we also are the pioneers in showing that bystander signals can be communicated between mammals. Our results are supported by previous work developed by Mothersill and O'Down<sup>71,85,86,101,108</sup> in which mice and fish were exposed to a whole body dose of radiation and signals were produced that affected unirradiated animals. This concept is corroborated by our findings; with the exception that synchrotron radiation was delivered specifically to the right cerebral hemisphere under radiosurgical doses of 17.5, 35, 70, and 350 grays.

Healthy and tumour-bearing Wistar rats were irradiated using two different beam modalities in order to study if bystander effects can be enhanced by the geometry of the radiation beam. The results showed that no significant differences in bystander signal production was observed between MRT and homogenous radiation when rats were kept alive 4, 8 and 48 hours after radiation exposure. The introduction of a tumour into our study model did not affect the production of bystander signals either. However, when it comes to the communication of bystander signals between rats, homogenous radiation



seems to produce a higher response in non-irradiated rats compared to the response induced by rats that received MRT.

Concerning to the production of bystander signals within each animal, our data suggests that the direct-irradiated tissue (right cerebral hemisphere) produces the highest amount of bystander factors. In addition, the production of bystander signals by the non-irradiated organs (left brain and bladder) decreases as we increase the distance from the radiation target. On the contrary, it is interesting to notice that when bystander factors are communicated to unirradiated rats, the production of bystander signals remains constant no matter if the brain or bladder are studied. Additionally, when analysing the clonogenic survival from bladder it is interesting to notice the marked error bars in all the charts. The author suggests that the cause may be the amount of urine that bladders were holding at the moment of tissue explant culture. Therefore, the introduction of urine in the media may induce a distinct apoptotic response in the reporter cells that is reflected in our charts.

Immunohistochemical analysis did not show conclusive data. However, this is the first step to develop a new technique to validate the detection of bystander proteomes in rat brains. In addition, it gave us a positive insight to know where we are standing on, in term of validating the bystander proteome.

Although the presence of radiation-induced bystander effects has been extensively studied *in-vitro*, this is the first time that bystander effects are produced after high doses of

radiation exposure. This suggests that the cellular response within an organism allows completely different cell behaviour and that the bystander effect may be a generalized response at the level of organism. Additional research needs to be done in order to decode the complexity of the mechanism involved in the production and transmission of bystander signals *in-vivo*. Moreover, it is imperative to find out what the extent of the communication of bystander signals to unirradiated animals is, and whether it is a protective or adverse innate response.

In a nutshell, the work showed in this thesis gives further evidence that bystander effects are produced in rats *in-vivo*, and challenges the current understanding about the transmission of bystander effects to unirradiated organisms within an animal group.

Major concerns are now whether the communication of bystander signals observed in rats could find analogies in other species including humans because if this is possible the impact in health and environmental regulations could be critical in terms of increasing awareness about non-targeted effects of radiation.

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