# 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*

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# 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 $^{3}$H-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, $5 \times 10^{5}$ radiolabelled cells ($0.33\ \mathrm{mBq.cell^{-1}}$) 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 $^{3}$H 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

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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)

# Chapter 1: Problem statement

## Problem Statement

Ionising radiation can cause damage to DNA. Radiation-induced DNA lesions can result in gene mutations that contribute 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 doses 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. Understanding the consequences of low-dose irradiation is crucial for evaluating risk-to-benefit ratios for our increasing exposures to radiation from medical, domestic, and industrial sources.

## Aim and Scope

This thesis explores phenomena known as radiation-induced bystander effects. The term bystander effects, as used here, describes biological responses to ionising radiation (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 aims to determine whether intercellular communication of radiation effects between irradiated and unirradiated cells also occurs *in vivo*, and if so, if it could alter current estimations of cancer risk following low-dose radiation exposures. The observation of systemic communication between irradiated tissues

and spatially distant, unirradiated tissues, known as abscopal or out-of-field effects (Mole, 1953) does not fall within the definition of bystander effects as used here. Bystander and abscopal effects, although analogous, are distinct phenomena (Kaminski *et al.*, 2005; Morgan and Sowa, 2007); and whilst the former is relevant to the general population, the latter is restricted to high-dose, partial-body exposures usually in the context of radiation therapy. Reinforcing the differences between bystander and abscopal effects, are demonstrations that high and low doses of radiation do not induce the same biological responses (Amundson *et al.*, 2003; Ding *et al.*, 2005) and in some cases can produce opposite effects (Liu *et al.*, 2004). Paying careful attention to this distinction is important, since much of the work on bystander effects conducted *in vitro* is more relevant to the issue of abscopal effects when considered *in vivo*.

## Overview of the Study

This thesis presents a new *in vivo* experimental method to elucidate and validate the *in vitro* phenomena known as radiation-induced bystander effects. The thesis will be presented as follows:

- **Chapter 2:** *Introduction and background,* begins by describing our radiation environment, defining some key terms and units as well as placing the physical occurrence of ionising radiations in a biological context. It then outlines the basis for current radiation risk-assessment practice, including the evidence forming and justifying the present risk model, as well as data concerning specific low-dose effects that now cast doubt on the assumptions inherent in the policy. The chapter proceeds to survey the prior art concerning radiation-induced bystander effects in particular, the questions which remain

unanswered, and concludes with the key research priorities in the bystander field as they existed at the outset of this project.

– **Chapter 3:** *Research intent and design*, outlines which of the identified priorities for bystander research were included in the study's objectives as related to the overall aim detailed in this chapter. It justifies the choice of an *in vivo* research design and explains the series of specific research questions posed and hypotheses tested.

– **Chapter 4:** *Development of the adoptive transfer method*, details the stages of selection, planning, testing and optimisation of the techniques used in the novel adoptive transfer method developed. It justifies the techniques chosen and describes the experimental method.

– **Chapter 5:** *Use of the adoptive transfer method to study bystander effects in vivo*, describes the experiments conducted using the adoptive transfer method to answer the specific research questions posed to determine if radiation-induced bystander effects occur *in vivo*. It details the course of the investigation and presents the results of each experiment.

– **Chapter 6:** *Evaluation of the adoptive transfer bystander method and its initial findings*, discusses the results of the experiments conducted using the adoptive transfer method and provides synthesis of these observations with the findings of both past, and most recent, bystander effect literature published since the commencement of the study. It assesses whether the aim and specific objectives of the study were met, and concludes with the significance of the findings in this study to our understanding of bystander effects and discusses future directions for bystander research.

# Chapter 2: Introduction and background

## Ionising radiation

### Electromagnetic radiation

Energy emitted from our Sun, other stars and from sources within the Earth's crust constantly bombards the Earth. Electromagnetic radiation spreads out as it travels in waves of discrete packets of energy known as photons. Photons can carry varying amounts of energy that affects the nature of the waves; higher energy photons travel with shorter wavelengths, lower energy photons with longer wavelengths. The electromagnetic spectrum describes the range of photon energies and their unique properties including the more familiar forms of energy such as visible light, microwaves, radiowaves and X-rays.

### Particulate radiation

Stable atoms are balanced composites of electrically charged and neutral particles. When an atom is unstable, it will undergo spontaneous transformations to form a stable, balanced atom. During this process (known as radioactive disintegration or decay), particles can be ejected from the atom. Particulate radiation is the kinetic energy carried by these ejected atomic and sub-atomic particles. Electrically charged particulate radiations including α-particles and β-particles are directly ionising particles as they can also interact with electrons through Coulombic forces. The emission of electromagnetic radiation can also accompany the release of particulate radiation from a radioisotope.

## Ionising radiation

Ionising radiation is radiation with enough energy to remove tightly bound electrons from the orbit of an atom and includes high-energy electromagnetic radiation (such as X-rays and γ-rays) as well as particulate radiation. Natural sources of ionising radiation include cosmic electromagnetic and particulate radiation, geological radioactive sources, and their permeation into the atmosphere, water systems and organic material. Man-made sources of ionising radiation include diagnostic medical exposure, therapeutic medical exposure, nuclear energy production, and fallout from nuclear weapon testing and use.

## Quantification and dosimetry of ionising radiation

As ionising radiation includes disparate forms of energy, radiation energy and exposure are quantifiable in a number of ways. For electromagnetic radiation, the emission of radiation energy is a multiple of both the number of photons per unit area and the energy incident on a unit area measured in units of electron volts (eV). For particulate radiation emissions, radioactivity, expressed in becquerel (Bq), measures the number of nuclear disintegrations per second in a given quantity of radioactive material with the energy of the emitted particles measured in electron volts (eV). The gray (Gy), equal to 1 joule of energy per kilogram of matter, expresses the *absorbed dose*, that is, the amount of energy deposited in a quantity of material, and is the same for all radiation types. The sievert (Sv) expresses the *equivalent dose*, that is, an estimate of the risk of biological damage from a radiation exposure, calculated by multiplying the *absorbed dose* by a radiation-weighting factor determined for each type of radiation exposure.

### Biological impact of ionising radiation

Radiation traverses our bodies at every moment and non-ionising radiations pass through with little consequence. However, ionising radiation has the potential to interact with our tissue at a molecular level. Deposition of radiation energy has the capacity to break chemical bonds and to ionise water within our cells producing reactive oxidative species. At first glance, the breaking of chemical bonds at the cellular level should be of little lasting consequence. At worst, a heavily exposed cell might suffer lethal damage and undergo cell death. Within a multicellular organism, this would render the damage contained and the surrounding healthy cells could quickly replace the exposed population. However, contrary to this benign scenario, the most menacing potential of radiation is that it can break chemical bonds within DNA molecules and compromise the genetic integrity of the cell. Rather than simple destruction, a cell can sustain genetic mutations that under the right conditions can lead to carcinogenesis, in this way, transmitting and multiplying the initial radiation damage as cells divide.

## The radiation risk assessment paradigm

### Radiation protection

Given the potential drastic consequences of radiation exposure, the most obvious course of action has been to determine the biological effects of varying doses of ionising radiation precisely (reviewed in Sankaranarayanana and Wassom, 2007). Radiation protection should be a simple matter of preventing hazardous exposures, once it is determined what levels of ionising radiation exposure are dangerous. This scenario has been complicated by two major issues: the assessment of risk at doses

where no immediate damage is observed and a possible compounding or diluting effect of chronic ionising radiation exposure (reviewed in Mason, 2004).

**Linear no-threshold model**

A linear relationship between radiation dose and relative damage or risk is intuitive; as the amount of energy deposited per volume of tissue increases, biological consequences should naturally escalate in synchrony. With mathematical adjustments made for simple differences in radiation *quality* (International Commission on Radiation Units and Measurements, 1970), a model can be devised on basic, fundamental principles: any exposure to radiation is harmful, relative risk is in linear proportion to absorbed dose and cumulative exposure results in cumulative risk.

This Linear No-Threshold (LNT) model has been developed based on principles used to regulate radiation risk since the 1950s and from the earliest acceptance of the proportionality of risk to accumulated dose (ICRP 6: International Commission on Radiological Protection, 1964; ICRP 9: International Commission on Radiological Protection, 1966). This instrument easily facilitates radiation protection; one simply decides on an acceptable level of risk and calculates the corresponding annual dose limit. At the core of validating this model (as with any mathematical modelling) is the generation of data along the relevant dose ranges. With long-term studies of survivors from the atomic weapon detonations over the cities of Hiroshima and Nagasaki (Preston *et al.*, 2007), exposed populations near the Chernobyl nuclear (Davis *et al.*, 2006) and other accidents (Chen *et al.*, 2007; Krestinina *et al.*, 2007) and occupationally exposed workers (Jacob *et al.*, 2007; Sokolnikov *et al.*, 2008), comes a plethora of data with which to validate the linear no-threshold model.

At issue, is the scarcity of data at low doses (<100 mSv) due to the difficulty of conducting epidemiological studies with suitable statistical power to detect significant changes in stochastic effects (National Research Council, 1995). In addition, difficulties in accurate dosimetry at exposures approaching normal background levels (2.4 mSv per year, worldwide average: United Nations Scientific Committee on the Effects of Atomic Radiation, 2000) complicate studies in the low-dose range. After analysis of data from studies of exposed human populations, Brenner and colleagues (2003) concluded that the lowest dose at which good epidemiological evidence existed to demonstrate an increased risk of cancer in any organ was a 10–50 mSv acute dose or a 50–100 mSv chronic dose. Consequently, validation or disputation of the linear no-threshold model at low radiation doses relevant to general community exposure (<100 mSv) has relied on a concerted effort employing a range of different methods, resulting in the establishment of a new scientific field: low-dose radiobiology.

**Low-dose radiobiology**

Identifying and quantifying detrimental health effects after high-level ionising radiation exposure is straightforward. Traditional measurements such as $LD_{50}$, cancer induction, co-morbidity incidence and lifespan studies can clearly evaluate the risk of deterministic and stochastic effects after exposure to high-dose radiation. Fortunately, for most of the population, ionising radiation exposure at high levels is unlikely to occur. For the public, the relevant risk assessment environment is low-level radiation doses from environmental sources and routine medical exposure. Characterising the stochastic effects of low-dose radiation exposure (<10 mSv) would require a controlled study with thousands to millions of participants exposed to precise radiation doses and vigilantly monitored over the following decades (ICRP

99: International Commission on Radiological Protection, 2006, pp. 26-7); however, ethical and practical limitations make such a study impossible. Instead, the risk-assessment community has two practical options: retrospective study of occupationally or accidentally exposed persons and *in vitro*/*in vivo* radiation-damage models. The use of *in vitro* and *in vivo* radiation-damage models has included the observation of naturally occurring phenomena as biological markers indicating induced/promoted carcinogenesis, as well as artificially constructed assay systems that exploit advances in molecular genetics. Genetically engineered cell lines and animal strains that express a radiosensitive or radioresistant phenotype represent a combination of these two approaches. In each case, the aim is to understand the ultimate biological responses to ionising radiation at doses where no obvious or immediate effect is observed.

**Findings of low-dose radiobiology research**

Use of *in vivo* and *in vitro* radiation-damage endpoints (such as DNA breaks, mutations and cell death) to study the effects of low-dose radiation on genomic and cellular integrity has provided mixed results. In some studies, results have seemed consistent with a linear extrapolation from high-dose effects (Chadwick and Leenhouts, 2000; Preston, 2003; Chadwick and Leenhouts, 2005); conversely, in contradiction to the linear no-threshold model are the repeated observations of four (not mutually exclusive) low-dose radiation phenomena:

- Radioadaptive responses;
- Low-dose hypersensitivity;
- Genomic instability; and,
- Radiation-induced bystander effects.

*Radioadaptive responses*

The radioadaptive response (reviewed by Cai, 1999; Bonner, 2003; Morgan, 2006) describes the finding that *in vitro* and *in vivo* systems that are 'primed' with a low dose of radiation can respond to a subsequent high dose of radiation with fewer detrimental effects than if the priming dose had not been administered. Radioadaptive responses have been observed for the induction of micronuclei and neoplastic transformation (Azzam *et al.*, 1994), mutation (Zhou *et al.*, 2004), apoptosis (Takahashi *et al.*, 2001a), cell death (Wang and Cai, 2000; Sawant *et al.*, 2001a) and chromosome changes (Olivieri *et al.*, 1984; Shadley *et al.*, 1987; Day *et al.*, 2006) in response to low-dose pre-irradiation. These observations are contrary to the assumption of the linear no-threshold model that cumulative doses result in cumulative effects. The adaptive response is hypothesised to reflect an overcompensation of DNA-repair and cell survival responses to the initial radiation insult that linger and provide a protection against the consequent radiation dose (Kadhim *et al.*, 2004; Mothersill and Seymour, 2004; Scott, 2004). Such an explanation would be consistent with the theory of hormesis, a concept more familiar in toxicology. Calabrese and Baldwin (2002, p. 92) have defined hormesis as:

> …an adaptive response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of the stimulatory response that are either directly induced (i.e., direct stimulation hormesis [DSH]) or the result of compensatory biological processes following an initial disruption in homeostasis (i.e., overcompensation stimulation hormesis [OCSH]).

The overcompensation stimulation hormesis model would predict that the shape of the dose-response curve is dependent on the post-irradiation time examined, and that the cumulative response is the superimposition of the damage induced and damage repaired at each dose (Burlakova *et al.*, 1999; Calabrese and Baldwin, 2002).

The presence of a finite subpopulation of radiosensitive cells *in vivo* or *in vitro* could also explain the radioadaptive response. Such cells would be eliminated by an initial low-dose irradiation and thus would not participate in the response to the following dose (Bodnarchuk, 2003; Mothersill and Seymour, 2005). Furthermore, the selective removal of genetically compromised cells following low-dose irradiation, that results in a reduction in genomic instability, mutations and neoplastic transformation following a high-dose challenge (coined the *protective apoptosis-mediated* process) has been included in the NEOTRANS$_3$ radiation dose-response model (Scott, 2004). It has also been proposed that the radioadaptive response induced by a conditioning dose exists independently of a subsequent dose, observed as a response in the opposite direction to that seen after the challenge dose (Day *et al.*, 2006; Day *et al.*, 2007b). In this way, the response to the conditioning and challenge dose is additive, but when the effects are superimposed they result in a relative decrease compared to the challenge dose alone. The radioadaptive response is increasingly considered a complex cooperation of DNA-repair, cellular defence mechanisms and selective removal of damaged cells (Feinendegen, 2003). The exact mechanisms underlying the radioadaptive response are still unclear, but thought to include at least DNA damage detection and repair pathways, cell-cycle checkpoints, chromatin packaging, and apoptosis (Zhou *et al.*, 2004; Brooks, 2005; Feinendegen and Neumann, 2006). The discovery that low-dose irradiation induces the expression of different sets of genes to high-dose irradiation (Amundson *et al.*, 2003; Ding *et al.*, 2005;

Sokolov *et al.*, 2006; Voy *et al.*, 2006), lends credence to the theory of a fundamental duality in the biological response to radiation exposure; again, in opposition to the linear no-threshold model.

However, there is not universal acceptance of the necessity of radioadaptive responses *in vitro* or in rodents to alter the linear dose-response relationship. There is debate regarding the translation of the response at the cellular level to the probability of tumour formation *in vivo*: 'since human tumor data are used as the primary data for establishing cancer risk estimates, cellular observations currently serve in a correlative or supportive role' (Preston, 2008, p. 544). In addition, there is doubt as to whether cellular radioadaptive responses would do more than change the slope of the dose-response curve for cancer induction at low doses; 'no version of an adaptive response appears to require departure from a LNT response for radiation-induced chromosome aberrations, or for cancer...' (Preston, 2003, p. 266). Some have questioned the data showing radioadaptive responses, highlighting the inter-individual, inter-replicate and inter-experimental variation observed in many studies (Schwartz, 2007). There is also a concern that a reporting bias of adaptive responses exists (cases of additive effects are not as interesting to publishers) and that meta-studies of adaptive response literature fail to factor in the 'lower than control' results that might be expected by chance (Zapponi and Marcello, 2006) and cases of supra-additive synergistic effects (Mortazavi and Ikushima, 2006). The only consensus appears to be that before radioadaptive responses can be included in radiation risk models, a greater understanding of the cumulative outcome of observed cellular effects on tumour induction/promotion is required, especially factoring in the differences between acute and chronic exposure scenarios.

*Low-dose hypersensitivity*

There is an overlap between the induction of the radioadaptive response and a phenomenon known as low-dose hypersensitivity. Specifically, low-dose hypersensitivity refers to observations that low-dose irradiation is sometimes more damaging per unit dose than high-dose irradiation (Joiner *et al.*, 1996; Joiner *et al.*, 2001; Marples *et al.*, 2002). Low-dose hypersensitivity is often observed as a deviation from the cell survival curve predicted by linear no-threshold modelling at low doses which returns, often abruptly, to the expected values as the dose increases, attributed to an induced radioresistance (Bonner, 2004).

Cell-cycle progression and arrest have been implicated in low-dose hypersensitivity (Marples *et al.*, 2003; Short *et al.*, 2003) with the postulation that cells exposed to low radiation doses do not halt their progression through the cell-cycle and thus suffer from replication failure. Conversely, higher dose irradiation may trigger cell-cycle checkpoint proteins and a resultant delay in cycle progression allowing the error-free repair of ionising radiation–induced damage. This hypothesis is further supported by findings that suspending cell cultures in a nonproliferative state for a period of time after irradiation – allowing potentially-lethal damage repair – results in increased cell survival (Cox *et al.*, 1981; Arlett and Priestley, 1983).

The hypersensitivity observed at low doses appears to be overcome by an increasing radioresistance above a threshold-dose, possibly the result of the same induction of DNA-repair responses seen in the radioadaptive response (Bonner, 2004). By its very definition, low-dose hypersensitivity represents a fundamental contradiction to the linear no-threshold model; however, as with radioadaptive responses, low-dose hypersensitivity is essentially a cellular effect, and the evidence does not yet support the extrapolation of the effect to a non-linear induction of tumours.

*Genomic instability*

Genomic instability is a phenomenon whereby the progeny of cells surviving exposure to a DNA damaging agent exhibit a long-term increase in the rate of spontaneous damage. The increased levels of damage observed in the distant progeny of the exposed cells are non-clonal and not caused by the exposure itself. Genomic instability in the progeny of irradiated cells, in the form of an increased rate of *de novo* chromosomal aberrations, has been observed at doses down to even a single α-particle traversal (Kadhim *et al.*, 2001), although the existence of the radiation-induced phenomenon is not universally accepted (Koterov, 2006). Endpoints other than chromosome aberrations have also been reported as examples of genomic instability, such as micronuclei, SCE, mutations and cytotoxicity (reviewed in Morgan, 2003; Snyder and Morgan, 2004). The persistence of the unstable phenotype has been attributed to mitochondrial dysfunction (Kim *et al.*, 2006a; Kim *et al.*, 2006b), DNA hypermethylation (Kaup *et al.*, 2006), tumour necrosis factor-α (Moore *et al.*, 2005a), altered gene expression profiles and proteasome activity (Snyder and Morgan, 2004). The occurrence of genomic instability after low-dose irradiation is not consistent with a linear response due to the induction of damage in more cells than predicted by the exposure dose.

*Radiation-induced bystander effects*

Perhaps the most elemental incongruity between the linear no-threshold model and the results of low-dose radiation research in the past decade is the revelation that cells within a sparsely irradiated population that were not even exposed to ionising radiation can exhibit a biological response to the exposure. These findings form the basis of this thesis and are discussed in detail below.

## Radiation-induced bystander effects

### Discovery of the bystander effect

With current biological knowledge, it seems intuitive that the effects of ionising radiation on a population of cells would not be restricted to discrete intracellular events. In the past however, researchers had only sought the consequences of irradiation inside cells suffering a direct radiation insult. The idea was that cells have only one radiation vulnerability, such that if the nucleus escaped a direct hit, the cell would remain unperturbed. Due to mounting evidence to the contrary, there is now dispute regarding the assumptions that have formed the basis of radiation risk modelling since the 1950's. The term radiation-induced bystander effects refers to the extracellular responses of cells exposed to ionising radiation, as well as the downstream responses to these signals in unexposed 'bystander' cells. The term encompasses the ultimate fate of unexposed cells as well as the associated effector mechanisms and stimuli.

No single landmark experiment was responsible for discovering or characterising radiation-induced bystander effects. Instead, the concept formed with the accumulation of disparate data gathered from multiple research groups and using a variety of *in vitro* radiation damage models and endpoints. The research credited with triggering the detailed investigation of the bystander phenomenon measured sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells exposed to low fluences of α-particles (Nagasawa and Little, 1992). At the low doses used, less than 1% of the cells would be expected to be directly traversed by an α-particle, yet there was an induction of SCE in around 30% of cells. Similar results with SCE in normal human lung fibroblasts (Deshpande *et al.*, 1996) supported these initial

findings. In further studies, the tumour suppressor protein TP53 (Hickman *et al.*, 1994; Azzam *et al.*, 1998) was induced in more human lung fibroblasts than were traversed by an α-particle. Still, there was no direct evidence of a bystander effect, as these results could be explained as mere statistical discrepancies.

To convincingly prove the potential for transmission of biological effects to unirradiated cells, medium taken from an irradiated cell culture was transferred to unirradiated cells, which resulted in a drop in cloning efficiency (Mothersill and Seymour, 1997). From here, the transfer of irradiated cell–conditioned medium (ICCM) in combination with direct irradiation was used extensively to probe bystander effects under a variety of conditions (Seymour and Mothersill, 2000). The next major advance in bystander effect research came when Columbia University (Hei *et al.*, 1997) and the Gray Laboratory (Prise *et al.*, 1998) both established charged-particle microbeams capable of delivering exact numbers of $^{3}He^{2+}$ ions (essentially α-particles) with micron-level precision. For the first time, this enabled irradiation of specific cells, distinguishing irradiated and unirradiated populations with certainty. In 1998, Prise *et al.* reported the effects of targeting only four cells within a population of primary human fibroblasts with a single $^{3}He^{2+}$ ion. Their measurements of micronucleus induction and apoptosis provided definitive evidence *in vitro* for a bystander effect between ionising radiation–exposed and –unexposed cells. More recently, the development of an ultrasoft X-ray microprobe has allowed the photon irradiation of individual cells with sub-micron accuracy (Schettino *et al.*, 2002).

Several research groups have used co-cultures of irradiated and unirradiated cells to study bystander effects (Shao *et al.*, 2001; Geard *et al.*, 2002; Yang *et al.*, 2005). In

1999, Bishayee *et al.* reported the development of a new three-dimensional tissue co-culture model for studying bystander effects. The model involved incorporating tritiated thymidine into the DNA of Chinese hamster lung fibroblasts, and then preparing mixtures of irradiated and unirradiated cells. Self-irradiated cells induced a dose-dependent decrease in bystander cell survival when centrifuged to form multicellular clusters approximately 1.6 mm in diameter and incubated intact before disaggregating. Mixtures of irradiated and unirradiated cells have now also been studied after incubation *in vivo* using transplantation techniques (Xue *et al.*, 2002).

The bystander effect has been investigated and reported on in great detail in the past decade, and a mass of data need to be integrated and checked for reproducibility and relevance outside their test systems (Newman, 2006). Following is a review of published results on radiation-induced bystander effects, available at the outset of this investigation, described by the endpoint studied and the radiation source, doses and conditions used.

**Evidence for cell death and reduced reproductive potential in bystander cells**

Direct radiation energy deposition and the generation of reactive oxygen species by the radiolysis of water molecules can result in irreparable damage to cellular macromolecules leading to cell death. The term *oncosis* describes the failure of a cell to restore homeostasis following trauma or stress, resulting in cell death despite the cell's efforts to survive (Majno and Joris, 1995). On the other hand, *apoptosis* is a form of programmed cell death where the cell works actively to dismantle itself (Fink and Cookson, 2005). *Necrosis* refers to the post-mortem events in a tissue following the point of cell death, regardless of the mechanism. Quantification of cell death both *in vivo* and *in vitro* usually incorporates the combined outcome of

oncosis, apoptosis, and transient or permanent cell-cycle arrest. Whilst these phenomena are mechanistically distinct, they each result in an immediate decrease in viable cell numbers or a reduction in the number of viable cells remaining after a defined post-irradiation period. Measurements of cell death include counting the number of remaining viable cells, the accumulation of dead cells, cloning efficiency, clonogenic survival, colony-forming efficiency, or morphological indications of necrosis. Although overlapping with simple measurements of cell death, the specific measurement of apoptosis is described later.

*Exposure to irradiated cell–conditioned medium*

Much of the evidence for the death of bystander cells has come from experiments that removed culture medium from irradiated cells some time after exposure and transferred it to unirradiated cells. From the outset, the transfer of irradiated cell–conditioned medium (ICCM) clearly decreased bystander cell survival (Mothersill and Seymour, 1997) but even the earliest results made it clear that not all cells would exhibit a bystander effect, and, that some cell-types were capable of sending, or responding to, a bystander signal but not necessarily both. The robust response of a human keratinocyte cell line in these experiments encouraged the further use of these cells as a reporter cell line for future medium transfer experiments.

Data from further investigations conducted using the keratinocyte medium transfer system, supported the theory of a sustained release by irradiated cells of a long-lived soluble mediator into the culture medium, which if above a threshold level could fully induce a bystander killing effect in unirradiated cells (Mothersill and Seymour, 1997; Mothersill and Seymour, 1998). The hypothesis was that this could occur by triggering a signalling cascade in the bystander cells, which once initiated, continued

even after the removal of the original signal. The magnitude of the bystander cell death was dependent on the number of cells present in the irradiated flask; but, the degree of cell–cell contact in the irradiated cells did not affect the bystander response (Mothersill and Seymour, 1998). Reduced cell survival in cells directly exposed to 0.01–0.5 Gy was attributed to a dose-independent bystander effect, whilst above 0.5 Gy, the saturated bystander response was then overwhelmed by the dose-dependent direct effects (Seymour and Mothersill, 2000).

In order to begin to relate these data to the situation *in vivo*, further experiments involved the transfer of medium from human tissue explants irradiated *ex vivo* to the HPV-G human keratinocyte reporter cell line, in which the bystander effect had been well characterised (Mothersill *et al.*, 2001). Medium from irradiated human explants from smokers, males, and patients with malignant tissue produced a diminished response (Mothersill *et al.*, 2002), whilst medium from explants of C57BL/6 but not CBA/Ca mice (irradiated *in vivo*) significantly reduced HPV-G plating efficiency (Mothersill *et al.*, 2005b). These studies indicated that results from *in vitro* ICCM experiments might not represent the complexity and individual variation in any equivalent phenomenon *in vivo*.

Co-cultures of irradiated and unirradiated mouse fibroblasts (Mitchell *et al.*, 2004a), mouse embryonic stem cells (Zhu *et al.*, 2005), and human lymphoma/salivary gland cells (Shao *et al.*, 2004a), have shown significant decreases in bystander cell survival for X-rays, α-particles and carbon ions. In each of these cases, the use of radiation doses greater than 0.5 Gy (up to 15 Gy α-particles or 300 Sv) simulates a heavily irradiated tissue, making the effects more analogous to abscopal effects rather than

the effects of rare, isolated hits in a tissue. This is further supported by the dependence of ICCM–mediated effects on irradiated-cell density.

The transfer or sharing of ICCM provides a convenient system for exploring the nature of bystander effects and allows easy manipulation of dose and exposure conditions; however, the relevance of the results obtained using medium-transfer experiments remains in doubt. Even ICCM from cells irradiated with low doses (below one traversal per cell) may not represent sparse irradiations *in vivo*, since the dependence of ICCM–effects on cell density may simply represent a reliance on the accumulation and concentration of the signal (simply mimicking higher dose exposures). The signalling and/or effector molecule(s) involved in the ICCM effect have yet to be identified and a clearer understanding of the responsible mechanism and the range, longevity and efficacy of the signal *in vivo* is required if results from medium-transfer experiments are to be generalised to the field of radiation risk-assessment.

*Irradiation of individual cells*

Unlike media-transfer experiments, the use of an ultrasoft X-ray microprobe allows the irradiation of individual cells within an unirradiated cell population, closer to the bystander situation that would occur in a tissue exposed at low dose-rate. At cellular doses below 200 mGy, the decrease in cell survival was not significantly different whether all cells (≈160) or only a single cell was targeted (Schettino *et al.*, 2003) suggesting that very few irradiated cells might be required to initiate full-scale bystander signalling. Above cellular doses of 200 mGy, the bystander cell killing reached a plateau but the killing of directly irradiated cells continued to increase relative to dose. Later indications of a binary induction of bystander effects, with an

increasing probability of triggering a bystander response (rising to 100% by approximately 300 mGy) explained this saturation point (Schettino *et al.*, 2005). Microbeam irradiation of only 10% of mouse fibroblast cells with $^{3}He^{2+}$ ions (effectively α-particles) resulted in a dose-dependent reduction in bystander clonogenicity (Sawant *et al.*, 2001b; 2002); an effect later shown to be proportional to the degree of cell–cell contact (Mitchell *et al.*, 2004b). Experiments with α-particle irradiated fibroblasts suggest that in addition to bystander cytotoxicity, reductions in bystander clonogenicity may also be due to permanent or transient cell-cycle arrest in bystander cells (Azzam *et al.*, 2000). All of these results were obtained from individual irradiation of fibroblasts, which have previously not shown ICCM–mediated bystander effects (Mothersill and Seymour, 1997), but have shown a sensitivity to bystander toxicity in co-cultures with shared medium (Mitchell *et al.*, 2004a); such discrepancies have yet to be resolved.

*Three-dimensional cell culture of radiolabelled and unirradiated cells*

Another attempt to mimic the situation in a low-dose irradiated tissue *in vivo*, involves irradiating a proportion of the cells in a multicellular cluster by the incorporation of internal emitters. In multicellular clusters assembled from mixes (100%, 50% and 10%) of radiolabelled and unirradiated cells, increasing radioactivity resulted in cell death in both the irradiated and bystander populations (Bishayee *et al.*, 1999; Bishayee *et al.*, 2001; Howell and Bishayee, 2002). The nature of the toxic signal in such experiments is unclear, as both the free radical scavenger dimethyl sulphoxide and lindane (an inhibitor of gap junctions) reduced the bystander effect, with the highest protection using both agents together. These three-dimensional culture experiments come closer to representing the stochastic traversal of ionising tracks through rare, isolated cells in a body exposed to a low

dose-rate of low-LET radiation; however, to be of relevance to public radiation protection, experiments need to study lower proportions of irradiated cells.

*In vivo growth of radiolabelled and unirradiated tumour cells*

*In vivo* experiments similar to the three-dimensional culture of radiolabelled cells involve implanting mixes of radiolabelled and unirradiated human colon adenocarcinoma cells subcutaneously into immunodeficient mice (Xue *et al.*, 2002). Lethally irradiating a proportion of the tumour cells with $^{125}$I-iododeoxyuridine significantly decreases tumour volume (proxy for bystander cell death) compared to controls adding the same proportion of dead cells (thrice freeze–thawed). Radiolabelling 50%, 20%, or 10% of the tumour cells resulted in a similar reduction in tumour growth. Although this experiment represents an initial step towards studying bystander effects *in vivo*, the results warrant careful interpretation. The irradiated cells received a fourfold higher dose than required to kill the cells, a dose inconsistent with isolated irradiated cells within a tissue. The high dose to the irradiated cells also resulted in a cross-dose to the *unirradiated* tumour cells of 10–70 mGy (although tumours irradiated directly with these doses showed no change in growth-rate). There was no tumour growth effect upon repeating the same experiment using *ex vivo* γ-radiation of 5 or 20 Gy to the irradiated tumour cells (even though these doses would likely be fatal if delivered to an individual). The necessity to lethally irradiate the tumour cells (to ensure tumour growth is solely from bystander cells), to use immunodeficient mice and to balance the tumour cell mixes with dead ‘spacer’ cells makes this method of limited utility for exploring relevant bystander conditions *in vivo*.

*Summary*

The evidence that irradiated cells can reduce survival or depress growth of neighbouring unirradiated cells *in vitro* is overwhelming. Fibroblasts, epithelial cells, embryonic stem cells, leukaemic cells, and cells derived from carcinomas and lymphomas exhibit both cytocidal and cytostatic bystander effects. However, a cell-type that responds using one method does not necessarily exhibit a bystander effect using other methods. Evidence for these effects following high- and low-LET irradiation does not mean that within any one system, both are effective at inducing a bystander response. Similarly, the requirement of direct cell–cell contact and the protection afforded by antioxidants, free radical scavengers or inhibitors of gap-junctional intercellular communication (GJIC), varies widely between systems. The observation that the magnitude of ICCM–mediated bystander effects is dependent on irradiated cell number is at odds with results using partially irradiated tumours that show the same effect after radiolabelling 10% or 50% of the cells, and is inconsistent with the substantial responses seen after the irradiation of single cells. Likewise, different systems report that bystander cell death is either dose-dependent or dose-independent. The firmly established potential for bystander cell killing *in vitro* is yet to be fully characterised, and as such, these contrasting reports necessitate exercising caution when basing future work on assumptions gleaned from any one study, and using care when quoting facts *known* about the bystander effect.

## Evidence for the induction of apoptosis in bystander cells

Metazoans are equipped with a highly organised and strictly controlled method of cellular self-destruction known as apoptosis. Although apoptosis will have contributed in part to the measurements of bystander cell death described in the

previous section, the following discussion is based on bystander cell death occurring specifically via apoptosis. Triggered by intercellular signalling, cellular damage, or stress, apoptosis describes an active, energy-dependent signalling cascade that ultimately results in cell death.

Cells undergoing apoptosis initiate a number of distinct pathways aimed at preventing the release of inflammatory stimuli. The morphological and cellular changes associated with apoptosis include chromatin cleavage, nuclear and cytoplasmic condensation, the maintenance of the cell membrane during formation of membrane-bound apoptotic bodies and the externalisation of cell-surface signalling molecules targeting the cellular remains for phagocytosis (Samali *et al.*, 1999). One of the stimuli known to induce apoptosis is DNA damage caused by exposure to ionising radiation, a mechanism believed to prevent the accrual of genetically compromised and potentially neoplastic cells (Hu and Hill, 1996; Hasegawa *et al.*, 2002; Kaina, 2003; Lee *et al.*, 2005a; Mendonca *et al.*, 2005). Ionising radiation–induced apoptosis is thought to be triggered by the accumulation of complex or irreparable DNA lesions (Radford, 2002a), regulated by protein complexes formed at foci proximal to DNA double-strand breaks (Bree *et al.*, 2004; Itamochi *et al.*, 2005) with some reports suggesting that even a single DNA double-strand break can be lethal (Huang *et al.*, 1996).

*Exposure to irradiated cell–conditioned medium*

ICCM from cells receiving γ-radiation doses of 0.5 or 5 Gy, or their progeny, specifically induced apoptosis in HPV-G cells (Lyng *et al.*, 2000; Lyng *et al.*, 2002a; Maguire *et al.*, 2005). However, other studies have demonstrated persistent non-apoptosis-mediated reduced plating efficiencies in bystander populations, lasting at

least 20 population doublings despite continual replenishment with fresh medium (Lewis *et al.*, 2001). The growing acceptance of the HPV-G cell line as a sensitive reporter system for detecting bystander effects allowed the progression to combined *in vivo* / *in vitro* experiments using irradiated mouse bladder epithelium explants (Mothersill *et al.*, 2005b). The dependence of bystander apoptosis on the donor mouse-strain seen in those experiments was also confirmed in bladder explants treated with explant-derived ICCM (Mothersill *et al.*, 2005b). ICCM from human leukaemic cells irradiated with 4 Gy induced apoptosis 12–48 h after exposure (Konopacka and Rzeszowska-Wolny, 2006), but unexpectedly, the presence of the antioxidants vitamin C or vitamin E did not reduce the effect as might have been expected from other studies (Lyng *et al.*, 2002a). Results from experiments using ICCM thus indicate a role for apoptosis in bystander cytotoxicity, but also suggest that this is not the sole mechanism responsible for reductions in bystander survival or reproductive potential.

*Irradiation of individual cells*

The earliest observations of bystander apoptosis in plated cells targeted with $^{3}He^{2+}$ ions using a charged-particle microbeam (Prise *et al.*, 1998) have been followed by more complex cell models in the move towards *in vivo* experimentation. $^{3}He^{2+}$ ion irradiation of 1–10 cells in porcine ureter explants increased bystander apoptosis after three (Belyakov *et al.*, 2003) and seven days in culture (Belyakov *et al.*, 2002), demonstrating the multiplicative effect of bystander signalling, affecting thousands of cells after targeting only a few cells with radiation. A pivotal study in a three-dimensional human skin model confirmed this far-reaching signalling potential, measuring apoptosis in sequential tissue slices parallel to a plane of $^{3}He^{2+}$ irradiated cells (Belyakov *et al.*, 2005). Induction of apoptosis in the bystander tissue over 1

mm away from the irradiated plane indicated the potential range of bystander signals in a tissue for the first time. Similar to the findings of general cell death/reduced reproductive potential in bystander cells, reactive oxidative species and gap-junctional communication appear to play complimentary, overlapping or distinct roles in inducing bystander apoptosis depending on the cell-type(s) and bystander system.

**Evidence for the stimulation of cell growth in bystander cells**

The demonstration of pro-survival bystander effects contrasts with the cell death, apoptosis, and growth-arrest in bystander cells already described. In one theory, these proliferative bystander effects represent a subsequent tissue repair response to radiation-induced cell death in order to repopulate the eliminated cells (Bijwaard *et al.*, 2006). If this is the case, cell death and enhanced proliferation may be sequential, not opposing, bystander effects. It is important to remember that pro-survival bystander effects do not necessarily equate to beneficial effects. Even if inducing proliferation is a tissue repair response, an increase in growth-rate or selection pressure favouring highly proliferative cells both have the potential to drive carcinogenesis (Tomlinson and Bodmer, 1999). The interpretation of cell death as detrimental to a tissue and cell growth as beneficial, needs to be balanced with the perspective that cancer is a disease of uncontrolled proliferation, and that dead cells do not form cancers (Barcellos-Hoff, 2001; Mothersill and Seymour, 2006a).

Medium from human lung fibroblasts exposed to a low fluence of α-particles can induce an increase in proliferation (Iyer *et al.*, 2000); the authors suggesting that bystander effects might be responsible for the lung hyperplasia associated with exposure to inhaled radon progeny. ICCM from α-particle and γ-radiation exposed

cells has also induced a bystander survival adaptive response to subsequent high-dose direct irradiation (Iyer and Lehnert, 2002a, 2002b). Antigen-presenting cells irradiated with 75 mGy stimulated proliferation in co-cultured, unirradiated T lymphocytes; whilst high-dose irradiation with 2 Gy inhibited proliferation in the co-cultured lymphocytes (Liu *et al.*, 2004). The proliferative effect in bystander cells is not always limited to low doses, since exposure to high doses of X-rays or a carbon ion beam increases bystander plating efficiency in co-cultured human salivary gland cells (Shao *et al.*, 2001), however these responses are dependent on the LET of the radiation (Shao *et al.*, 2002; Shao *et al.*, 2003a). Likewise, cells irradiated with a range of doses from 0.5 to 20 Gy of γ-rays, or radiolabelled with $^{3}$H-thymidine, induce an increase in proliferation in unirradiated co-cultured cells in multi-cellular clusters (Gerashchenko and Howell, 2003b, 2004, 2005).

The repeated observation of bystander proliferation in a number of systems has confirmed it as a major bystander response, even though the number of results showing bystander proliferation is fewer than the number showing bystander toxicity. In some cases, the same system shows a bystander cell death effect in fibroblasts (Howell and Bishayee, 2002) and bystander proliferation with epithelial cells (Gerashchenko and Howell, 2003b). This contrast affirms that there is not one *bystander effect* but a range of effects differentially induced based on dose, dose-rate, cell-type, and exposure system. It is likely that competition between the effects determines the outcome in any one experimental system.

**Evidence for DNA double-strand breaks in bystander cells**

Since direct exposure to ionising radiation causes DNA breaks, the question has arisen as to whether bystander cells show the same types of DNA damage. This

would be consistent with the identification of clastogenic factors in serum of individuals exposed to high doses of radiation (Lloyd and Moquet, 1985). The γ-H2AX assay provides a method sensitive enough to detect small numbers of DNA double-strand breaks in single cells. Phosphorylation of the H2AX histone to form the γ-variant H2AX (γ-H2AX) was originally noticed in irradiated cells (Rogakou *et al.*, 1998) and was later observed in foci flanking the sites of DNA breaks induced by a microbeam laser (Rogakou *et al.*, 1999). In addition to DNA damage sites, the formation of γ-H2AX at endogenous DNA double-strand breaks is seen in the Immunoglobulin Heavy Chain and T Cell Receptor genes during thymocyte development and class-switch recombination (Chen *et al.*, 2000; Petersen *et al.*, 2001); and is prevalent in spermatogenic cells during meiosis (Mahadevaiah *et al.*, 2001). It is now known that γ-H2AX formation plays a role in the recruitment of DNA-repair proteins to the sites of DNA double-strand breaks (Paull *et al.*, 2000; Bassing *et al.*, 2002; Shroff *et al.*, 2004).

Increases in γ-H2AX staining in 48% of the population after α-particle traversal of ≈ 9% of cells (Hu *et al.*, 2005) suggested the release of a clastogenic factor into the medium, later confirmed by a twofold induction of γ-H2AX staining in shielded cells up to 7.5 mm away from irradiated cells (Hu *et al.*, 2006). Irradiating cells with a higher dose of α-particles (20 per cell) induced an even larger induction of γ-H2AX foci in unirradiated cells mixed with, or sharing medium with, the irradiated cells (Sokolov *et al.*, 2005). The potential significance of DNA double-strand break induction in bystander cells is that if unirradiated cells receive DNA damage, they too might produce a bystander signal resulting in a multiplicative cascade. Determining whether DNA damage in bystander cells can trigger these cells to initiate their own bystander signal has become a research priority.

## Evidence for chromosomal damage in bystander cells: Chromosomal aberrations

Downstream of DNA damage is the misrepair of, or failure to repair chromosomal breaks. Chromosomal fragments left unjoined or incorrectly ligated, can result in large amounts of coding sequence being lost, duplicated, or placed under promiscuous transcriptional control. Unlike isolated mutations, which may affect only a single gene product or even a non-coding region, chromosomal aberrations can trigger mitotic catastrophe or produce an unstable phenotype that promotes carcinogenesis.

Mouse cell cultures with small fractions of cells (<10%) traversed by α-particles, show a larger than expected fraction of cells exhibiting chromosomal aberrations, and, this fraction greatly increases in cells in which either of the mouse non-homologous end-joining enzymes Xrcc5 or Prkdc are knocked out (Little *et al.*, 2003). When non-homologous end joining is disabled in Chinese hamster cells (DNA-PKcs$^{-/-}$: equivalent to knocking out mouse Prkdc), traversal of 1% of nuclei with α-particles results in a significant induction of chromosomal aberrations in bystander cells, an effect not seen in wild-type or homologous recombination deficient cells (Nagasawa *et al.*, 2005). These findings suggest routine repair of the induced DNA effects in normal cells, with conversion to toxic lesions only in cells with compromised DNA-repair. However, cells with normal DNA-repair have been shown to exhibit bystander chromosomal damage, but this was observed from 3 h after irradiation rising to a maximum by 48 h (Suzuki *et al.*, 2004), suggesting an induction of changes in DNA maintenance and repair rather than a directly toxic, clastogenic effect.

**Evidence for recombination in bystander cells: Sister chromatid exchange**

The balanced exchange of DNA between original and newly-replicated strands (as opposed to homologous chromosomes during meiotic recombination) is known as Sister Chromatid Exchange (SCE), and is greatly increased after radiation exposure (Marin and Prescott, 1964). The interest in sister chromatid exchanges stemmed from observations that doses of DNA damaging agents which did not induce classical cytogenetic endpoints such as chromosome aberrations, did significantly induce the formation of SCE (Perry and Evans, 1975). Screening of a raft of proven and suspected mutagens compared SCE and chromosome/chromatid aberrations as endpoints, the results showing that low doses of mutagens could cause a tenfold induction in SCE before an induction in chromosomal aberrations was detected. Studies have used the SCE assay to evaluate DNA damage from many suspected chemical mutagens (Beek and Obe, 1975; Solomon and Bobrow, 1975; Natarajan *et al.*, 1976) and from radiation (Graves and Kellow, 1983; Nagasawa *et al.*, 1991).

The induction of SCE in ≈ 30% of cells irradiated with low α-particle fluence (1% nuclear traversals) demonstrated the earliest potential for radiomimetic effects in bystander cells (Nagasawa and Little, 1992). In similar experiments using human lung fibroblasts, 8.6-fold more cells than calculated to be traversed by an α-particle showed induction of SCE (Deshpande *et al.*, 1996). The induction of bystander SCE later proved dependent on homologous recombination, and was increased in non-homologous end joining–deficient cells (Nagasawa *et al.*, 2005). Thus, whilst SCE are not the direct result of radiation-induced DNA breaks, they can act as a marker representing a change in the regulation of DNA-repair processes. If bystander cells do experience such intercellular regulation of DNA-repair, this could be a

mechanism for radioadaptive responses and might help cells to cope with subsequent DNA insults.

**Evidence for chromosomal damage in bystander cells: Induction of micronuclei**

A common surrogate marker for chromosomal aberrations is the formation of micronuclei. The interest in scoring the occurrence of micronuclei came with the observation that when a chromosomal aberration resulted in a chromosome fragment without a centromere, this was not always incorporated into the dividing daughter nuclei (Carrano and Heddle, 1973). These lost chromosomal fragments formed *micronuclei* that were small and distinct from the nucleus but still exhibited a nuclear staining pattern. Further investigation validated the scoring of micronuclei as a method to quantify chromosomal damage induced by radiation and chemical mutagens (Matter and Schmid, 1971; Heddle, 1973; Obe *et al.*, 1975; Countryman and Heddle, 1976). The ease and speed of quantifying micronuclei formation over manual methods for analysing chromosomal aberrations resulted in the widespread use of the *Micronucleus Test* and its acceptance as a classic DNA damage endpoint (Jenssen and Ramel, 1980; Kliesch *et al.*, 1981; Bertsche, 1985). Refinements of the technique include the cytokinesis-block micronucleus assay (Fenech and Morley, 1985, 1986), which prevents the passage of cells through the first mitotic division, thus precluding an underestimation of micronucleus frequency through the proliferation of undamaged cells. Since apoptosis and micronuclei can both be scored from nuclear morphology, they are often each quantified within the same experiment.

Fibroblasts irradiated with α-particles showed a non-linear induction of micronuclei above that predicted from nuclear traversals (Azzam *et al.*, 2001; Azzam *et al.*, 2002) and at low fluences, lindane (an inhibitor of GJIC), superoxide dismutase, catalase

and diphenyliodonium (inhibitors of free radicals) were able to block the induction of micronuclei in bystander cells, implicating both GJIC and oxidative metabolism. Fibroblasts, tissue explants and human skin reconstructions which have previously shown bystander apoptosis after microbeam irradiation have also shown the induction of micronuclei in unirradiated bystanders (Prise *et al.*, 1998; Belyakov *et al.*, 2001; Belyakov *et al.*, 2002; Belyakov *et al.*, 2003; Belyakov *et al.*, 2005), highlighting the link between irreparable DNA damage and apoptotic cell death.

Kashino *et al.* (2004) determined the yield of micronuclei in nonhomologous end joining–deficient (Ku80 mutant), base excision repair–deficient (XRCC1 mutant) and normal CHO cell lines after irradiation of a single nucleus with 1 Gy X-rays. In the nonhomologous end joining–deficient line, single-cell irradiation induced micronuclei after 24 h, but micronuclei were only induced after 48 h in base excision repair–mutants. This delay in formation and the lack of response in cells with normal DNA-repair, indicated that the chromosomal damage causing the formation of micronuclei was the result of sustained perturbation of DNA-maintenance and -repair mechanisms and not of immediate induction of DNA lesions.

Unirradiated neighbours of $^{3}He^{2+}$ ion irradiated cells (Shao *et al.*, 2003d) (Ponnaiya *et al.*, 2004b), or unirradiated cells co-cultured with $^{3}He^{2+}$ ion irradiated cells (Shao *et al.*, 2005) both show increased micronuclei, even down to irradiating a single cell with a single helium ion. However, the magnitude of the bystander response was cell-type dependent and could be abrogated by the addition of a nitric oxide scavenger (Shao *et al.*, 2004b). The bystander micronuclei induced by X-rays or carbon ions has been shown to be dose-dependent (Shao *et al.*, 2001; Shao *et al.*,

2002), conversely, α-particle doses from 0.1–6 Gy each induced a 50% increase in the frequency of micronuclei in co-cultured bystander cells (Wang and Coderre, 2005). Addition of dimethyl sulphoxide completely blocked this bystander effect, and bystander cells must have been in contact with the medium during irradiation for the effect to occur, implicating short-lived free radicals (Wang and Coderre, 2005) as has been observed for bystander micronuclei in other studies (Konopacka and Rzeszowska-Wolny, 2006).

A cautionary finding comes from experiments where fibroblasts exposed to cell culture medium that was irradiated with >10 Gy of α-particles (without cells), show a significant increase in micronuclei (Geard *et al.*, 2002); an effect not seen in epithelial cells. The finding that irradiated medium alone can induce effects in unirradiated cells confirmed the need to pay careful attention to appropriate controls in such ICCM–transfer experiments. It might also be prudent to confirm whether the irradiation of dead (or frozen) cell cultures is able to induce an ICCM effect, implicating the presence of irradiated cellular contents/debris in the generation of a signal.

**Evidence for the induction of mutations in bystander cells**

The two mutation endpoints that have been commonly used to explore bystander effects are the HPRT and CD59 mutagenesis assays. The HPRT assay is based on detecting mutations in the gene encoding the Hypoxanthine-Guanine Phosphoribosyl Transferase (HPRT) enzyme that is involved in DNA synthesis. When a wild-type gene encodes a functional HPRT enzyme, cells are poisoned by the nucleoside analogue 6-thioguanine. Conversely, cells become resistant to 6-thioguanine when a mutation results in an inactivation of the HPRT enzyme. Surviving colonies after

exposure to a dose of ionising radiation represent spontaneous plus ionising radiation–induced mutants.

Cells exposed to low fluences of α-particles have shown an increased number of mutations at the HPRT locus above that expected from the number of nuclear traversals (Nagasawa and Little, 1999) and HPRT mutations have since been detected specifically in unirradiated bystander cells (Zhou *et al.*, 2005). One study has shown that the HPRT mutations induced by α-particles when 44% of cells were traversed, primarily deletions, were distinct from those observed when only 3% of cells were traversed), nearly all point mutations (Huo *et al.*, 2001). The indications of a unique bystander-induced mutation pathway may be a key to elucidating the differences in direct radiation damage and radiomimetic effects initiated in unirradiated cells.

The CD59 mutagenesis assay uses a hybrid mammalian cell line and an introduced reporter gene to detect DNA changes. The cells in the $A_L$ cell line contain a full complement of Chinese hamster chromosomes, plus a single copy of the human chromosome 11, which encodes the cell-surface antigen CD59. A monoclonal antibody against CD59 selectively eliminates cells expressing a wild-type CD59 marker via complement-mediated toxicity. The spectrum of CD59-inactivating mutations ranging from point mutations to large deletions and even chromosome loss is then measured in the surviving cells.

In experiments using low fluences of α-particles, the frequency of CD59 mutations was increased above that assuming no bystander effects (Zhou *et al.*, 2000), and exposing 10% or 20% of $A_L$ cells induced the mutant frequency even higher than in

the 100% irradiated population (Zhou *et al.*, 2002). Exposing $A_L$ cells to a low-dose of X-radiation four hours prior to exposing to $^{3}He^{2+}$ ions significantly reduced this bystander effect (Zhou *et al.*, 2003). An increasing radioactivity of tritium added to CHO cells induced a dose-dependent increase in CD59 mutations (up to 14-fold), and decreased survival, in co-cultured bystander $A_L$ cells (Persaud *et al.*, 2005). The bystander mutations observed exhibited a higher rate of deletions than in spontaneous mutants (Persaud *et al.*, 2005), opposite of the characteristic HPRT– point-mutations observed in the bystander range using α-particles (Huo *et al.*, 2001). Thus, the results from examining mutations in bystander cells point towards mechanisms distinct from mutations caused by direct radiation exposure, and that differ between cell-types and mutation assays.

**Evidence for the initiation of genomic instability in bystander cells**

It has become apparent that genomic instability, as well as being a low-dose radiation phenomenon, may also be an endpoint in itself, induced in bystander cells (reviewed in Lorimore *et al.*, 2003; Morgan, 2003; Kadhim *et al.*, 2004). Even a single α-particle traversal can induce genomic instability in bystander cells, whereby the progeny of bystander cells exhibit a long-term increase in the rate of *de novo* chromosomal damage (Kadhim *et al.*, 2001; Ponnaiya *et al.*, 2004a; Moore *et al.*, 2005b). In experiments utilising an *in vitro* mouse bone marrow transplantation model, a mixture of irradiated and unirradiated male bone marrow cells, which can be distinguished by the presence of a cytogenetic marker, are transplanted into lethally-irradiated (10 Gy) female mice. Following a period to allow re-population of the haemopoietic stem cell compartment, bone marrow cells are analysed for chromosome-aberrations indicative of genomic instability. In these experiments, chromatid breaks, minutes and chromosome fragments significantly increased in the

short-term (<100 days) and long-term (>1 year) progeny of unirradiated cells in donor marrow cells irradiated with 0.5 Gy neutron radiation (Watson *et al.*, 2000) or 4 Gy γ-radiation (Lorimore *et al.*, 2005) and mixed with unirradiated marrow cells prior to transplantation. The doses used in both of these experiments, however, represent extreme radiation exposures outside the range where bystander effects are likely to predominate.

As the long-term progeny of bystander cells are further studied, other endpoints reported as examples of genomic instability such as increased spontaneous rates of micronuclei, SCE, mutations and cytotoxicity (reviewed in Morgan, 2003; Snyder and Morgan, 2004) may also be detected.

**Evidence for altered protein expression in bystander cells**

Specific up- or down-regulation of proteins in unirradiated bystander cells shows extensive variability (*Table 2.1*); a difficulty inherent in cell culture systems. Like many other bystander endpoints, there appear to be differences in the expression of certain proteins (TP53, CDKN1A) in bystander cells according to cell-type. However, the understanding of bystander protein-induction is still limited. Although the proteins induced or suppressed in bystander cells appear related to DNA-repair, cell-cycle control and stress-responses, this may simply represent a bias in the candidate proteins chosen for study.

**Evidence for neoplastic transformation in bystander cells**

The first indication of bystander neoplastic transformation came when irradiating 100% or 10% of unstable fibroblasts with up to eight $^{3}He^{2+}$ ions per cell induced the same level of transformation (Sawant *et al.*, 2001b). ICCM from HeLa/skin

fibroblast hybrid cells exposed to the very high doses of 5 or 7 Gy of X-rays, induced a fourfold increase in neoplastic foci in bystander cells (Lewis *et al.*, 2001). Conversely, neoplastic transformation was induced by 5 Gy α-particles (but not 5 Gy X-rays) in co-cultures of bystander unstable fibroblasts (Mitchell *et al.*, 2004a; Mitchell *et al.*, 2004b), whilst in other systems, neoplastic transformation has been induced in bystander cells co-cultured with cells exposed to as little as 100 mGy γ-rays (Weber *et al.*, 2005). It is only with these results, showing an increase in carcinogenic transformation that the induction of cell death, proliferation, and mutation, compete to give an overall indication of the final carcinogenic outcome in bystander cells.

**Table 2.1: Summary of protein expression changes in bystander cells**

| | Protein | Change | Species | Cell-type | Reference |
|---|---|---|---|---|---|
| Tumour suppressor p53 | TP53 | ↑ | Human | Skin fibroblasts, Glioblastoma cells | (Azzam *et al.*, 1998; Azzam *et al.*, 2001; Matsumoto *et al.*, 2001; Azzam *et al.*, 2002; Little *et al.*, 2002) |
| Tumour suppressor p53 | TP53 | ↓ | Human | Lung fibroblasts | (Iyer *et al.*, 2000; Iyer and Lehnert, 2002b) |
| Cyclin-dependent kinase inhibitor1a | CDKN1A | ↑ | Human | Skin fibroblasts | (Azzam *et al.*, 1998; Azzam *et al.*, 2001; Azzam *et al.*, 2002; Little *et al.*, 2002; Yang *et al.*, 2005) |
| Cyclin-dependent kinase inhibitor1a | CDKN1A | ↓ | Human | Lung fibroblasts | (Iyer *et al.*, 2000; Iyer and Lehnert, 2002b) |
| Cyclin-dependent kinase 1 | CDC2 | ↑ | Human | Skin fibroblasts | (Azzam *et al.*, 1998) |
| G2/mitotic-specific cyclin-B1 | CCNB1 | ↑ | Human | Skin fibroblasts | (Azzam *et al.*, 1998) |
| DNA repair protein Rad51 | RAD51 | ↑ | Human | Skin fibroblasts | (Azzam *et al.*, 1998) |
| Nuclear factor NFκB | NFκB | ↑ | Human | Skin fibroblasts | (Azzam *et al.*, 2002) |
| p38 mitogen-activated protein kinase | p38 MAPK | ↑ | Human | Skin fibroblasts | (Azzam *et al.*, 2002) |
| Proliferating cell nuclear antigen | PCNA | ↑ | Rat | Primary tracheal cells | (Hill *et al.*, 2005) |
| DNA-directed RNA polymerase | RPA | ↑ | Human | Foetal lung fibroblasts | (Balajee *et al.*, 2004) |
| Apurinic-Apyrimidinic endonuclease | APE | ↑ | Human | Foetal lung fibroblasts | (Balajee *et al.*, 2004) |
| Heat shock protein 72 | HSP72 | ↑ | Human | Glioblastoma cells | (Matsumoto *et al.*, 2001) |

Increases (↑) or decreases (↓) in the levels of proteins, compared to controls, were observed in bystander cells under a variety of experimental conditions. Note the opposite changes in TP53 and CDKN1A observed between studies.

**Evidence against bystander effects**

Published studies showing no bystander effects are hard to find. However, rather than an indication of the universality of bystander effects following radiation exposures, the lack of evidence against bystander signalling is likely due to publication bias. Experiments that show a linear relationship between biological effects and the number of cells hit are unlikely to pique interest; and systems that show robust effects are favoured (Seymour and Mothersill, 2000), whilst those that do not are abandoned. Some authors have directly reported a failure to detect bystander signalling; unirradiated human fibroblasts grown in three-dimensional culture with $^{3}H$ radiolabelled cells do not show any evidence of the cell-cycle arrest seen in the irradiated cells (Pinto *et al.*, 2006). However, most evidence that does not support bystander effects is couched amongst pro-bystander findings, cast as examples of conditions not conducive to bystander signalling. For example,

- No effect of ICCM on fibroblasts: cell-type dependence (Mothersill and Seymour, 1997)
- No effect in CBA/Ca mice: genotype dependence (Mothersill *et al.*, 2005b)
- Bystander effects not occurring in all replicates: binary on/off induction (Moore *et al.*, 2005b; Schettino *et al.*, 2005)
- No bystander effect after γ-radiation: LET-dependence (Xue *et al.*, 2002)
- Bystander effects not occurring in wild-type cell lines: extreme sensitivity of DNA-repair deficient cells (Kashino *et al.*, 2004; Nagasawa *et al.*, 2005)

Whether each of the cases in which bystander effects were not observed are truly indications of particular dependence on cell-type, genotype or LET is not clear. What is clear is that some irradiated cells, under some circumstances can induce radiomimetic effects in neighbouring unirradiated cells *in vitro*.

## Bystander effects: moving from phenomenon to risk

Over fifteen years since research began in earnest, bystander effects still retain the status of intriguing, yet mysterious phenomena. In part, this is because there is no single bystander 'effect', but rather an entire class of non-targeted effects that were previously outside the scope of the classical double-strand break–induced mutation paradigm. The inclusion of bystander effects in risk assessment means the sensitive target for radiation-induced carcinogenesis would grow from the size of a single cell nucleus, to whole organs and beyond. The expansion of the target volume introduces further complexity and poses the risk of turning the currently parsimonious linear dose-response relationship into a tangled web of networks interacting on multiple levels, i.e. systems biology.

The recent ICRP 99 report *Low-dose Extrapolation of Radiation-related Cancer Risk* explained why the evaluation of carcinogenic risk did not incorporate bystander effects (and other low-dose phenomena), saying that:

> … a better understanding of the mechanisms for these phenomena [*adaptive response, genomic instability and bystander effects*], the extent to which they are active in vivo, and how they are inter-related is needed before they can be confirmed as factors to be included in the estimation of potential risk to the human population of exposure to low levels of ionizing radiation. (International Commission on Radiological Protection, 2006, p.76).

The maturation of bystander effects from low-dose curiosities to assessable risks, or, conversely, their debunking as *in vitro* artefacts, rests on the answering of a number of unresolved questions.

*Is there a parallel effect in vivo?*

The primary question is one of relevance. Without translation to the human context, an intimate understanding of bystander signalling, dose-responses and effector mechanisms is inaccessible for risk-assessment. Extrapolation to the *in vivo* exposure situation is difficult without the observation of a single, predictable bystander effect across a wide range of *in vitro* systems. The variable nature of bystander effects, casts doubt over the validity of modelling *in vivo* scenarios based on *in vitro* derived parameters. The initial step in incorporating bystander effects into risk assessment is to determine whether anything like the bystander effects observed *in vitro*, truly do occur *in vivo*. This is the basis of *Research Need 3* of the BEIRVII committee (Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation, 2006, p.16) and is regarded by many as the key question in the bystander effect field: '…it must first be determined if the bystander effects can [*sic*] observed *in vivo'* (Brooks, 2004, p.68). If an *in vivo* bystander effect can be seen, it will then be important to ascertain whether the effect falls into the realm of public radiation protection or is limited to high-dose, partial body exposures such as medical radiotherapy (if the effect is closer to abscopal effects).

*Are we at risk?*

If bystander effects do occur *in vivo*, the next question is whether they pose a real, significant risk above current estimates at exposures relevant to the general population. Key to this determination will be an understanding as to whether the effects are restricted to high-LET exposures or are relevant to chronic, low dose-rate, low-LET irradiation. This specificity will affect the types of tissues and cells that are at risk, since the quality of the radiation also determines tissue penetration. The

modifying effects of dose and dose-rate will determine the contribution of bystander effects to the carcinogenic risk compared to the direct effects.

Discussion of bystander effects in terms of their potential to propagate damage to unirradiated cells overlooks a number of biological endpoints induced in bystander cells that have the prospect of providing a beneficial effect:

> It is unclear at this time whether the bystander effect would have a net positive or net negative effect on the health of an irradiated person (Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation, 2006, p.9).

Bystander-induced killing of normal cells might be detrimental to a tissue, but will not increase cancer risk; yet, secondary induction of proliferation to replace any eliminated cells could potentially promote carcinogenesis. Similarly, exploitation of bystander-induced tumour killing could provide novel cancer therapeutic strategies that avoid high-dose radiation side effects.

*How do we quantify the risk?*

If bystander effects do increase carcinogenic risk, future research will need to establish the hazardous exposure range and assess the associated risk levels in order to justify any risk-mitigation policies. *In vitro* data already suggest that this may be the most difficult aspect of dealing with bystander effects, as the scenario of a unified, predictable, dose-proportionate induction of bystander effects seems unlikely: 'If bystander effects do exist, it may be necessary to alter both the calculation of dose and the prediction of risk' (Brooks, 2004). Some studies have shown the induction of bystander effects *in vitro* to be dose-dependent but the magnitude of the response to be independent of dose (Schettino *et al.*, 2005). A

stochastic initiation of bystander signalling is the most favoured model to date (Brenner *et al.*, 2001), with an all-on/all-off response triggered with saturable dose-dependent probability (Schettino *et al.*, 2005).

Radiation quality–dependent induction of bystander effects could further complicate risk-estimation when dealing with exposures to mixed radiation fields or sequential exposures. All of these considerations may result in much the same approach used today; that is, acceptance of bystander effects *in vivo* after low-dose exposures may simply prompt more conservative exposure limits and lower safe-working levels. Whether accurate models can be used to estimate additional risk from a few, known exposure parameters, may decide whether bystander effect–inclusive regulation is possible, or if it will prompt the addition of yet another modifying factor to the current LNT model.

*Are there interactions with other low-dose effects?*

Finally, it is becoming increasingly obvious from experimental data, that bystander effects may be only one manifestation of a larger set of interrelated low-dose phenomena including radioadaptive responses, genomic instability and low-dose hypersensitivity (reviewed in ICRP 99: International Commission on Radiological Protection, 2006, p. 76). The propagation of radiomimetic effects to unirradiated cells may include the transmission of one or more of these low-dose effects, which would truly mark the end of the cell's era as the radiation biological target. Future experiments will continue to investigate how bystander signalling interconnects with these other non-linear responses in an effort to understand the collective outcome of the low-dose radiation phenomena on carcinogenic risk.

## Summary

The presence of radiation-induced biological effects in unirradiated cells, the noncumulative effects of repeated exposures and the difference in effective damage caused per unit dose at low doses – as well as potential interactions between these phenomena (Mothersill and Seymour, 2006b; Shankar *et al.*, 2006; Schwartz, 2007) now challenge the validity of the linear no-threshold model (Edwards and Bouffler, 2005; Breckow, 2006). The predictive value of risk models is increasingly important, especially since our exposure to radiation in everyday life seems set to increase, particularly from diagnostic imaging modalities such as computed tomography scanning (Kai, 2005; Brenner and Hall, 2007; Mayo, 2008). Currently, the use of the linear no-threshold model for radiation protection standards is recognised by most as a pragmatic measure rather than a biological reality (Breckow, 2006), the 'best we can do—today' (Preston, 2003).

However, the key to moving forward both pragmatically and scientifically, is to consolidate the large amount of data gathered on mechanisms, pathways, endpoints, and phenomena, to inform the ultimate question of risk. Risk will ultimately be the superimposition of a range of signals, mechanisms and effects (Breckow, 2006), to create a response greater than the sum of its parts – an emergent property of a system (Mothersill *et al.*, 2005a; Barcellos-Hoff and Costes, 2006). Risk is thus, a quantity necessarily determined *in vivo* where the various elements of the *system* converge to produce effects that can predict the response of humans to radiation exposure. In this context, the next chapter of this thesis describes the way the investigations conducted here aimed to contribute to the understanding of radiation-induced bystander effects.