

**Development and use of an
adoptive transfer method for
detecting radiation-induced
bystander effects *in vivo***

A thesis submitted in fulfilment of the requirements of the

Doctor of Philosophy

Benjamin John Blyth, B. Biotech (Hons)

March 2009

Department of Haematology and Genetic Pathology
School of Medicine, Faculty of Health Sciences
Flinders University

Contents

FIGURES	III
TABLES	V
SUMMARY	VI
CANDIDATE'S DECLARATION	IX
ACKNOWLEDGEMENTS.....	X
ABBREVIATIONS	XII
PUBLICATIONS & ABSTRACTS ARISING DURING CANDIDATURE	XIII
CHAPTER 1: PROBLEM STATEMENT.....	1
Problem Statement.....	1
Aim and Scope	1
Overview of the Study	2
CHAPTER 2: INTRODUCTION AND BACKGROUND	4
Ionising radiation	4
The radiation risk assessment paradigm	6
Radiation-induced bystander effects	15
Bystander effects: moving from phenomenon to risk	40
Summary	44
CHAPTER 3: RESEARCH INTENT AND DESIGN	45
Précis	45
Research questions	45
Research method and hypotheses.....	46
Summary	49
CHAPTER 4: DEVELOPMENT OF THE ADOPTIVE TRANSFER METHOD FOR DETECTING BYSTANDER EFFECTS <i>IN VIVO</i>	50
Précis	50
Initial decisions in the development of the <i>in vivo</i> bystander method	50
Donor splenocytes: Chronic radiolabelling experiments	59
Donor splenocytes: Acute X-irradiation	90
Recipient Mice	95

Pilot adoptive transfer experiments	99
Analysis of recipient mouse spleen tissues.....	105
Biological endpoints as candidates for induction in bystander cells	116
Experimental design	139
CHAPTER 5: USE OF THE ADOPTIVE TRANSFER METHOD TO STUDY BYSTANDER EFFECTS <i>IN VIVO</i>	143
Précis	143
Detection of bystander effects from chronically irradiated lymphocytes lodged in unirradiated mouse spleens	144
Detection of bystander effects from increased numbers of chronically irradiated lymphocytes	157
Detection of bystander effects from chronically irradiated lymphocytes after longer-term lodging <i>in vivo</i>	164
Detection of bystander effects from high dose-rate chronically irradiated lymphocytes	174
Detection of bystander effects from splenocytes exposed <i>ex vivo</i> to X-radiation.....	178
Summary	181
CHAPTER 6: EVALUATION OF THE ADOPTIVE TRANSFER BYSTANDER METHOD AND ITS INITIAL FINDINGS	184
Précis	184
Assessing the research findings	184
Suitability of the adoptive transfer method	186
Reliability of the adoptive transfer method	198
Generalisability of the results obtained using the adoptive transfer method.....	216
Significance and implications of not detecting a bystander effect <i>in vivo</i>	221
Recent developments	240
Conclusions.....	244
REFERENCES	248

Figures

Figure 4.1:	Starting method for chronic radiolabelling experiments.....	59
Figure 4.2:	Proliferative response to concanavalin A in donor splenocytes	64
Figure 4.3:	Viable cell numbers of donor T lymphocytes after ConA stimulation.....	71
Figure 4.4:	Cell-cycle progression of donor T lymphocytes after ConA stimulation	72
Figure 4.5:	Effect of cell density on donor cell growth in culture	73
Figure 4.6:	Effect of atmospheric CO ₂ on donor cell growth in culture	74
Figure 4.7:	Representative growth of donor cells in culture	75
Figure 4.8:	Incorporation of BrdU into donor T cells	78
Figure 4.9:	³ H-thymidine incorporation into donor cells	81
Figure 4.10:	CMRA labelling of donor cells.....	87
Figure 4.11:	Final protocol for the chronic radiolabelling adoptive transfer method	89
Figure 4.12:	Initial strategy for the acute irradiation adoptive transfer method	90
Figure 4.13:	Final protocol for the acute irradiation adoptive transfer method	94
Figure 4.14:	Pre-warming of cages to dilate mouse tail veins.....	97
Figure 4.15:	Recipient mouse tail vein injection setup.....	97
Figure 4.16:	Radiolabelling and fluorescent labelling of donor cells prior to adoptive transfer.....	100
Figure 4.17:	³ H-thymidine radiolabelled donor cells identified lodged in recipient mouse spleen.....	101
Figure 4.18:	Locations of donor cell lodging in representative spleen section.....	102
Figure 4.19:	Z-stack projection of 3-dimensional cluster of lodging donor cells	104
Figure 4.20:	Representative global field.....	111
Figure 4.21:	Area surveyed in 20 representative global fields	112
Figure 4.22:	Donor cells surveyed in 25 representative local fields	114
Figure 4.23:	Region included in the area around donor cells in a representative local field.....	115
Figure 4.24:	Pseudo-coloured overlay of a local screening field from TUNEL-stained spleen section	121
Figure 4.25:	Fragmented TUNEL-stained cell nucleus	123
Figure 4.26:	Phagocytic cell and ingested TUNEL-positive debris	124
Figure 4.27:	Manual apoptosis scoring in local screening field from TUNEL-stained spleen section	125
Figure 4.28:	Estimating total number of cells per field	126
Figure 4.29:	Pseudo-coloured overlay of a local screening field from Ki-67 assay.....	131
Figure 4.30:	Levels of Ki-67 staining in local fields.....	132
Figure 4.31:	Automatic proliferation scoring in local screening field from Ki-67 stained spleen section	133

Figure 4.32:	pKZ1-encoded β -galactosidase activity detected in X-gal stained spleen sections	138
Figure 4.33:	Workflow for adoptive transfer experiments	141
Figure 4.34:	Data analysis workflow.....	142
Figure 5.1:	Donor cell lodging frequencies in the spleens of recipient mice.....	146
Figure 5.2:	Local and global apoptosis frequencies in mice receiving sham-radiolabelled or radiolabelled donor cells	148
Figure 5.3:	Local and global proliferation indices in mice receiving sham-radiolabelled or radiolabelled donor cells	151
Figure 5.4:	Very high levels of Ki-67 staining in Mouse #44	153
Figure 5.5:	Apoptosis and proliferation within mice receiving radiolabelled donor cells in local fields with only ^3H -negative donor cells, versus fields with confirmed ^3H -positive donor cells	155
Figure 5.6:	Lodging patterns in spleens of mice receiving 5 or 50×10^5 donor cells	159
Figure 5.7:	pKZ1 chromosomal inversion frequencies in mice receiving sham-radiolabelled or radiolabelled donor cells	161
Figure 5.8:	Effect of donor cell lodging density on proliferation index in global fields.....	162
Figure 5.9:	Equivalent donor cell radiolabelling in duplicate trials.....	168
Figure 5.10:	Correlation between apoptosis frequency and donor cell lodging frequency for analysis of spleen tissues at one or three days.....	172
Figure 5.11:	Donor cell lodging frequencies in the spleens of recipient mice.....	180
Figure 6.1:	Variation in bystander apoptosis and proliferation of mice receiving sham-irradiated cells.....	206
Figure 6.2:	Model of ^3H decay and accumulated dose over 22 hours at one disintegration per hour	227
Figure 6.3:	Model of time elapsed since last tritium disintegration at an average exposure rate of 1 disintegration per hour	237

Tables

Table 2.1:	Summary of protein expression changes in bystander cells	38
Table 5.1:	Number of mice used in the initial three experiments using the adoptive transfer method.....	145
Table 5.2:	Ki-67 status of radiolabelled and non-radiolabelled lodged donor cells in mice receiving radiolabelled donor cell preparations	147
Table 5.3:	Summary of results from local and global screening of apoptosis	149
Table 5.4:	Summary of results from local and global screening of proliferation.....	152
Table 5.5:	Summary of results from global screening of apoptosis, proliferation and pKZ1 inversions	160
Table 5.6:	Summary of local and global screening results for apoptosis and proliferation	170
Table 5.7:	Correlations between number of donor cells and proliferation/apoptosis in local and global fields.....	170
Table 5.8:	Correlations between donor cell lodging frequency and global and local apoptosis frequencies	171
Table 5.9:	Summary of global screening results for apoptosis and proliferation.....	177
Table 5.10:	Summary of local and global screening results for apoptosis and proliferation	179
Table 5.11:	Summary of experimental conditions.....	182
Table 5.12:	Effect sizes for apoptosis and proliferation across the series of experiments conducted to detect a radiation-induced bystander effect <i>in vivo</i>	183
Table 6.1:	Number of bystander cells and fields scored in the local screens	203
Table 6.2:	Number of bystander cells and fields scored in the global screens	203
Table 6.3:	Predicted sample sizes based on set prospective statistical power	209
Table 6.4:	95% confidence limits on effect sizes	211
Table 6.5:	95% confidence limits on effect sizes, Acute X-ray Experiment	214
Table 6.6:	Bystander effect experiments conducted <i>in vitro</i> with low-LET radiations	225

Summary

Ionising radiation can cause damage to DNA that can result in gene mutations contributing to carcinogenesis. Radiation-protection policy currently estimates cancer risks from exposures to radiation in terms of excess risk per unit dose. At very low radiation dose-rates, where not all cells are absorbing radiation energy, this formula carries the inherent assumption that risk is limited to those cells receiving direct energy depositions. Numerous studies have now called this assumption into question. Such low dose-rates are in the relevant range that the public receives from natural background and man-made sources, and, if this fundamental assumption proves unfounded, current estimations of radiation-induced cancer risk at low doses will be incorrect. Accurate predictions of stochastic cancer risks from low-dose radiation exposures are crucial to evaluating the safety of radiation-based technologies for industry, power generation and the increasing use of radiation for medical diagnostic and screening purposes.

This thesis explores phenomena known as radiation-induced bystander effects. The term bystander effects, as used here, describes biological responses to ionising radiation (hitherto observed *in vitro*) in cells not directly traversed by an ionising track, due to intercellular signals received from neighbouring cells that did receive energy depositions. This study aimed to determine whether radiation effects are communicated between irradiated and unirradiated cells *in vivo*, and if so, whether this effect alters current estimations of cancer risk following low-dose radiation exposures. In order to answer these questions, an *in vivo* experimental system for studying bystander effects in mice was developed. The method was based on the adoptive transfer of irradiated splenocytes into unirradiated hosts with simultaneous

identification of irradiated donor cells, and biological endpoints in unirradiated bystander cells *in situ* using fluorescence microscopy and image analysis.

Splenocytes from donor mice were radiolabelled with ^3H -thymidine or received an acute X-ray dose. The irradiated donor cells, labelled with a fluorescent probe, were then adoptively transferred into unirradiated recipient mice via the tail vein, whilst control mice received sham-irradiated donor cells. A proportion of the cells lodged in the recipient mouse spleens where they remained for a period before the tissues were cryopreserved. The locations of donor cells were identified in frozen spleen sections by the fluorescent probe, and the levels of apoptosis and proliferation were simultaneously evaluated *in situ* in the surrounding unirradiated bystander cells using fluorescence-based assays. Transgenic pKZ1 recipient mice were also used to quantify chromosomal inversions in bystander cells. Since three-dimensional spatial relationships were preserved, responses could be measured in the local area surrounding irradiated cells as well as further afield. Following the development of the irradiated-cell adoptive transfer protocol and validation of the sensitivity and reproducibility of the biological assays *in situ*, a series of experiments was performed. In the initial experiments, 5×10^5 radiolabelled cells ($0.33 \text{ mBq}\cdot\text{cell}^{-1}$) were injected into recipient mice and the spleen tissues were isolated 22 h later. No changes in apoptosis or proliferation were detected in local bystander spleen cells or throughout the spleen, compared to mice receiving sham-radiolabelled donor cells. In subsequent experiments, the effects of a number of experimental conditions were explored including the injection of tenfold more donor cells, analysis of spleen tissues after three days lodging *in vivo*, radiolabelling of donor cells with 100-fold higher ^3H dose-rate and irradiation of donor cells *ex vivo* with 0.1 or 1 Gy X-rays. In each case, no changes in apoptosis or proliferation were observed.

The *in vivo* method described here was designed to simulate the conditions of a bystander scenario from low dose-rate exposures relevant to public radiation protection. Contrary to the many reports of bystander effects *in vitro*, experiments using this sensitive method for examining the *in vivo* responses of unirradiated cells to neighbouring low-dose irradiated cells, have so far shown no changes in bystander cells in the spleen. This adoptive transfer method is the first *in vivo* method for examining the effects of known irradiated cells exposed to low radiation doses at low dose-rates, on neighbouring cells *in situ* that are truly unirradiated. Both the irradiated and bystander cells are normal, non-transformed primary spleen cells functioning in their natural environment. This *in vivo* experimental system allows the examination of tens of thousands of bystander cells and has shown a remarkable sensitivity, with statistical power to rule out changes in apoptosis >10% from the control.

The relevance of *in vitro* bystander findings is unclear. Many reported bystander effects are more analogous to the systemic communication of abscopal effects from highly irradiated tissues. Disagreement between experimental systems and difficulty in reproducing key results between laboratories further complicate the translation of bystander data *in vitro* to human risk-estimation. The radiation protection community has expressed its need for *in vivo* validation of the bystander phenomenon before it can be included into the appraisal of carcinogenic risk. This adoptive transfer method is now available to study a range of bystander endpoints and potential signalling mechanisms *in vivo*, and provides a way to translate the wealth of data previously collected *in vitro* into findings directly relevant to human risk-estimation.

Candidate's Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Benjamin Blyth

Acknowledgements

I would like to pay principal acknowledgement to Associate Professor Pam Sykes for her superb supervision of the project, my PhD candidature and the laboratory. Pam's concern for my project, career, professional development in science and teaching, as well as my personal life, provided the perfect role model for an all-round mentor and advisor. I am indebted to her for teaching me to challenge my assumptions, for reminding me to always keep-in-mind the reason why you are doing an experiment, and for letting me make and learn from my mistakes. I would also like to thank my co-supervisor Dr David Turner and my assessor Dr Keryn Williams for their assistance, advice and perspective at various points throughout my candidature.

Acknowledgement goes to all past and present members of the Department of Haematology and Genetic Pathology for assistance with experiments, for advice and for their companionship. In particular, I wish to directly acknowledge Dr Tony Hooker, Dr Tanya Day, Dr Rebecca Ormsby, Monica Dreimanis, Ami-Louise Cochrane and Alex Staudacher; and all members of the laboratory who have assisted with the animal experiments. I further acknowledge Dr Edouard I Azzam and Dr Roger W Howell from the University of Medicine and Dentistry, New Jersey for their contributions to the inspiration and initial design of the adoptive transfer method described in this thesis. Thanks also goes to the Adelaide Radiotherapy Centre (Flinders Private Hospital) for assistance with the *ex vivo* irradiations; Flinders Microscopy and Image Analysis Facility for technical support; and, the Flinders Medical Centre Animal House for care and maintenance of the mouse colonies. Funding for the work presented in this thesis was provided by the Low Dose Radiation Research Program, Biological and Environmental Research, US Department of Energy (Grant #DE-FG02-05ER64104). Assistance for conference travel was also received from Flinders University and the Australian Institute for Nuclear Science and Engineering.

Personal thanks go first to my wife Alyce, who selflessly agreed to share me with my PhD thesis during our first six months of marriage, and whose patience, love, understanding and assistance enabled me to perform at my best. Special thanks to my best friend Andrew, who shared much of my PhD candidature with me as my housemate, confidante and companion during late nights of image analysis and statistics. Thanks to all my friends and family whose company, distraction and support kept me going over the years.

Most warm thanks go to my parents, whose example throughout my life has inspired me to work hard and always put in my best effort, who made sacrifices to give me opportunities to learn and mature, and who found a way to love, support and encourage me from thousands of kilometres away (with prayers overcoming the distance when I needed them most).

Final thanks go to my Dad, whose curiosity and fascination with the world inspires my love of science; and to whom I dedicate this thesis.

Abbreviations

BrdU	Bromodeoxyuridine
CHO	Chinese hamster ovary
CMRA	CellTracker™ Orange CMRA
ConA	Concanavalin A
DAPI	4',6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dUTP	Deoxyuridine triphosphate
FITC	Fluorescein isothiocyanate
GJIC	Gap-junctional intercellular communication
HPRT	Hypoxanthine-Guanine Phosphoribosyl Transferase
ICCM	Irradiated cell-conditioned medium
ICRP	International Commission on Radiological Protection
LD ₅₀	Lethal dose (50%)
LET	Linear energy transfer
LNT	Linear no-threshold
LPS	Lipopolysaccharide
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
ROS	Reactive oxidative species
RPMI 1640	Rose Park Memorial Institute cell culture medium #1640
SCE	Sister chromatid exchange
SCM	Splenocyte culture medium
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside
γ-H2AX	γ-variant of histone H2AX

Publications & abstracts arising during PhD candidature

Publications

Zeng, G, Day, TK, Hooker, AM, Blyth, BJ, Bhat, M, Tilley, WD and Sykes, PJ. 2006. "Non-linear chromosomal inversion response in prostate after low dose X-radiation exposure." *Mutation Research* 602(1-2): 65-73.

Hooker, AM, Grdina, D, Murley, J, Blyth, BJ, Ormsby, R, Bezak, E, Giam, K and Sykes, PJ. 2009. "Low doses of amifostine protect from chromosomal inversions in spleen *in vivo* when administered after an occupationally relevant X-radiation dose." *International Journal of Low Radiation* 6(2).

Publications in preparation

Blyth, BJ, Azzam, EI, Howell, RW and Sykes, PJ. "A Novel *in vivo* Method to Detect Radiation-Induced Bystander Effects in Normal Mouse Spleen"

Blyth, BJ, Ormsby, RJ, Staudacher, AH, Dreimanis, M and Sykes, PJ. "Chronic low-dose irradiation from incorporated radionuclides does not alter the fate of bystander cells in mouse spleen"

Oral Presentations

'Identifying the fate of low dose irradiated cells *in vivo*' BJ Blyth & PJ Sykes, *Radiation 2006- Australian Institute for Nuclear Science and Engineering*, Sydney, Australia (April, 2006)

'Can bystander signalling really change the carcinogenic risk of unirradiated cells *in vivo*?' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *DOE Low Dose Radiation Research Investigators' Workshop VII*, Washington DC, USA (January, 2008)

'Bystander Effects: A Risk or an Opportunity?' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *Modelling of Tumours Meeting*, Adelaide, Australia (June, 2008)

'Determining the impact of radiation-induced bystander effects on low dose radiation protection *in vivo*' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *Australian Radiation Protection Society Conference*, Canberra, Australia (September, 2008)

'Bystander Signalling *In Vivo*: Rising above the noise' BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *LOWRAD 2008*, Lisbon, Portugal (November, 2008)

Poster Presentations

'Identifying Non-linear Radiation Dose Responses *In Vivo*: Exploring Bystander Effects' BJ Blyth, TK Day, PJ Sykes, *BELLE Conference*, Amherst, USA (May, 2006)

'Low dose radiation exposure: exploring bystander effects *in vivo*' BJ Blyth & PJ Sykes, *DOE Low Dose Radiation Research Investigators' Workshop VII*, Washington DC, USA (August, 2006)

'An *in vivo* model for detecting radiation-induced bystander effects: shedding light on tissue responses to low dose radiation' BJ Blyth & PJ Sykes, *Australian Society for Medical Research SA Scientific Meeting*, Adelaide, Australia (June, 2007)

‘Low dose radiation-induced bystander effects in the spleen’ BJ Blyth, EI Azzam, RW Howell & PJ Sykes, *International Conference of Radiation Research*, San Francisco, USA (July, 2007)

‘Low dose radiation-induced bystander effects in the spleen’ BJ Blyth & PJ Sykes, *Conference on the Normal Tissue Radiation Effects*, Las Vegas, USA (July, 2007)

‘Determining cancer risks after low-dose radiation exposures’ BJ Blyth, AL Cochrane & PJ Sykes, *Lorne Cancer Conference*, Lorne, Australia (February, 2008)

‘Studying intercellular signalling after low dose radiation exposures *in vivo*’ BJ Blyth, RJ Ormsby, AH Staudacher & PJ Sykes, *Australian Society for Medical Research SA Scientific Meeting*, Adelaide, Australia (June, 2008)