Chapter 6: Evaluation of the adoptive transfer bystander method and its initial findings

Précis

The method described in this thesis allows the introduction of irradiated cells into an unirradiated organ in order to monitor the reactions of the surrounding recipient cells. This chapter assesses the suitability, reliability and generalisability, of the adoptive transfer method, as used in the current study, and addresses the significance and implications of failing to detect a bystander effect in this *in vivo* system. An analysis of whether the adoptive transfer method was able to meet the aims of the study is provided, along with proposals for future use of the method to answer remaining questions. Included throughout are updates on the literature published in the bystander field since the commencement of the study.

Assessing the research findings

The aim of this research was to determine whether intercellular communication between radiation hit and unhit cells changes the risk of radiation-induced cancer from a linear dependence on radiation dose. Achieving this aim required exploration of radiation-induced bystander effects *in vivo*, posing three research questions:

1. Do radiation-induced bystander effects of the nature seen in cell culture investigations occur *in vivo*; if so,
2. Do the biological effects induced in unhit cells change the fate of those cells, so as to result in an altered risk of developing cancer; and,
3. Can such bystander effects be triggered at the low radiation dose-rates required to result in non-uniform dose exposure?

Efforts to resolve these questions involved establishing an adoptive transfer method in mice. Answering the first question began by investigating the existence of bystander effects *in vivo* in a series of experiments mimicking conditions previously conducive to the observation of such effects *in vitro*. The experiments started on the *a priori* assumption of no bystander effect, that is, that radiation-induced effects are limited to directly irradiated cells. The experiments were carried out under the conditions considered most relevant to the application of bystander effects to human radiation risk-estimation. The initial experimental data obtained using the adoptive transfer method did not identify a bystander response for apoptosis or proliferation.

From the outset, the scope of this study was limited to searching for bystander effects *of the nature seen in cell culture investigations*. Thus, with no evidence for bystander effects in the adoptive transfer system using the initial experimental conditions, the experiments progressed to test other conditions or factors known to be crucial to previous observations of bystander effects *in vitro*. Alterations in the dose-rate or radiation source, the timing of analysis or the proportion of irradiated cells within the unirradiated spleen tissue, still provided no evidence of a bystander effect. Since no bystander effects were observed, the second and third research questions (concerning characterising any bystander effects that were detected) were not answered in this study.

Although detecting a bystander effect using the *in vivo* model would have provided an affirmative answer to the first research question, the failure to observe one does not prove that bystander effects do not occur. This research endeavoured to meet the need of radiation risk-assessors and -regulators to determine whether current radiation risk-management policy is adequate, or, if bystander effects pose an

additional risk for including in future risk-assessments. Without confidently answering the bystander question, the *status quo* of bystander effects as curious phenomena will remain. Thus, achieving the overall aim requires assessing confidence in the results of this study.

If confident that the findings of this research are suitable, reliable, and generalisable, it is important to determine the meaning and significance of these results. Irradiated cells clearly have the capability to induce biological effects in neighbouring, unirradiated cells; the evidence for this ability *in vitro* is overwhelming. The reasons why this might not be the case *in vivo* (whether only in this system or generally) are crucial to the decision of whether bystander effects are relevant to human radiation exposures. Without demonstration of bystander effects *in vivo*, regulators are not willing to include them in risk assessments; conversely, without a satisfactory explanation as to why they might not occur *in vivo*, it is unlikely that the many *in vitro* observations of bystander effects can be easily dismissed.

Suitability of the adoptive transfer method

The suitability of the adoptive transfer method described here, essentially pivots on whether the experimental design precluded the generation of an effect that would otherwise have occurred given optimal conditions. This question is, from the outset, unanswerable; that is to say, it provides us *propter hoc* with no testable hypothesis. Without a proven bystander effect, the existence of optimal conditions is merely hypothetical. However, it is possible to determine whether the experimental design and implementation was capable of providing the normal *in vivo* conditions it sought to replicate.

Were the recipient bystander cells perturbed from normal behaviour?

The advantage of the adoptive transfer method was that unlike other attempts to study bystander effects *in vivo*, the unirradiated cells were:

- not manipulated and were native to the host mouse;
- not immortalised or tumour cells;
- comprised of a range of cell-types;
- non-clonal;
- not cell-cycle synchronised;
- truly unirradiated; and,
- examined *in situ*.

The ability to study unirradiated cells in such a natural state is unique amongst the attempts made thus far to detect bystander effects *in vivo*. Other *in vivo* experimental methods have examined human tumour cells in mice (Xue *et al.*, 2002; Kishikawa *et al.*, 2006) or repopulation of lethally irradiated hosts with mixes of irradiated and unirradiated congenic bone marrow cells (Watson *et al.*, 2000; Lorimore *et al.*, 2005). The conditions used in the adoptive transfer method gave the best chance of observing the normal behaviour of unirradiated cells in a tissue exposed to isolated, irradiated cells, as would occur in a tissue exposed to a very low-fluence of photons or charged particles.

Were the donor cells perturbed from normal behaviour?

Results from experiments using very low whole-body radiation exposures (Hooker *et al.*, 2004b; Day *et al.*, 2006; Zeng *et al.*, 2006) provide indications of bystander signalling in tissues where the majority of cells do not receive radiation

deposition. However, these experiments, whilst providing the ideal conditions of irradiating unmanipulated cells *in situ,* cannot prove that the observed effects are not occurring solely in the irradiated cells, as the hit and unhit cells are not distinguishable. Currently, this limitation applies to all *in vivo* attempts to study bystander effects, that is, technology at present does not allow the precise irradiation of living cells *in situ*, in a whole animal with certain identification of the irradiated cells. Thus, whilst the use of *ex vivo* cultured and irradiated/radiolabelled cells is not ideal, it is one of few options available at present.

It is reasonable to assume that removing the donor cells from the mouse spleen alone would have somewhat perturbed their behaviour. This can be seen in the sharp decline in cell numbers during the first day in culture. Further, measured apoptosis levels in the spleen vary considerably depending on whether the cells are analysed *in situ* or after removal from the spleen (Komarova *et al.*, 2000; Takahashi *et al.*, 2001b; Wang *et al.*, 2002). It is for this reason, that the time in culture was kept to a minimum to avoid the instabilities known to affect long-term cultured primary spleen cells (Hu *et al.*, 1992). However, despite the changes induced by *ex vivo* manipulation, donor splenocytes once lodged back in a recipient spleen, do continue to function normally (Manfra *et al.*, 2001) suggesting the changes are only transient.

The use of concanavalin A was necessary, and posed the greatest potential for altering the response of the donor cells. Within minutes of concanavalin A binding to the cell-surface, increases are detectable in the cellular constituents necessary for cell growth and division, such as glucose, phospholipids, cyclic adenosine and guanosine 3’:5’ monophosphates and potassium (Hadden *et al.*, 1976). Concanavalin A causes an increase in lymphocyte volumes even before the first division, and produces large

lymphoblasts from 180–600 μm^3 (resting splenic lymphocyte volume ≈ 120 μm^3 (Sanderson *et al.*, 1980)). Concanavalin A stimulated spleen cells show a decreased immune response *in vitro* to erythrocyte antigens (Dutton, 1972), and changes in TGF-β (Ellingsworth *et al.*, 1989), glucocorticoids and nitric oxide (Ramirez and Silva, 1997). Thus, mitogen stimulation of the donor cells induces significant changes from their resting state, and is perhaps, the largest uncertainty in the attempt to re-create a normal, bystander environment.

However, the large lymphoblasts induced by the lectin-stimulation are similar to those which arise in culture from cells whose division began *in vivo*, and are naturally found at low levels in unstimulated spleen cell cultures (Piguet *et al.*, 1976), suggesting that the lectin-stimulated phenotype and associated changes in cell growth constituents are merely an induction of a natural activated T cell blast state (Tutt *et al.*, 1995). Further, the acute X-ray experiment did not use mitogen-stimulated donor cells and like the other experiments, showed no bystander effects. Given the alternative (use of an immortalised or tumour cell line), primary spleen cells, even with the stress of *ex vivo* manipulation and the changes induced by mitogen stimulation, still represent the closest model to date for studying the response of normal spleen cells to radiation.

Did the radiation stimulus applied to the donor cells adequately simulate the desired scenario?

As explained above, the ideal scenario for studying bystander effects *in vivo* would examine the immediate and long-term effects of cells irradiated *in situ*; although current technological limitations made this impossible. It was necessary instead to decide which *ex vivo* irradiation method would most closely mimic both the relevant

situation *in vivo* and the conditions used in previous experiments *in vitro*. Two methods were chosen, a chronic self-irradiation begun 18 h before adoptive transfer, and a single acute dose applied 1–2 h before injecting the donor cells.

For the chronic irradiations, it is necessary to consider a number of possible confounding factors. Firstly, did initiating the radiation dose *in vitro* alter the later response of the donor cells *in vivo*? It is important to remember that almost all observations of bystander effects have been in cultured cells or tissues. It is thus reasonable to assume that whatever the initiating events in irradiated cells might be, they must be able to occur under standard *in vitro* conditions. One scenario that might make such chronic radiolabelling unsuitable for bystander experiments is if after initiating a bystander signal *ex vivo*, an irradiated cell becomes permanently or temporarily refractory to re-signalling. If this was the case, the prior *ex vivo* exposure might hinder any response to later ^{3}H-disintegrations occurring *in vivo*.

Other bystander experiments involving chronic self-irradiation of target cells use similar periods of radiolabelling prior to introduction into the animal (Xue *et al.*, 2002), cell cluster (Gerashchenko and Howell, 2004) or transfer of conditioned-medium (Boyd *et al.*, 2006); and in these experiments, the *in vitro* or *ex vivo* irradiations do not prevent the later transmission of a bystander effect. The fact that introduction of pre-radiolabelled cells induces a bystander response at all indicates that either: there is no refractory period, the refractory period is short and does not last into the post-labelling period, or, if there is a substantial refractory period, the original signal persists for the length of the refractory period.

Chronic radiolabelling experiments have shown a dose-independence for bystander proliferation after ^{3}H-thymidine dose-rates from 2.5 – 12.5 ^{3}H disintegrations per cell per hour (Gerashchenko and Howell, 2004). Since each β-particle deposition represents the same mean dose (whatever the radioactivity of the labelled cells), high and low radioactivity (dose-rates) only represent the frequency of repetition of the same nominal radiation insult. No increased effect with increasing radioactivity suggests that the continuing, repeated radiation depositions do not alter the response to the initial decays. In other chronic radiolabelling studies, the degree of bystander cell killing (Persaud *et al.*, 2005) and bystander mutagenesis (Persaud *et al.*, 2007) do show a dependence on ^{3}H radioactivity and thus in some cases, dose repetition does provide an enhancement.

When chronically irradiated cells are sensitive to further energy depositions, it is still unclear how the continually applied dose-rate affects the duration or magnitude of the initial signal. A number of scenarios are possible following initiation of the signal, where after a given number of energy depositions:

- subsequent decays continue to increase disruption to the donor cell, which increases the level or nature of the signal;
- subsequent decays continue to increase (or maintain) the initial disruption resulting in an uninterrupted continuation of the signal; or,
- subsequent decays repeat the initial disruption, each time resulting in re-transmission of the bystander signal (of like, diminishing, or increasing intensity).

The data are not currently available to evaluate which of these scenarios is the case, or whether the radiation type, energy, dose-rate, or other interacting factors determine the outcome. The difference between chronic irradiation of the same cells, and the presence of multiple irradiated cells due to chronic irradiation of a tissue, can confuse discussion of bystander signalling at low-dose rates. In the former case, the same subset of cells receive repeated radiation-stimuli; in the latter, the chances of the same cell receiving a second radiation dose is minimal, the continuation of the dose-rate merely results in a steady-state number of cells irradiated within a given window of time. The relevant human situation is chronic, low-dose radiation exposures, for which bystander effects would only be of concern in the range of single hits, rarely occurring multiple hits at the same instant (*Poisson* effect at low mean instantaneous doses), or rare occasions of multiple hits separated by time (determined by the dose-rate). As such, the effect of chronic cellular irradiation on maintenance or escalation of bystander signalling is largely an experimental concern and is unlikely to play a significant role in risk assessment. Nevertheless, the potential difference in effects from chronic versus acute dose-rates to the directly irradiated cells should be taken into account when interpreting these results.

The same concerns over the duration of bystander signalling or the longevity of the signal itself are also relevant to the acute irradiation experiment conducted in this study. Due to practical constraints, there was a necessary delay between the irradiation of the donor cells and their lodging in the recipient mouse spleen. This delay was comprised of the time for transporting the donor cells from the site of irradiation to the animal facility, the time to inject the donor cells and the time taken to lodge in the spleen (total of 1–2 h). There is evidence suggesting that the delay between irradiation and contact with the bystander environment would impede a

bystander effect, and conversely, evidence that an effect would still be induced. Evidence supporting the former scenario, are the findings in human prostate carcinoma cells showing micronuclei in co-cultured bystander cells (Wang and Coderre, 2005) but only when bystander cells were in contact with the irradiated-cell medium during irradiation (not when co-cultured immediately after). In medium-transfer experiments, ICCM could induce DNA breaks in bystander cells when collected as early as 2.5 min after irradiation, with the signal decreasing when collected after 10 min and lasting only out to 30 min (Han *et al.*, 2007). Also supporting the immediacy of the effect are findings that exposure to ICCM induces calcium signalling in bystander cells in a little as 30 s (Lyng *et al.*, 2002a; Shao *et al.*, 2006).

Experimental evidence is also available to support the hypothesis of a long-lived activation state in irradiated cells. Conditioned medium collected from cultured mouse embryonic stem cells, two weeks after irradiation, induced DNA breaks in bystander cells (Rugo *et al.*, 2007). Even more surprising, was the finding that medium collected from these bystander cell cultures three weeks after exposure to ICCM, was able to induce DNA damage in so-called *secondary* bystander cells. ICCM collected from 30 min up to 60 h after irradiation is still able to reduce clonogenic survival in bystander epithelial cells (Mothersill and Seymour, 1997) and elevations in ROS in directly irradiated cells persist up to 60 h after irradiation and 30 h after exposure to ICCM in bystander cells (Yang *et al.*, 2005). Cells exposed to γ-rays, then washed twice, trypsinised, re-plated and co-cultured with unirradiated cells are still able to induce a bystander effect despite the delays (Gerashchenko and Howell, 2003a; Gerashchenko *et al.*, 2004). Ideally, an acute irradiation scenario should aim to reduce the delay between irradiation and exposure to the bystander

cells. Future experiments using this adoptive transfer system should endeavour to streamline the protocol to ensure the detection of short-term signalling events.

Did the donor cells appropriately interact with the indigenous recipient spleen environment?

A number of studies showing that the bystander effect is dependent on the plating density of the irradiated cultures have implicated cell contact–mediated communication in bystander signalling (Azzam *et al.*, 1998; Mitchell *et al.*, 2004b). The use of cells deficient in the protein connexin 43 (an essential component of the GJIC apparatus) and the use of lindane (an inhibitor of gap-junctions) has further supported a direct role for GJIC (Azzam *et al.*, 2001; Persaud *et al.*, 2005). The addition of lindane reduces or removes the bystander effect measured by γ-H2AX foci formation (Hu *et al.*, 2006), CD59 mutagenesis (Zhou *et al.*, 2002; Persaud *et al.*, 2005), protein induction (Azzam *et al.*, 1998), the induction of micronuclei (Little *et al.*, 2002) and proliferation (Bishayee *et al.*, 2001). The cause of this suppression is not merely a toxic effect specific to lindane; other GJIC inhibitors also have the ability to block bystander signalling, including 1,19 bis(chlorophenyl)-2,2,2-trichloroethane (DDT), dieldrin, 12-*O*-tetradecanoylphorbol-13-acetate and phorbol myristate acid (Azzam *et al.*, 2001; Shao *et al.*, 2003c; Mancuso *et al.*, 2008). Curiously however, phorbol myristate acid has also been noted to enhance bystander killing in epithelial cells (Mothersill and Seymour, 1998) raising the possibility of negative feedback controls within bystander signalling pathways. Despite clear demonstrations of the involvement of GJIC, a number of findings have confused its role in bystander signalling. Some bystander effects are dependent on cell–cell contact, but not on GJIC (Gerashchenko and Howell, 2003a; Mitra and Krishna, 2007). Blocking GJIC removed the bystander effect observed in

cell-mixing experiments but had no effect in media-transfer experiments using the same cells and radiation doses (Sokolov *et al.*, 2005); within a single experiment, some bystander endpoints were shown to be dependent on GJIC whilst others were not (Shao *et al.*, 2003b). Together, these findings suggest a role for GJIC in transmission of certain bystander signals, but one that can exist in concert with overlapping, soluble-mediator signalling pathways.

Cells in the human (and mouse) spleen are well known to communicate via gap-junctions (Betoulle *et al.*, 2000), between like (homocellular signalling) and differing (heterocellular signalling) cell types (reviewed in Wong *et al.*, 2004). It is thought that GJIC plays an important role in stromal regulation of leukocyte maturation and differentiation during development and periods of heightened haematopoiesis. Further, lymphocytes activated with concanavalin A (as were the donor cells in this study) are known to increase the formation of, and their communication via, gap junctions (Sáez *et al.*, 1998). The spleen, as used in the current study, therefore, offers the ideal environment for studying bystander signalling, whether via GJIC, soluble mediators or a combination of the two.

Another question is whether the donor cells remain stationary within the spleen in the position where they first lodged (and are eventually recorded during the local screen) or if they are in regular motion, or even re-circulating. This issue is important for two reasons: firstly, did the cells interact with the indigenous environment long enough to engage in intercellular signalling, and secondly, have the bystander cells actually been under the influence of the donor cell(s) present at the moment of cryopreservation?

Data from many adoptive transfer experiments provide confidence that most of the donor cells identified in the spleen have remained *in situ* since first arriving (Butcher and Ford, 1986; Pabst and Binns, 1989; McEvoy *et al.*, 1997; Albright *et al.*, 1998; Manfra *et al.*, 2001; Moeller *et al.*, 2003), allowing plenty of time to interact with their local environment; those that have not remained stationary have most likely left the spleen. The global screening method (which does not rely on the presence of a donor cell within the field) is not affected by these concerns, and can detect if recipient cells are responding to the signals sent by previously lodged donor cells that are no longer visible. Further, since the donor cells lodge loosely in three-dimensional clusters, local screening fields may in fact be recording the effects of the donor cells that are still present as well as from those that are no longer there. This means that although it is not certain that in any one local field, the recipient cells have been engaged with the donor cell in question for the duration of the experiment, it is very likely that in all cases, they have been in proximity to at least one donor cell for an extended period.

Summary

The adoptive transfer method in mice described here, by no means perfectly replicates the human *in vivo* situation. However, the method represents the study of normal, unirradiated cells within a living, functioning organ and monitors the response to introduced normal irradiated cells. Although the donor cells did undergo *ex vivo* manipulation and mitogen-stimulation, the effect of these procedures on the behaviour of the cells can certainly be no more than that exhibited by virus or cell fusion immortalised cells, or cultured tumour cells, as currently used to perform most bystander effect experiments. In addition, no effects were induced by unstimulated donor cells exposed to an acute X-ray dose. The adoptive transfer method is the most

suitable technique to date for the detection of bystander responses *in vivo* and future improvements in the protocols should enable the realistic human situation to be even more closely modelled. Techniques for concentrating radiolabelled compounds within cells without the need for DNA incorporation (Boyd *et al.*, 2006) could help alleviate concerns over mitogen-stimulation and enable complete, rapid radiolabelling of donor cells; and refinements of the acute irradiation protocol should allow the time between irradiation and lodging in the spleen to be reduced.

Reliability of the adoptive transfer method

The question of the reliability of the adoptive transfer method, essentially asks whether the experimental design precluded the detection of any bystander effects that may have occurred. Even given optimal experimental conditions and a robust bystander effect, an insensitive assay, flawed screening technique or inadequate controls, could each compromise the detection of a bystander effect *in vivo*. By definition, answering this question fully is not possible; one only knows that an effect was not detected if one is sure it was actually there. However, the sensitivity of the method can be determined and the power of the statistics assessed, in order to evaluate the degree of confidence that can be placed in the results. Likewise, understanding the screening protocol and use of controls will help to define exactly what was measured and what was not observed.

Were the controls adequate?

An *in vivo* method of the kind used here involves a great deal many more parameters than equivalent methods *in vitro*. In order to control for all aspects of the protocol, each experiment included recipient mice injected with sham-radiolabelled or sham-irradiated donor cells, thus comparing responses of unirradiated tissues to donor cells differing only by their irradiation. For the chronic radiolabelling experiments, the sham-radiolabelled cells even received the same molar concentration of exogenous thymidine as the irradiated cells. Most bystander experiments using chronic radiolabelling do not include this control, either in the sham-radiolabelled groups or to keep the total thymidine concentration consistent between ^{3}H-thymidine dose groups (Bishayee *et al.*, 2001; Gerashchenko and Howell, 2004, 2005; Persaud *et al.*, 2005; Persaud *et al.*, 2007). The maintenance of a consistent total thymidine

concentration for both radiolabelled and control cells is important given the known potential for toxicity from exogenous thymidine alone (Cleaver, 1967; Fox *et al.*, 1980; Ferraro *et al.*, 2005).

The use of sham-donor cells was crucial for the local screening method. Since adoptively transferred cells do not lodge randomly in the spleen, apoptosis and proliferation in the local area around irradiated donor cells might be expected to differ naturally from the average frequencies across an unirradiated mouse spleen. In these experiments, the local and global frequencies (for apoptosis and proliferation) were significantly different from each other, possibly due to the non-random lodging of donor cells, confirming the need for such parallel controls. Although not included in each of the experiments, mice receiving only PBS without donor cells (vehicle control) and mice undergoing anaesthesia but no injection (procedure control) were included in the most recent of the experiments conducted in this study. Although the number of animals was small, $n = 3$ (PBS) and $n = 2$ (anaesthesia only), the frequencies of apoptosis and proliferation were similar to those in the sham-radiolabelled recipient mice. The routine inclusion of such control mice in the future will add further assurance that the handling and injections do not significantly alter the biological endpoints in the spleen.

Did the chosen screening protocols adequately survey the populations of interest?

The bystander cells studied using this adoptive transfer method, using either the local or global screening methods, included all of the cell-types normally resident within the mouse spleen. Although biased towards areas with lodged donor cells, the local screen still examined biological endpoints in all non-donor cells in each field. This indiscriminate method did introduce possible variation between fields (and between

mice) in terms of which bystander cells were actually present. However, no study has been conducted to show a difference between spleen cell-types in the potential to respond to bystander signalling *in vitro*, as has been performed with fibroblasts and epithelial cells (Mothersill and Seymour, 1997). Normal direct cell–cell communication of lymphocytes in the spleen occurs not only between lymphocytes, but also from lymphocytes to antigen-presenting and endothelial cells (reviewed in Wong *et al.*, 2004). It is reasonable to assume that if *in vivo* bystander signalling exists, its role should not be confined to the cell-type that received the energy deposition; in fact, intercellular signalling in other biological contexts often permits the regulation of one cell-type by another (Rosendaal *et al.*, 1991). In one experimental system, irradiated antigen-presenting cells induced a bystander effect in a T lymphocyte line *in vitro* (Liu *et al.*, 2004). Since tissue-level responses to sparse ionisations in isolated cells pose the greatest potential to modify carcinogenic risk, it is prudent to examine the responses of the spleen tissue as a whole. Upon detecting a bystander effect, interrogating the individual cell-types to profile the response would not be a difficult task.

The use of the local screening method allows the analysis of the immediate area vicinal to the irradiated cells for short-range effects, possibly diluted if examined without respect to the topography. Permanently recording the staining of every field permits later changing of the spatial resolution to re-examine a radius anywhere from 100 µm down to a single cell-diameter from the irradiated cell(s); and this re-analysis could be performed in the future. Use of the global screening method allows a very sensitive examination of the tissue, surveying > 160,000 bystander cells across two tissue sections. The adoptive transfer method used here, could also be adapted to analyse the response of the whole spleen tissue via flow cytometry. However, as

noted above, preserving the spatial relationships between the irradiated and bystander cells is of great advantage whilst first exploring bystander effects *in vivo*. If a reproducible, robust bystander response is characterised, as has happened with certain *in vitro* methods, incorporating flow cytometric analysis of fixed bystander cells might represent a high-throughput method to quantify bystander responses *in vivo* using the adoptive transfer method.

How sensitive were the chosen screening protocols and biological measurements?

Sensitivity is a multi-faceted concept in reviewing the performance of an assay system, the term covering measurements of precision, inherent population variability, and prospective and retrospective statistical power. A great number of metrics are available for assessing each of these qualities.

Precision

The precision of the apoptosis, proliferation, and inversion assays primarily relates to the number of *events* scored compared to the number of cells surveyed. For the local screening assays, the number of cells surveyed varied due to the number of local fields available in the tissue sections (related to donor cell lodging frequency and spleen cross-sectional area) and the number of cells in each field (*Table 6.1*). Compared to the initial experiment, the longer-term lodging, higher radioactivity and acute X-ray experiments each resulted in a lower donor cell lodging frequency, fewer fields scored, and therefore less cells surveyed. At the lowest cell numbers surveyed (13,336 cells: 1 Gy X-rays) this equates each apoptotic cell to a 7.5×10^{-5} change in the apoptosis frequency; at the highest, (28,526 cells: Sham) each apoptotic cell represents a 3.5×10^{-5} change in the apoptosis frequency. Thus, the conservative

precision for the local apoptosis assay is 1×10^{-4}. Since the proliferation index uses two exact area measurements for each field, the precision of each arbitrarily truncated at 1×10^{-4}, the quotient of the two parameters is then truncated further, giving a precision of 1×10^{-3} or 0.1%.

Despite the much larger number of total number of cells scored in the global screens (*Table 6.2*), the precision of the global apoptosis frequency (10^{-4}) and global proliferation index (0.1%) was set as for the local screens, given that the variation between individual mice was relatively larger than between the fields of each mouse. For the pKZ1 chromosomal inversion assay, which surveyed a mean number of 187,962 cells per replicate section, a single inversion event equated to a change of 5×10^{-6} in the inversion frequency; thus, giving a conservative precision of 1×10^{-5}.

Table 6.1: Number of bystander cells and fields scored in the local screens

Experiment	Mean number of bystander cells scored per spleen section			Mean number of local fields per spleen section [a]		
	Sham-radiolabelled		*Radiolabelled*	*Sham-radiolabelled*		*Radiolabelled*
Initial Experiments	28,526		27,112	25.7		24.5
Longer-term lodging experiments	22,001		21,592	20.6		20
High dose-rate experiment	26,251		17,179	24.9		16.8
	0 Gy	*0.1 Gy*	*1 Gy*	*0 Gy*	*0.1 Gy*	*1 Gy*
Acute X-ray experiment	16,984	23,787	13,336	15.8 [b]	21.9 [b]	12.6 [b]

[a] A maximum of 25 local fields were photographed for the initial experiments, 20 thereafter. Where the mean number of fields is substantially below the maximum, this is indicative of a low donor cell lodging frequency.

[b] The unusual pattern for the Acute X-ray experiment is the result of underlying differences in the donor cell lodging frequencies; and, low and high outliers in the 0 Gy and 0.1 Gy groups respectively. Analysis of the median values for number of fields screened shows less difference between the 0.1 Gy and control groups (18.3, 20.9), although the lower lodging in the 1 Gy was still clear (median = 13).

Table 6.2: Number of bystander cells and fields scored in the global screens

Experiment	Mean number of bystander cells per replicate	Mean number of global fields per replicate
Initial experiments	158,771	37 [a]
Increased donor cells experiment	85,881	20
Longer-term lodging experiments	85,613	20
High dose-rate experiment	85,259	20
Acute X-ray experiment	83,548	20

[a] In the initial experiments, a maximum of 40 global fields were photographed

Inherent population variation

Regardless of the precision of an assay, if the biological variation between individuals is large, it is difficult to detect subtle effects. In short, a lesser inherent variation in the population allows the detection of a smaller effect size; this is a major advantage to *in vitro* studies in clonal cell lines. The variance measured between individual mice is a composite of experimental error and true variation in the biology of each mouse. This biological variation in turn, depends on the normal degree of homeostatic control of the variable in question and the likelihood of an individual falling outside of this control. What is apparent from the control mice in the adoptive transfer experiments is the broad range of apoptosis (*Figure 6.1A*) and proliferation (*Figure 6.1B*) rates in the spleens of normal mice. Although the spread of apoptosis values was similar for each of the five experimental conditions, the mean and range did appear higher in those experiments using the pKZ1 recipient mice (Exp 2, 3 & 4, *Figure 6.1A)* compared to those in the C57BL/6J mice (Exp 1 & 5, *Figure 6.1A)*. Since the two mouse strains are obtained from different mouse colonies, the difference is most likely due to their origin/breeding history rather than the presence of the transgene.

The variation observed arises partly from the precision of the assay. Takahashi *et al.* (2001a) performed a study of apoptosis in the spleens of C57BL/6 mice after irradiation, counting only 500 cells to determine an apoptosis frequency, and unsurprisingly, found an average 0% apoptosis in the unirradiated mice. In the same study, their lowest dose of 1.5 Gy X-rays resulted in an apoptosis frequency of 7.94×10^{-2} (an effect size of >2000% above the baseline determined here in the adoptive transfer model). When the effect size to be tested is so large, the natural variation in

the population is of little concern, however, it can interfere with detecting smaller effects. A smaller effect does not equate to a less important one; the relevance of an effect size depends on the biological norm e.g., resting heart rate can safely double during exercise, but a 15% increase in core body temperature can be fatal. The question becomes, what increase in bystander apoptosis or proliferation would be of concern compared to the normal homeostatic range?

It has been shown that mice subjected to 2 × 12-h physical restraint periods over two days exhibited a 35–50% reduction in spleen numbers due to apoptosis (Yin *et al.*, 2000; Wang *et al.*, 2002). Conservatively, these stress-induced increases in apoptosis represent at least an extra 0.5-1% apoptosis per hour over the 48-h period (probably much higher at its peak) equivalent to a prolonged effect size of 100-400% above baseline. Hind-limb unloading (simulating low-gravity stress) in rats for 2 days induces similar drastic increases in apoptosis (Wei *et al.*, 2003) whilst rats exposed to hyperthermia (41.5°C for 2 h) undergo 400-900% increases in apoptosis in the spleen in the 6–8 h following the heat treatment (Sakaguchi *et al.*, 1995). The use of the heat lamp to dilate the mice tail veins before adoptive transfer in this study is unlikely to have had an effect on apoptosis. The temperature in the centre of the cage whilst exposed to the infrared lamp only reached 33°C after 15 min exposure (data not shown), whereas a 2.8°C increase in rodent core body temperature requires at least 25 min at 48°C (Wirth *et al.*, 2003) or 40 min at 42°C (Morrison *et al.*, 2000). Splenic B lymphocytes show a 1 h exposure heat-stress threshold of 42°C core temperature, whilst splenic T lymphocytes require at least 39°C (Gothard *et al.*, 2003; reviewed in Tolson and Roberts, 2005). In this study, mice had free access to water throughout the heating period, which also raises the ambient temperature required to induce a heat-stress response (Pritchard *et al.*, 2003).

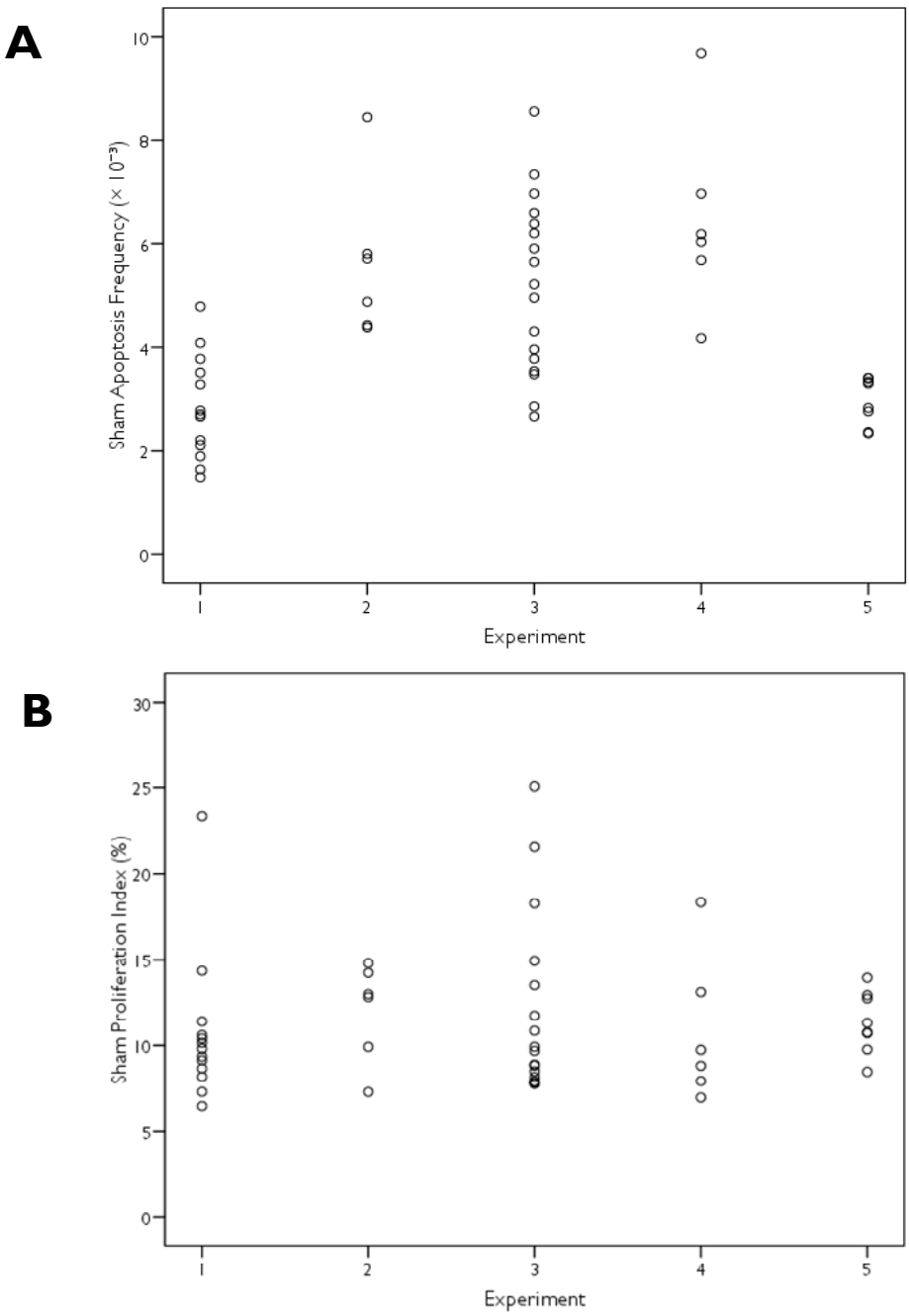

Figure 6.1: Variation in bystander apoptosis and proliferation of mice receiving sham-irradiated cells

Bystander apoptosis frequencies (A) and proliferation indices (B) were determined using the global screening method for each mouse receiving sham-radiolabelled or sham-irradiated cells. The mean frequencies are shown for each mouse for (1) the experiments conducted under the initially established conditions; and (2) the increased donor cell numbers; (3) longer-term lodging; (4) higher dose-rate; and, (5) acute X-ray experiments.

In vitro studies of bystander-induced apoptosis have demonstrated >1300% increases with ICCM from 0.5 Gy irradiated cells (Lyng *et al.*, 2000) and 100-700% increases in apoptosis in bystander cells co-cultured with X-ray or carbon-ion irradiated cells (Shao *et al.*, 2004a). Some studies have shown more modest inductions (≈60% increase) (Konopacka and Rzeszowska-Wolny, 2006).

An analysis of the variation in basal proliferation rates due to homeostatic control shows that proliferation rates in an individual mouse spleen can vary by up to 20% over the course of each day due to circadian control alone (Kovshik *et al.*, 2006). Environmental stressors such as cold stress (Aviles *et al.*, 2004) and burn injuries (Cho *et al.*, 2003) increase splenic proliferation, and restraint stress can suppress it (Fan *et al.*, 1995). The high spontaneous proliferation index in the spleen (compared to the number of cells undergoing apoptosis) reduces the relative effect size expected from any bystander induction. In addition, the heterogeneity of splenic proliferation increases the variation between mice, as in this study (particularly in the global screen) and screening of one extra follicular area with very high proliferation levels can drown out any subtle effects in the remaining fields. *In vitro*, bystander-signalling causes a 40% increase in lung fibroblast numbers after 3 days (Iyer *et al.*, 2000) an 80% increase in lymphoma cell proliferation over a 24-h period (Liu *et al.*, 2004), and can induce up to a 19% increase in bystander liver epithelial cell growth over 24 h (Gerashchenko and Howell, 2003a, 2003b, 2004, 2005). The *in vitro* data show that the effect sizes for proliferation are much smaller than for apoptosis, as expected *in vivo*.

These results suggest that a mean change in apoptosis of 50% from the baseline, would fall in the range of the most modest bystander effects that have been reported,

and might represent a change outside the range of normal homeostatic fluctuation (of the order induced by various environmental stressors). For proliferation, a 10% change from baseline falls in the range observed previously for bystander induction and would indicate a change inline with other environmental stimuli.

Prospective statistical power

The results from the first experiment conducted using the adoptive transfer method established the expected mean apoptosis frequency and proliferation index (and standard deviations) from the response of the mice receiving sham-radiolabelled cells in order to conduct power calculations. Using standard values of 80% power with 95% confidence (α=0.05, β=0.2) power calculations were performed (*Table 6.3*) for detecting effect sizes of ± 50% for apoptosis and ± 10% for proliferation as discussed earlier. As expected, whilst the variation and effect size predicted that groups of five mice would supply the desired statistical power for the apoptosis screens, the groups would need to be prohibitively large to give the desired power to detect a change of 10% in proliferation. As predicted above, the global screen for proliferation showed a higher variability between mice (coefficient of variance = 21%) than the local screen. To accommodate practical group sizes for the adoptive transfer experiments (5–10 mice per group) the predicted sample sizes were recalculated for detecting a 20% change from the sham-recipient level (*Table 6.3*).

Raising the threshold for the effect size reduced the number of mice per group to within acceptable limits for the local proliferation screen, and predicted that including eight or more mice would give >50% power to detect a 20% effect with the global proliferation screen. Although the effect size that could be considered

significant using this method was predicted to be higher than desired, the actual effect size could be evaluated *post hoc* to determine if further investigation was warranted.

Table 6.3: Predicted sample sizes based on set prospective statistical power

	Sham-level	Effect size	Sample size
Local apoptosis	$4.4 \pm 1.0 \times 10^{-3}$	± 50%	4
Global apoptosis	$3.7 \pm 0.3 \times 10^{-3}$	± 50%	>1
Local proliferation	9.7 ± 0.9%	± 10%	14
	9.7 ± 0.9%	± 20%	4
Global proliferation	9.1 ± 1.9%	± 10% [a]	69
	9.1 ± 1.9%	± 20% [a]	18

Sample size calculations were based on 80% prospective statistical power and 95% confidence. Predictions were based on the mean ± standard deviations from sham-recipient mice from the first experiment. [a] Two different effect sizes were modelled for the proliferation assays.

Retrospective statistical power

The issue of retrospective analysis of sensitivity is a controversial one (Goodman and Berlin, 1994; Zumbo and Hubley, 1998). This is particularly due to the common misuse of power calculations on gathered data to compute a *post hoc* β-value from the observed effect size (i.e., although no effect was observed, there was only a 40% chance of detecting one, therefore the effect might still exist). The flaw in such analysis is that whilst prospective predictions of power use a predicted effect size, expected variation, and have no knowledge of the outcome, retrospective statistical power actually represents a Bayesian statistical question (what was the likelihood of

detecting an effect knowing now that no effect was detected?). Instead, Goodman and Berlin (1994) and Zumbo and Hubley (1998) advocate the retrospective analysis of sensitivity by comparing the observed effect size and its confidence intervals, i.e. given the data and the effect size actually observed, what effect size can we rule out with 95% confidence?

Such an analysis is shown below for each of the experiments (*Table 6.4*). For the apoptosis screens in the initial experiments, a 10.5% (local) and 7.1% (global) decrease from the sham-control level was actually observed, although this was not enough evidence to reject the null hypothesis. However, given that previous *in vitro* data in the literature would predict an increase in bystander apoptosis, the data gathered here rules out (with 95% confidence) an increase greater than 9%. Similarly, the bystander proliferation index decreased by 3.2% and 5.6% in the local and global screens respectively; thus, precluding an increase in bystander proliferation (as predicted by the *in vitro* data) of greater than 18%. These analyses of the effect size confidence intervals show the difference between prospective and retrospective analysis of statistical power; given that the bystander apoptosis and proliferation actually decreased, the size of the increase that can be ruled out is better than that predicted before the experiment was conducted.

Table 6.4: 95% confidence limits on effect sizes, chronic radiolabelling experiments

Experiment	Screening Method	Observed Effect Sizes and 95% Confidence Limits [a]					
		Apoptosis Assay			Proliferation Assay		
		Lower 95% CL	*Observed Effect Size*	*Upper 95% CL*	*Lower 95% CL*	*Observed Effect Size*	*Upper 95% CL*
Initial experiments	*Local*	-28%	-10.5%	+9%	-37%	-3.2%	+18%
	Global	-15%	-7.1%	+8%	-28%	-5.6%	+17%
Increased donor cell experiment	*Global*	-26%	+10%	+43%	-23%	+27%	+77%
Longer-term lodging experiments	*Local*	-34%	+1.6%	+28%	-20%	+1.0%	+21%
	Global	-28%	-1.9%	+23%	-20%	+12.6%	+46%
Higher dose-rate experiment	*Global*	-26%	+4.6%	+35%	-52%	+0.9%	+53%

[a] The differences are expressed as a percentage increase or decrease from the sham-recipient frequency.

Injecting ten times as many donor cells (Increased donor cell experiment) only caused an effect size of +10% for the apoptosis frequency, but, +27% for the proliferation index, although there was not enough evidence to reject either null hypothesis. The sensitivity of the experiment was such that an increase in apoptosis of >43% (more *sensitive* than the target of ± 50%) can be confidently excluded (*Table 6.4*). However, the experiment could only rule out an increase of greater than 77% in the proliferation index. The experiment was thus not *sensitive* enough to detect the target effect size of ± 20% (a change of >20% was observed but could not be proven). This suggests that especially in regards to the global proliferation index, the greater natural variation in proliferation *in situ* and *in vivo* requires studying larger numbers of mice to detect the same changes in proliferation that are readily detected *in vitro*. The precision of the proliferation assay already far exceeds what is required to detect a change of 10-20%, and thus, increasing the number of cells or fields screened per mouse would have no effect on the sensitivity of the assay.

When examining spleen tissues three days after the adoptive transfer (Longer-term lodging experiments), the observed effect sizes were smaller than the nominated target sizes and neither were significant. For apoptosis, not only was there no increase observed, but the data can confidently reject any increase of >23%, twice as *sensitive* as the target level (*Table 6.4*). Although the experimental data give no evidence for an increase in proliferation greater than the nominated effect size, it should be acknowledged that the experiment was powered only to exclude an increase >46% above the sham-level, and thus was less *sensitive* than planned.

Injecting high-dose radiolabelled cells (Higher dose-rate experiment), resulted in effect sizes of +4.6% for apoptosis and +0.9% for proliferation (neither significant).

Both of these effect sizes were well below the target threshold and the data thus give no evidence to increases above these levels. The confidence limits on the effect sizes (*Table 6.4*) show that the experiments provide greater sensitivity than the original target level for the apoptosis screens, but that they are underpowered to detect significant changes in the target range for proliferation (although no such changes were actually detected).

When adoptively transferring acute X-irradiated donor cells, effect sizes ranged from +2.4% up to +10% for apoptosis using either screening method and from -9.1% up to +11.5% for proliferation; none were significant. Thus again, the experiment showed no evidence for changes above the target levels. In this experiment, however, the reduced variation in the response of the 1 Gy irradiated donor cell-recipients increased the sensitivity of the proliferation assay to within the target range (*Table 6.5*). The target sensitivity was approached in the 0.1 Gy irradiated donor cell-recipients (*Table 6.5*).

In summary, the precision of the assays was proportional to the expected mean values and the variation seen between individual mice. The assays were designed to measure the biological endpoints to the desired precision, in order that low frequencies were not falsely reported as zero, nor were variations between mice hidden by *rounding* effects. The statistical power of the experiments was based on target effect sizes derived from the known homeostatic variability in the biological endpoints, and observed *in vitro* bystander effects reported in the literature. As such, any bystander effects occurring that were below the limit of detection would be negligible when compared to day-to-day or individual variation, or the effects of common environmental stresses.

Table 6.5: 95% confidence limits on effect sizes, Acute X-ray Experiment

Dose	Screening Method	Observed Effect Sizes and 95% Confidence Limits [a]					
		Apoptosis Assay			Proliferation Assay		
		Lower 95% CL	*Observed Effect Size*	*Upper 95% CL*	*Lower 95% CL*	*Observed Effect Size*	*Upper 95% CL*
1 Gy	*Local*	-31%	+2.4%	+34%	-27%	-9.2%	+7%
	Global	-11%	+10%	+33%	-20%	-1.8%	+16%
0.1 Gy	*Local*	-26%	+7.3%	+40%	-16%	+2.8%	+21%
	Global	-9%	+10%	+32%	-10%	+11.5%	+33%

[a] The differences are expressed as a percentage increase or decrease from the sham-recipient frequency.

Retrospective statistical power analysis, conducted according to accepted statistical practice, demonstrated that for apoptosis, effect sizes >43% could be excluded in all cases, with sensitivity to discount an effect size >8% in the experiment conducted using the initial conditions. In fact, the greatest observed effect size for apoptosis, in any experiment, with either screening method was only 10.5% and this was a decrease from the sham-level. The results for proliferation were not as powerful; despite a +27% effect size for global proliferation when the number of donor cells

injected was increased, the data were not sufficient to reject the null hypothesis. However, regardless of the theoretical ability of the assay to narrow the potential effect sizes, all other effect sizes for proliferation were measured at <12.6% and in some cases showed differences of <1% from the control. Thus, the failure to reject the null hypotheses in each of the experiments using the adoptive transfer system was not the result of the method being insensitive to detect the types of changes that might be expected.

Were there any confounding factors?

The use of an *in vivo* experimental system added a number of variables that are not relevant or easily controlled in experiments conducted *in vitro*. The sex, age and health/immunity of the mice could each contribute to the variability of the response to the irradiated donor cells (Mothersill *et al.*, 2001). Experimental variables such as the order in which the recipient mice were injected could also provide additional sources of error. Although the study was not designed to detect effects of possible confounders, the variables mentioned above were each tested for their influence on the apoptosis or proliferation responses; none was found ($P > 0.05$ in all cases). The sensitivity of the bystander effect observed *in vitro* to the responder cells' source strain (Mothersill *et al.*, 2005b), or to the sex and health of the irradiated cell donor (Mothersill *et al.*, 2001) suggests that significant inter-individual differences may affect the bystander response. This further supports the logic behind examining bystander responses *in vivo*, where such modifying factors can be taken into account.

Generalisability of the results obtained using the adoptive transfer method

Usually, the testing of any hypothesis necessitates the selection of a model or representative system. The results acquired using the model system are then generalised to the wider population of interest. Generalisability refers to the extent of extrapolation that is reasonable given the peculiarities of the model system. The generalisations required for interpreting the adoptive transfer model include translating the results in a controlled experimental system to the real *in vivo* scenario, drawing conclusions about the responses of other cell/tissue types based on examining effects in the spleen, and the extent to which responses in mice inform us of the response in humans.

How well do the experimental conditions represent the realities of bystander irradiation scenarios?

The adoptive transfer model described here was designed with the intent to study the potential risks of bystander effects from a range of exposure scenarios relevant to humans. The potential risks for humans from putative bystander effects *in vivo* arise from a few, specific irradiation scenarios. Ionising radiation exposure for the public (excluding radiation workers) is largely due to inhaled radon (and progeny), cosmic radiation passing through the Earth's atmosphere, exposure to radioactive elements in our environment (in soil, water and organic matter), medical diagnostic irradiations and radiotherapy. Medical diagnostic techniques usually use local radiation doses whereby all cells within an exposed tissue will be irradiated. Radiotherapy uses very high doses, often to small, defined areas, and as such is a prime candidate for the occurrence of abscopal effects, not bystander effects (as described here). Newer radiotherapy techniques (and anticipated future techniques)

involve the delivery of radiation doses to more defined targets within the body, even to certain cell-types within a tumour; such uneven dose distributions could blur the boundary between abscopal and bystander signalling. The remaining exposures relevant for the public are low dose-rate exposures from natural sources, consumer products and potentially, sources of environmental contamination.

The main source of α-particle exposure for human tissue is from inhaled radon progeny trapped in the lung (Harley and Robbins, 1992). Low fluences of α-particles from a sealed external source do not have the range in tissue to penetrate the dead skin layer; however, ingested or inhaled α-emitters can irradiate sensitive tissues. Since α-particles are densely ionising, the radiation energy is confined in fewer discrete hits than for an equivalent dose of photons, and as such, a higher dose is delivered to the few irradiated cells even at low mean absorbed doses. If bystander signalling propagates carcinogenic damage to unirradiated cells in the lung, the effect would multiply the biological consequences of the exposure above the estimate based on the absorbed dose.

Thus, single α-particle traversals through rare cells in the lung *in vivo* would make an excellent model for detecting bystander effects relevant to human risk-estimation, and the *in vivo* method described here could be adapted to test such a scenario, since adoptively transferred lymphocytes are known to traffic to the lung (Tseng *et al.*, 1989). Donor lymphocytes could be radiolabelled with an α-particle-emitting radionuclide and the unirradiated lung tissue surrounding lodged, irradiated donor cells could be examined. However, since the range of an α-particle in tissue is at least a few cell diameters, the radiation would not be contained in the radiolabelled cell, and effects within an extended radius of the donor cell would need to be excluded.

Given the heterogeneous morphology of lung tissue and the dynamic movement of air, the obstacles to a spatial analysis are substantial, however, such an experiment is crucial to *in vivo* confirmation of the potential for radon-induced bystander effect in the lung.

Other high LET sources such as high atomic mass, high energy (HZE) particles, such as accelerated carbon, iron or titanium ions have the range in tissue to pose an external hazard and can deliver large doses within very small volumes (Brenner and Elliston, 2001; Ballarini and Ottolenghi, 2003). Fortunately, such HZE exposures only occur outside the Earth's magnetosphere precluding the possibility of HZE bystander effects except in the case of astronauts. The occurrence of such effects may prove important for space radiation risks; however, detecting an HZE-induced bystander effect *in vivo* would not inform risk-assessment for regulatory purposes. Additionally, such an effect is likely to differ from any putative low LET bystander effect given the vastly different nature of the radiation and history of exposure throughout biological history.

For the rest of the body, other than the lung (Harley and Robbins, 1992), the main potential for bystander-like exposures comes from irradiation by photons or other low-LET radiations where the dose-rate spares the majority of cells at any one time. If the majority of cells in the tissue are to remain as unirradiated bystander cells, Poisson statistics dictates that the dose range of concern for the irradiated cells is the traversal of single electron tracks (Feinendegen, 1990) delivering nuclear absorbed doses on the order of a few milligray (Roeske and Hoggarth, 2007), simulated in the present study by low activities of DNA-incorporated ^{3}H. Such exposures mimic the type of radiation insult received by rare, individual cells within an irradiated tissue,

unlike many experiments that deliver 0.5–5 Gy of low-LET radiation. Thus, the experimental conditions considered in the current study are directly relevant to one of the types of human radiation exposures for which any putative bystander effect would pose a real risk.

Given that no effect was observed in bystander splenocytes, what conclusions can be drawn on the responses of other cell/tissue types?

Previous experiments to determine the presence of a bystander effect in the spleen have been limited to the detection of abscopal effects after irradiation of distant tissues (Koturbash *et al.*, 2007; Koturbash *et al.*, 2008a; Koturbash *et al.*, 2008b). However, there are a number of studies that have investigated bystander effects in primary or immortalised lymphocyte lines (Mothersill *et al.*, 2002; Liu *et al.*, 2004; Moore *et al.*, 2005a; Shankar *et al.*, 2006; Ermakov *et al.*, 2007). The available data suggest that lymphocytes/splenocytes are capable of initiating and responding to bystander signalling in a similar manner to epithelial and fibroblast lines. It is possible that the small number of published studies represents reporting bias against experiments that do not show a significant bystander effect. However, it is more likely that since lymphocytes/splenocytes are more difficult to study *in vitro* with some irradiation techniques, the requirement to use only adherent (Moore *et al.*, 2005a) or immobilised (Kadhim *et al.*, 2001) lymphocytes prevents their wider study. Given the cell-type variability observed *in vitro*, it is reasonable to assume that other cell-types might respond in a different manner. If a bystander effect had been observed, it would have served as a proof-of-principle that radiation effects could be propagated by intercellular signalling *in vivo*, however that was not the case in this study. Thus, it is important that bystander responses in other cells/tissues each be examined in the context of their relevant bystander exposure scenarios.

What do these findings in mice tell us about the bystander response in humans?

The induction of a radiation-induced bystander effect (or at least the underlying capacity to conduct bystander signalling *in vitro*) seems to be a highly conserved phenomenon across species and cell-types; however, the nature of the effect(s) appears to be more variable. Clearly, experimental data gathered in mice is an imperfect model, and within the low-dose radiobiology scientific community it has long been recognised that effects detected in surrogate systems for the human do not automatically imply that the same effect occurs in humans (Trosko *et al.*, 2005).

However, recently developed mouse models of human tumours (reviewed in Abate-Shen, 2006; Dennis, 2006) have shown that when human carcinogenic lesions are induced in mice, the resulting tumour development and progression is remarkably similar to human tumour pathology (Johnson *et al.*, 2001; Huysentruyt *et al.*, 2008). Given the current state of bystander research, which is almost exclusively *in vitro*, each move towards complex or *in vivo* experimentation takes the field one step closer to results directly relevant to human risk-estimation. Any bystander effects observed in a mouse model would need to be closely studied to determine if the same pathways and effector mechanisms are likely to operate in humans. Conversely, if bystander effects that are observed *in vitro* do not occur *in vivo* using a mouse model, the cause of the difference in response will need to be scrutinised to establish whether the same differences would apply to the human situation.

Significance and implications of not detecting a bystander effect *in vivo*

If it is plausible and justifiable that the *in vivo* method described here can simulate a bystander exposure scenario, and if the results are reliable and generalisable, the question becomes, what is the significance and implications of the findings presented here? No bystander effects were observed in any of the experiments conducted in this study for apoptosis or proliferation (or chromosomal inversions, where measured). The possible reasons why the types of bystander effects observed *in vitro* were not observed in these experiments include:

1. The bystander signal that is initiated *in vitro* was not initiated *in vivo*;

2. The bystander signal that is initiated *in vitro* was initiated but not propagated *in vivo*; or,

3. The response induced in unirradiated cells by bystander signals *in vivo* was not the same as that induced *in vitro*.

These theories represent significant *in vitro*/*in vivo* differences in each of the three nodes in the bystander system as is currently proposed: the response of the irradiated cell to the radiation, the mechanism of signal transmission, and, the response of the unirradiated cells to the signal.

Was the signal that is commonly initiated *in vitro* not initiated here *in vivo?*

It is possible that the irradiated cells in these experiments simply did not produce a bystander signal, or did not produce the same signals as have been previously

observed *in vitro*. A failure to initiate a bystander signal could be due to the cells themselves, or the applied radiation stimulus.

Do lymphocytes irradiated in vivo not produce the same signal as cultured cells, given the same stimulus?

The ongoing efforts to identify and characterise the bystander signal(s) *in vitro* (Hamada *et al.*, 2007) makes this question difficult to answer at present. Newman (2006) suggests a chemists' approach to identifying the bystander signal apparent in ICCM experiments. Newman concluded that the most likely candidate that met the criteria of the bystander signal (as described by Mothersill and colleagues) was the small molecule nitric oxide. In recent work published by Dr Mothersill's own research group (Ryan *et al.*, 2008), the authors proposed instead that a proteinaceous factor such as TGF-β or TNF-α might be the signal. Curiously, the implication of TGF-β as the bystander signal by both Ryan *et al.* and Iyer *et al.* (2000) is at odds with the fact that the former group measure bystander-induced cell death whilst the latter report bystander-induced proliferation (indication that both may be facets of the same effect, or that both are artefacts of the experimental system).

In addition to nitric oxide and TGF-β, other reports suggest calcium signalling (Lyng *et al.*, 2006; Shao *et al.*, 2006), reactive oxidative species (Han *et al.*, 2007), membrane signalling pathways (Nagasawa *et al.*, 2002; Burdak-Rothkamm *et al.*, 2007; Tartier *et al.*, 2007), interleukin-8 (Facoetti *et al.*, 2006) and even small DNA fragments (Ermakov *et al.*, 2007) as candidate bystander signalling messengers. Whatever the extracellular mechanism, it is reasonable to expect that conditions in the resting cell at the time of radiation exposure affect the initiation of that signal. The inducible nitric oxide synthase (iNOS) is subject to hypoxic regulation

(Melillo *et al.*, 1995), complicating the interpretation of *in vitro* bystander experiments conducted under non-physiological oxygen tensions. Further modifying factors could include the irradiated cell's cell-cycle state (Ballarini *et al.*, 2006), methylation status (Rugo *et al.*, 2007), levels of oxidative metabolism (Azzam *et al.*, 2003) or pre-existing factors in the culture medium (or tissue environment) (Newman, 2006). Differences in any of these factors *in vivo* could affect the way irradiated cells respond to radiation depositions.

The revelation that cytoplasmic irradiation can also induce bystander effects *in vitro* (Wu *et al.*, 1999; Shao *et al.*, 2004b; Tartier *et al.*, 2007) further complicates the issue; and since cytoplasmic irradiation results in bystander mutations distinct from those caused by nuclear irradiation, the possibility of multiple initiating and effector pathways for the bystander effect is now raised. In some experiments, bystander effects are only generated by cells with mutant p53 status (Matsumoto *et al.*, 2001; Matsumoto and Ohnishi, 2003), whilst in others, p53–wild-type, –null and –mutant cells each initiate a bystander signal (Zhang *et al.*, 2008). In recent studies, cell lines which accumulated mutant forms of p53 did not show a bystander response, whereas p53–null or p53–deficient cell lines were able to generate bystander signals (Ryan *et al.*, 2008), indicating a suppression effect by mutant TP53 protein.

Although a more complete understanding of the initiating events is required to determine whether the irradiated donor cells do in fact send a bystander signal *in vivo*, some clues might be available from studies that could be conducted *in vitro*. After the radiolabelling period, the irradiated donor cells could be washed and then incubated in fresh medium for a period to allow the secretion of any soluble bystander signals, before removing the irradiated cell–conditioned medium and

transferring it onto a test cell line as in the approach used by Ryan *et al.* (2008). Such an experiment would allow the investigation of whether or not the cells were capable of producing the classic media-transferable bystander signal *in vitro* or whether the cells are one among a group of cell-types known not to produce the signal in the media-transfer system. However, in such an experiment, failure to observe a media-borne signal might result from inadequate cell density, incubation time or a range of other factors already known to influence the production of bystander signals *in vitro*. Similarly, confirmation that the donor cells produce a media-borne bystander signal *in vitro* is no guarantee that the same effect occurs *in vivo*, thus leaving the initial question unanswered. A failure of the irradiated donor cells even to initiate a bystander signal *in vivo* is thus possible, but not discernable at this time.

Is the stimulus (dose, dose-rate or LET) in this experiment sufficient to initiate the signals observed in vitro?

Despite the early concentration on high-LET bystander effects, due to the interest in radon-induced carcinogenic risk and the earlier development of high-LET microbeams, the results of many *in vitro* experiments have now demonstrated bystander effects from low-LET radiations over a wide range of radiation sources, doses, and experimental systems (summarised in *Table 6.6*).

Table 6.6: Bystander effect experiments conducted *in vitro* with low-LET radiations

Radiation Type	Lowest dose *(mGy)*	Study
Carbon k-shell ultra-soft X-rays	50	(Schettino *et al.*, 2005)
200 kVp X-rays	75	(Liu *et al.*, 2004)
^{137}Cs γ-rays	500	(Gerashchenko and Howell, 2003a)
^{60}Co γ-rays	10	(Mothersill and Seymour, 1997) (Seymour and Mothersill, 2000)
Uptake of ^{131}I	1000	(Boyd *et al.*, 2006)
Radiolabelling with ^{3}H-thymidine	200	(Gerashchenko and Howell, 2004; Persaud *et al.*, 2005; Persaud *et al.*, 2007)

Some bystander effect investigations have shown that the LET of the radiation source affects the magnitude of the response (Shao *et al.*, 2003a; Shao *et al.*, 2003c; Boyd *et al.*, 2006), while others show no dependence on LET (Kanasugi *et al.*, 2007). In some cases, the LET of the radiation determines the nature of the induced effect (Baskar *et al.*, 2007), or even whether an effect occurs at all (Xue *et al.*, 2002; Frankenberg *et al.*, 2006). Published reports show bystander effects that are dose-dependent (Hei *et al.*, 1997; Sawant *et al.*, 2002; Persaud *et al.*, 2007) and those that are dose-independent (Seymour and Mothersill, 2000; Gerashchenko and Howell, 2004; Ponnaiya *et al.*, 2004b; Facoetti *et al.*, 2006). Thus, it seems drawing conclusions from any one study as to what the bystander effect 'is' or 'is not' is likely to be flawed. The possibility that the dose/dose-rate used in the current study was too low, is at odds with the same lack of response even with a dose-rate that was one hundred-times higher. In the initial experiments, the radioactivity of the ^{3}H-

radiolabelled donor cells delivered just over one tritium disintegration per hour per cell on average. The mean nuclear absorbed dose per DNA-bound tritium decay is estimated at 2.61 mGy (Goddu *et al.*, 1997). Modelling the stochastic occurrence of the radioactive decays shows that the radiolabelled donor cells could accumulate a dose between 30 and 90 mGy over the 22 hours *in vivo* (*Figure 6.2*). In the higher dose-rate experiment, the radiolabelled cells received an average of just under a hundred ^{3}H decays per hour. Cells surviving the 22 h *in vivo* period could have accumulated a nuclear dose of ≈ 5.7 Gy. Despite 51% of the irradiated donor cells being radiolabelled before adoptive transfer, only 6% of the donor cells identified in the local screens were radiolabelled after 22 h, indicating substantial radiation-induced cell death. Despite these very high doses, there was still no hint of a change in global apoptosis (P = 0.7) or proliferation (P = 0.99) in bystander cells. Similarly, in the acute X-ray experiment, there was no difference between the apoptosis or proliferation in mice receiving donor cells irradiated with 0.1 or 1 Gy.

The possibility that the doses/dose-rates or LET used in this study were insufficient to induce a bystander effect is supported by such dependencies in many studies *in vitro* (often peculiarities of the individual experimental system) but is countered by the growing acceptance that ‘bystander effects appear to predominate at low doses of low LET radiation’ (Morgan and Sowa, 2007). The ^{3}H-thymidine dose-rates (and thus accumulated doses) and the acute X-ray doses used in this study fall within the ranges used previously in bystander experiments *in vitro* and mirror the types of exposures that would result in non-uniform dose distributions *in vivo* under real-life conditions.

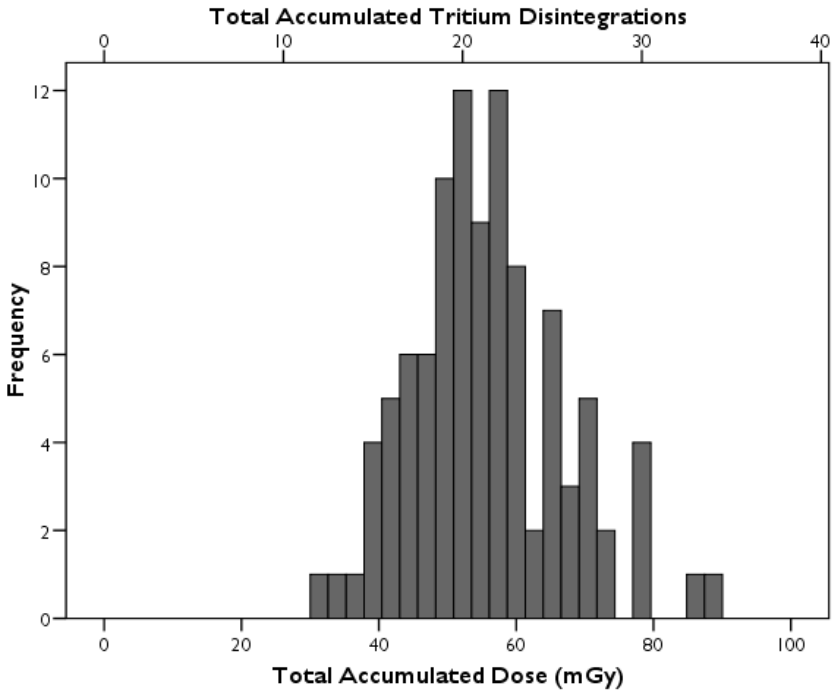

Figure 6.2: Model of ^{3}H decay and accumulated dose over 22 hours at one disintegration per hour

In this simulation, for each minute over a 22-hour period a cell was randomly assigned to receive a ^{3}H decay (with a probability of 1 in 60) or to remain unhit, representing a dose-rate of one ^{3}H decay per hour per cell. The total number of tritium disintegrations over the 22-hour period is shown above for 100 randomly generated cells. An estimate of the equivalent total accumulated dose is shown, calculated by assigning a mean nuclear absorbed dose of 2.61mGy.Bq^{-1}.

Conclusion

There is no convincing evidence to suggest that a failure of the irradiated cells to initiate a bystander signal can explain the results obtained with this system. It is likely that the initial radiation-injury to the donor cells that occurred *in vivo* would be indistinguishable from the same dose applied *in vitro*. Until the signal(s) is identified or the initiating events are characterised, it is not possible to determine whether the lack of bystander effects observed here is due to the irradiated cells or if a signal is produced and interrupted further downstream.

Was the signal propagated *in vitro* initiated but not propagated *in vivo?*

It is possible that the irradiated donor cells initiated a bystander signal as is seen *in vitro*, but that due to *in vivo* fluid dynamics, dilution, competing signals, redox status, signal counteraction or any other *in vivo*–specific challenge, the signal did not reach the unirradiated target cells sufficiently to induce a response.

Does the mechanism responsible for propagating in vitro bystander effects operate in vivo?

The intercellular-signalling responsible for bystander effects observed *in vitro* must operate either via direct cell–cell contact (gap junctions or membrane signalling) or indirect message transmission (soluble small molecule, radical, gas, ion or protein). It is unlikely that any signalling pathway that operates *in vitro*, would be absent from the same cells *in vivo*, in fact, the opposite is more likely true. The occurrence of bystander effects in the intact tissues studied to date (Belyakov *et al.*, 2002; Belyakov *et al.*, 2003, 2006), suggests that the signalling pathway responsible for *in vitro* bystander effects does operate *in vivo*, and similar studies in artificial tissues (Belyakov *et al.*, 2005) indicate that they likely work much as they do in the same cells grown in disaggregated culture.

Is bystander signal propagation possible but not as efficient in vivo?

If interruption or impedance of the putative bystander signal is responsible for the failure to observe a bystander effect in the adoptive transfer system, there are a number of possible causes. The example of nitric oxide signalling is used here to illustrate. Cell cultures grown in static tissue culture medium have the opportunity to accumulate secreted factors normally diluted by fluid flow *in vivo*. In addition to dilution, fluid flow can aid in the consumption or metabolism of potential signalling

agents. *In vitro* studies of nitric oxide have tended to over-estimate its biological reactivity since *in vivo*, nitric oxide rapidly diffuses over hundreds of micrometres into the vasculature and irreversibly reacts with the oxyhaemoglobin in erythrocytes to form methaemoglobin and nitrate (Beckman, 1996; Kim, 2001). The continual replenishment of erythrocytes (and hence oxyhaemoglobin) *in vivo* ensures that nitric oxide levels remain low. This has two consequences: firstly, nitric oxide reacts with molecules under *in vitro* conditions that it does not react with *in vivo* (e.g. combining with oxygen to form nitrogen dioxide) and secondly, the half-life *in vitro* is on the order of minutes, compared to only a number of seconds *in vivo*. Interaction between signalling pathways will thus also differ under physiological conditions, such as the regulation of TGF-β by nitric oxide and its reciprocal counter-regulation (Vodovotz *et al.*, 1999).

To continue the same example, many cell-types (e.g. lymphocytes) generate only low levels of nitric oxide, whilst other neighbouring cell-types (e.g. macrophages) can generate large nitric oxide bursts (Mannick *et al.*, 1994). *In vitro* cultures of a single cell-type will fail to represent the dynamic signalling occurring *in vivo*. To further complicate signal transmission, nitric oxide is known to be hypoxia-inducible (Melillo *et al.*, 1995), which has implications for *in vitro* culture at non-physiological oxygen tensions. Thus, for each putative signalling pathway, there will be key differences between the *in vivo* and *in vitro* situations and the connection between irradiated and unirradiated cells seen experimentally may be broken in real-life.

Was the response induced in unirradiated cells by bystander signals *in vivo* not the same as that induced *in vitro?*

An inherent difficulty in attempting to detect a low-dose radiation–induced bystander effect *in vivo* is knowing where and when to look, and, knowing what to look for. In the present study, the aim was to detect bystander effects of the nature seen *in vitro*. Although this does narrow the search to a degree, the observed variability of bystander effects *in vitro* still leaves a wide range of endpoints, times and experimental conditions to evaluate. Further, the same underlying bystander signalling phenomena may induce entirely different effects *in vivo*. Finally, it is possible that tissue culture conditions, peculiarities of the cell line in question, and high radiation doses, converge to produce a robust effect *in vitro* that would be undetectable in the equivalent *in vivo* scenario.

Do unirradiated cells exposed to bystander signals in vivo receive a different net 'signal' to those exposed in cell culture due to concurrent endogenous, homeostatic signalling?

Intercellular signalling does not occur through discrete, isolated pathways; instead, cells use 'elaborately branched signalling pathways' (Barcellos-Hoff and Costes, 2006) that co-ordinate a variety of inputs and stimuli within a tissue, resulting in a multicellular network response. Each signal is received upon a background of endogenous, homeostatic signalling and is interpreted in the context of simultaneous messages that can be synergistic or antagonistic. The maintenance of monoclonal cell populations in exponential or contact-inhibited growth cannot fully represent the complexity or co-ordination of the tissue environment it models. The diversity of cell-types and the microenvironment formed by a tissue *in vivo* means that even the signalling molecules can be intercepted and the message modified en route.

Transforming growth factor–β (TGF-β) is secreted in latent form bound to a latency-associated peptide (LAP) which can dissociate in the extracellular environment to produce active TGF-β; the LAP can later re-associate to inactivate the signal (Barcellos-Hoff, 2005). However, a single amino acid residue in the LAP acts as a redox-sensitive switch, allowing reactive oxidative species (endogenous or radiation-induced) to modify the site and prevent binding of LAP to TGF-β (Barcellos-Hoff and Dix, 1996; Jobling *et al.*, 2006). Thus, oxidation-sensitive sites in signalling molecules can act as sensors of the levels of oxidative stress in cells or tissues. Deposition of large latent TGF-β complexes in the extracellular matrix (Koli *et al.*, 2000) and binding of TGF-β to extracellular matrix proteins (Yamaguchi *et al.*, 1990) provides a tightly controlled source of the cytokine within tissues, which allows rapid induction of TGF-β signalling within a localised area, a signalling mode not present in cell culture.

The bioactive gas nitric oxide can follow several chemical pathways depending on the existing reactive oxidative species milieu (Brune *et al.*, 1996), such that release of nitric oxide from irradiated cells might be received as one of a variety of reaction products, each inducing unique responses. For example, under the right extracellular conditions, a nitric oxide signal can induce apoptosis directly, bypassing p53 regulation (Messmer and Brune, 1996b; Heigold *et al.*, 2002; Portess *et al.*, 2007) via a reaction with endogenous superoxide anions to produce highly toxic peroxynitrite. The ability of nitric oxide signals to both induce and suppress apoptosis has been widely studied *in vitro* (Beckman and Koppenol, 1996; Wink *et al.*, 1996; Kroncke *et al.*, 1997; Kim *et al.*, 2001; Stuart-Smith, 2002; Li and Wogan, 2005). This duality is also observed *in vivo* since inhibiting nitric oxide synthase activity or

administering nitric oxide generating compounds can both induce radioprotection in mice (Liebmann *et al.*, 1994).

The response of cells to hormones has been shown to take one of two routes depending on the occurrence of a simultaneous intracellular calcium flux (Mulvaney and Roberson, 2000), an example of signal transduction pathways that have been termed concurrent reactive systems (Täubner and Eckstein, 2008). These examples demonstrate that signals released from an irradiated cell might not show strict fidelity in eliciting a particular response in target cells; the signal(s) could be altered or even hijacked en route. In addition, the molecules proposed for communicating bystander effects are all involved in multiple, overlapping signalling pathways within living tissues. Signals released from irradiated cells may fail to induce a response if the basal levels of the signal are already saturating, a similar signal was already induced by independent means (e.g. an immune response) or, if a counteracting signal is present. Such modifying factors are commonly observed in bystander experiments conducted *in vitro*, whereby addition of vitamins, antioxidant enzymes or reducing agents can enhance or suppress an observed response (Bishayee *et al.*, 2000; Bishayee *et al.*, 2001; Konopacka and Rzeszowska-Wolny, 2006; Kashino *et al.*, 2007).

It is possible that any of these scenarios has occurred in the present adoptive transfer system. Such potential interruptions to bystander signalling pathways highlight the need to study bystander effects using an *in vivo* system that can mimic the situation relevant to human exposure. It is more important to determine what signals released from irradiated cells do in a living, functioning tissue system than what they might do when cultured in isolation. It may be that the nature of the bystander effect that is

induced at any one time is dependent on the resting state of the tissue, immune status of the individual, age, nutrition and diet, and even inter-individual genetic differences. The *in vivo* method described here is capable of exploring each of these factors.

Do unirradiated cells exposed to bystander signals in vivo receive the same 'signal' as those exposed in cell culture but respond differently?

Although elucidating cellular signalling often requires studying particular pathways in isolation, cells respond to multiple, simultaneous inputs and signal balances converging on molecular 'switches' that ultimately regulate the cell's response (Bree *et al.*, 2004; Hornberg *et al.*, 2004). Thus, cells *in vivo* and equivalent *in vitro* cell lines do not necessary respond in the same way to a given stimulus. TGF-β, which promotes angiogenesis *in vivo* can inhibit proliferation of endothelial cells *in vitro* (Iruela-Arispe and Sage, 1993). Even between *in vitro* bystander experimental systems, TGF-β has been proposed to induce both cell death (Ryan *et al.*, 2008) and mitogenic responses in bystander cells (Iyer *et al.*, 2000; Iyer and Lehnert, 2002b). Signals from α-particle irradiated cells can either induce TP53 and CDKN1A in bystander normal human fibroblasts (Azzam *et al.*, 1998; Azzam *et al.*, 2001), or down-regulate them (Iyer *et al.*, 2000; Iyer and Lehnert, 2002b).

A possible explanation for such contradictory results is the way in which the status of oxidative metabolism in a cell, and other stressed states, can modify the cell's response to a signal. Perhaps the best known example is TP53, which regulates both pro-survival and pro-apoptotic pathways simultaneously, the final outcome dependent on total active TP53 levels, post-translational modifications and the presence of various co-factors (Sionov and Haupt, 1999; Bree *et al.*, 2004).

Application of the same stimulus can induce alternate responses in cells depending on the coalescing of the extracellular signal with internal signal balances (Christophorou *et al.*, 2005). This type of control is particularly relevant since many published *in vitro* bystander studies are conducted in cells that are p53-null, -mutant or -deficient, potentially misrepresenting the response of normal cells. However, a recent report has demonstrated *in vitro* that human lymphoblastoid cell lines from the same donor but with p53-wild-type, -null or -mutant status are each capable of generating and responding to a bystander signal (Zhang *et al.*, 2008), suggesting a p53-independent response.

The internal balance of pro- and anti-oxidative agents in cells can regulate the response to an extracellular signal (de Toledo *et al.*, 2006). Oxygen concentrations directly affect the way mitochondria respond to nitric oxide signals (Balakirev *et al.*, 1997) and differences are seen in basal antioxidant enzyme levels between cells maintained in cell culture or physiological oxygen tensions (Chen *et al.*, 2008). Data from *in vivo* and *in vitro* experiments with nitric oxide and glutathione (GSH) 'indicate a multifaceted and complex involvement of GSH in responses of cells and tissues to toxic levels of NO' (Li *et al.*, 2005).

It is possible that in the *in vivo* bystander method used here, the irradiated cells generated a classic bystander signal (as is observed *in vitro*), that the signal was transmitted with fidelity to the bystander cells, but that differences between cell culture conditions and the physiological environment *in vivo* resulted in a different response. Perhaps instead of undergoing apoptosis, or enhanced proliferation, the same bystander signal induced changes in DNA methylation (Rugo *et al.*, 2006; Koturbash *et al.*, 2007; Rugo *et al.*, 2007; Koturbash *et al.*, 2008a; Kovalchuk and

Baulch, 2008), DNA double-strand breaks (Rugo *et al.*, 2007; Sedelnikova *et al.*, 2007; Mancuso *et al.*, 2008; Ojima *et al.*, 2008; Zhang *et al.*, 2008) or homologous recombination (Ermakov *et al.*, 2005; Ermakov *et al.*, 2007). Thus, the failure to observe a bystander effect for apoptosis or proliferation, both severe changes in cell fate, does not preclude the possibility of other, subtle changes in bystander cells. The adoptive transfer method described here is capable of being utilised to study a variety of cell biology endpoints *in situ* using fluorescence-based assays and to study molecular endpoints, by using flow cytometry to separate donor and recipient splenocytes and analyse DNA or RNA changes. Future experiments are required to continue to explore the range of responses that have been reported hitherto in bystander cells.

Do unirradiated cells exposed to bystander signals in vivo respond in the same manner as those exposed in cell culture but in a timeframe not measured in this experiment?

The issue of timing is complex when discussing radiation exposures at chronic dose-rates. At the low dose-rates expected to produce rare, isolated tracks through an exposed tissue, timing also becomes dependent on the spatial range considered. For a single cell, the relevant period might begin at the first or most recent moment of irradiation, whilst for the spleen as a whole, multiple cells may be being hit simultaneously at any one time producing multiple overlapping time-frames. It could be hypothesised that each irradiated cell initiates a definable series of events within its local region on an independent time-scale; or, that bystander signalling is a constant, ever-present communication within a tissue that is maintained by continual, yet random ionisations.

The first hypothesis would suggest that when analysing the recipient mouse spleen tissues with the local screening method, the local fields surrounding irradiated donor cells represent a range of time-points along the continuum, each depending on the stochastic distribution of times since the last (or first) irradiation. Although the ^{3}H activity used in the initial experiments here results in a dose-rate averaging to ≈ 1 tritium disintegration per cell per hour, the time elapsed since the last disintegration can vary significantly. In a simulation of stochastic tritium decay over 22 h in 100 model cells, most simulated donor cells had received a ^{3}H disintegration in the past hour, although for some cells, 3–4 hours had passed since the last ^{3}H decay (*Figure 6.3*). Thus, in experiments with a 22-h lodging period, it would be inaccurate to compare responses observed in the spleen tissues to 22 hours post–whole-body irradiation. Further, as discussed earlier, the timing of any bystander response may be related to the time of the initial radiation exposure, or the most recent. If bystander signals are effective *in vivo* over large distances, the dose-rate is high enough and the duration of the response is long enough, after the initiation of a bystander response, the tissue may enter a steady-state equilibrium where the signal is replenished by each new random cell that receives a radiation dose. In such a scenario, the time-frames for each irradiation–signalling–response event would be overlapped to effectively remove the time-scale altogether.

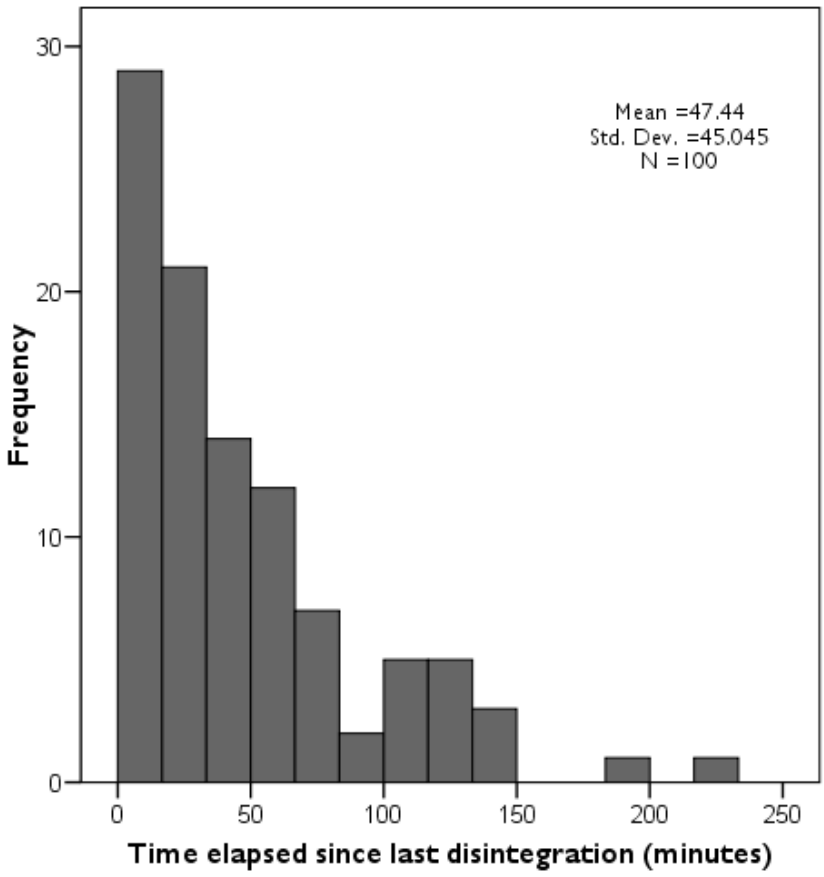

Figure 6.3: Model of time elapsed since last tritium disintegration at an average exposure rate of 1 disintegration per hour

In this simulation, for each minute over a 22-hour period a cell was randomly assigned to receive a ^{3}H decay (with a probability of 1 in 60) or to remain unhit, representing a dose-rate of one ^{3}H decay per hour per cell. At the 22 h time-point, representing the cryopreservation of the spleen tissue, the time elapsed since the cell's last ^{3}H decay was calculated for 100 randomly generated cells.

The choice of 22 h as the initial lodging period was designed to allow time for the donor cells to lodge, signal, and for the bystander cells to respond; the later experiments conducted over 3 days were performed to allow more time for a delayed signal or a protracted response time. It was expected that in both cases, the tissues would represent bystander cells exposed to cells most recently irradiated over a range of times. In the acute X-ray dose experiment, however, there was a single,

homogeneous irradiation-time; if the signals were propagated evenly and the response was similarly synchronised, it is possible that the time of analysis was too early or too late to identify the effect.

Judging the best time of analysis based on *in vitro* experiments is problematic, since the timing of apoptosis and proliferation is known to differ greatly between *in vivo* and *in vitro* studies. Again, such a strategy assumes that the timing of signalling and response, as well as the progression of the biological endpoint itself are similar in cell culture and in a living tissue. *In vivo*, radiation-induced apoptosis induces an immune response to clear the debris (Lorimore *et al.*, 2001), corresponding to a peak apoptosis induction <12 h after the irradiation (Komarova *et al.*, 2000; Takahashi *et al.*, 2001b; Takahashi *et al.*, 2003). In bystander experiments conducted *in vitro*, apoptosis responses are usually measured from 2–7 days after exposure to irradiated cells or conditioned medium (Mothersill and Seymour, 1997; Lyng *et al.*, 2000; Belyakov *et al.*, 2001; Belyakov *et al.*, 2002; Belyakov *et al.*, 2003, 2006), times when toxic by-products and cell debris from early apoptotic cells may have exerted a compounding effect on the health of the culture or explant. Given the uncertainties, it is perhaps prudent that future investigations examining bystander effects *in vivo* should monitor a wide range of times post-exposure to determine if a temporal response can be detected, and calculate the optimum time to measure a peak response.

Do unirradiated cells exposed to bystander signals in vivo respond in the same manner as those exposed in cell culture but to a lesser, undetectable magnitude?

An alternate explanation for the results seen in this study, is that the *in vivo* model faithfully reproduced the bystander effect as repeatedly demonstrated *in vitro*, but that the response in this system was less pronounced, and was thus below the limit of detection. This prospect will always exist, since any system, no matter how sensitive, will have a lower-threshold for detection. However, in this case, differences between the adoptive transfer method as used here, and experiments conducted using a variety of *in vitro* systems, lend credence to this hypothesis. This *in vivo* experimental system was designed to reproduce conditions relevant to human exposure scenarios where bystander effects might alter the risk of radiation-induced carcinogenesis. This aim directed the choice of lower dose-rates, lower ratios of irradiated-to-unirradiated cells, and longer exposure periods. Use of these experimental conditions carried the risk of producing a smaller effect than seen with *in vitro* experiments; however, the decision was taken in the light of overwhelming evidence that cellular responses to radiation are distinct between high and low doses (Amundson *et al.*, 2003; Ding *et al.*, 2005; Chaudhry, 2006; Sokolov *et al.*, 2006; Voy *et al.*, 2006; Short *et al.*, 2007).

The discovery of bystander effects was heralded as a prime example of important phenomena that might be overlooked by simple extrapolations from high to low radiation doses. However, the history of bystander effect research shows a familiar pattern of attempts to increase the signal-to-noise ratio by elevating radiation doses beyond the range relevant to radiation protection of the public. The assumptions inherent in the linear-no-threshold model, are called into action once again when the collective response of millions of cells to hundreds of ionising tracks each is

considered representative of sparse, single radiation hits within an unirradiated tissue. This has increased the confusion between bystander effects (as defined here) and abscopal effects (Kaminski *et al.*, 2005; Morgan and Sowa, 2007).

Promising developments are seen in experiments involving the irradiation of single cells (Belyakov *et al.*, 2001; Schettino *et al.*, 2003; Shao *et al.*, 2003c; Kashino *et al.*, 2004; Moore *et al.*, 2005a; Fournier *et al.*, 2007). The fact that these experiments still show robust bystander responses, suggests that equivalent dose-rates (which are relevant to human radiation protection of the public) should be used when trying to characterise the bystander response *in vitro*.

Recent developments

In the time since the adoptive transfer method was developed, several major bystander studies were published which continue to shed light on the relevance and mechanism of radiation-induced bystander effects. Continuing experiments to probe the nature of the medium-mediated bystander effect have found that bystander cells respond differently to autologous ICCM than medium conditioned by foreign cells (Vines *et al.*, 2008); and, when the irradiated and bystander cell-types differ, it is the irradiated cell-type that determines the magnitude of the bystander response. At odds with experiments showing robust effects after the irradiation of single cells was the perplexing finding that in some cases, the effect induced by ICCM can be removed by dilution factors as low as 1.2 (Ryan *et al.*, 2008). This provides further indication that medium-transferable bystander effects might at least represent a different class of bystander effect, if not a distinct phenomenon analogous to abscopal effects.

The question of the effect of radiation LET for the transmission of bystander effects remains unanswered with studies supporting LET-independence (Fournier *et al.*, 2007; Kanasugi *et al.*, 2007) or those observing the reverse (Boyd *et al.*, 2006; Frankenberg *et al.*, 2006; Nuta and Darroudi, 2008). Experiments repeating the landmark work of Xue *et al.* (2002) with ^{125}I radiolabelled human tumour cells injected into unirradiated mice, showed the same reduction in tumour growth, but when radiolabelling the same cells with ^{123}I (which has the same Auger electron spectrum but different dose-rate), tumour growth was increased (Kishikawa *et al.*, 2006). Given the variability of the responses, and the peculiarities of the system (lethally irradiated human tumour cells in immunodeficient mice), the interpretation of these results is difficult.

Dose-dependence continues to be a controversial issue, with dose-response studies using ICCM showing that very low doses of photons (down to 0.04 mGy) were ineffective at inducing a bystander response, with a threshold appearing at around 2 mGy – a dose equivalent to all cells within a tissue receiving an electron track (Liu *et al.*, 2006). For α-particle irradiation of individual cells, γ-H2AX induction in bystander cells was independent of dose and the proportion of cells hit (Smilenov *et al.*, 2006). Conversely, an inverse dose-response was observed for proton irradiation, where bystander cell death was induced when 10% of cells where irradiated with low doses of protons, but not higher doses (Frankenberg *et al.*, 2006). At the other extreme are 'bystander' studies using extremely high doses (15 Gy) still showing only modest inductions of bystander apoptosis (50% increase) (Gaugler *et al.*, 2007). When cells were radiolabelled with ^{3}H-thymidine up to dose-rates of 320 mGy.h^{-1}, no changes in cell-cycle distribution were observed in the co-cultured bystander cells (Pinto *et al.*, 2006). Together, these divergent results affirm

that extrapolation from high-dose bystander effects to low-dose exposures is unwise, upholding the strategy described in this study, to examine doses/dose-rates commensurate with realistic radiation exposures for the general public.

Investigations into the timing of DNA double-strand break induction in co-cultured and medium-transfer bystander cells has begun to narrow the time-frame of the bystander response (Hu *et al.*, 2006; Han *et al.*, 2007), which now seems to be quite rapid. Despite the apparent speed of the signal, microbeam α-particle irradiations of three-dimensional human tissue models have shown bystander γ-H2AX, micronuclei and apoptosis persisting 12–48 h after irradiation (Sedelnikova *et al.*, 2007). Confirming the value of the global screening method used in the present study, are data showing that CDKN1A was induced in bystander cells throughout a cell culture, but was found not to be spatially related to the locations of microbeam-irradiated cells (Fournier *et al.*, 2007). Such a result is perhaps explained by the finding that medium collected from long-term cultures of bystander cells (originally exposed to ICCM from 5 Gy irradiated cells) could induce DNA breaks in secondary bystanders (Rugo *et al.*, 2007), providing a mechanism for signal amplification. Persistent DNA methylation changes in bystander cells were implicated in these long-term secondary responses (Kaup *et al.*, 2006; Rugo *et al.*, 2007).

Much work has been performed to try to identify the bystander signal(s). Studies have continued to show the involvement of TGF-β, nitric oxide and reactive oxidative species, with more studies now implicating NFκB, DNA fragments and membrane signalling (Burdak-Rothkamm *et al.*, 2007; Ermakov *et al.*, 2007; Shao *et al.*, 2008a; Shao *et al.*, 2008b; Zhou *et al.*, 2008). The pathways activated in bystander cells seem as varied as the endpoints studied (Chaudhry, 2006;

Shankar *et al.*, 2006; Fournier *et al.*, 2007; Mitra and Krishna, 2007; Ponnaiya *et al.*, 2007), and what appears essential to one bystander endpoint can have no effect on others (Lyng *et al.*, 2006; Baskar *et al.*, 2007; Kashino *et al.*, 2007; Nagasawa *et al.*, 2008; Zhang *et al.*, 2008). Further interactions with other low dose effects have been investigated with studies that detected bystander genomic instability (Bowler *et al.*, 2006), those that did not (Huang *et al.*, 2007), and the observation of *anti*-adaptive responses that sensitise bystander cells to subsequent direct irradiation (Mothersill *et al.*, 2006).

Conclusions

The adoptive transfer method described here fulfils a need of the radiation protection community to have an *in vivo* model in which to validate the reports of bystander effects observed *in vitro*. Although communication of radiation effects between irradiated and unirradiated cells has been clearly established *in vitro*, after over 15 years of research, it has not been possible to demonstrate a true bystander effect in an animal model. The work that has been conducted *in vivo* thus far is difficult to interpret, since bystander and abscopal effects are often not clearly distinguished. The differences between bystander effects as described here and abscopal effects are important for a number of reasons. Firstly, the existence of low-dose radiation phenomena such as radioadaptive responses, low-dose hypersensitivity and the bystander effect itself should make investigators wary about extrapolating the responses of high-dose irradiations down to low doses. The biology of signalling between cells within a sparsely irradiated tissue and the communication of systemic effects from a highly necrotic tissue throughout the body is likely to be very different, and conclusions drawn from one scenario are unlikely to correspond to the other.

Most importantly for radiation risk-regulation, the effects arise from two very different exposure scenarios. Abscopal effects are likely to occur after therapeutic exposure of a tissue, which is planned, necessary, and balances the risks of a life-threatening illness and future secondary cancer. The risks of secondary cancers after radiotherapy can be determined directly in human patients; and ultimately, the risk decision will be left to doctors and their patients, not radiation regulators. In accidental exposures creating an abscopal situation, regulation is not a concern, since

post hoc analysis of separate risk from direct and abscopal effects is only of use in triage of the exposed individual.

Conversely, bystander effects, if they occur *in vivo*, will affect everybody. In fact, most of our natural radiation exposure occurs under bystander conditions, that is, with rare, isolated cells receiving radiation energy depositions at any one moment, whilst the remaining cells are unexposed. Highly refined biological defences against radiation-induced carcinogenesis are likely to have developed under these circumstances; the response to a tissue receiving 5 Gy is simply an uncoordinated trauma-induced series of events. The challenge of low-dose radiobiology is to determine the risks of radiation exposure below the levels where deterministic effects predominate, that is, where the cellular and tissue responses are not traumatic, but dynamic.

The bystander effect, as observed *in vitro*, displays enormous variability in its nature, magnitude and the conditions under which it can be induced. Taking the large amount of data gathered thus far, two simultaneous approaches need to be taken. Firstly, as Newman (2006) proposes, there is a need to develop a common, defined *in vitro* system for probing bystander effects that is reproducible between laboratories and is amenable to chemical and molecular biological analysis:

> ...in the absence of such a system, the field is doomed to continue in its current manner; viz, individual reports that are consistent within a group but cannot be (completely) confirmed in other laboratories. Newman (2006)

This common system should be developed based on its relevance to the question at hand, (i.e. lung cell lines exposed to low fluences of α-particles, or cells from other

tissue types exposed to very low fluences of photons) and not based upon convenient cell lines that give responses only at irrelevant doses, or cells that give robust effects clearly unrelated to normal tissue responses. Such an *in vitro* system could be carefully studied to identify the molecular signalling pathway(s) involved in propagating bystander effects.

Equally important, is the development of *in vivo* models to validate and confirm the results that have been observed *in vitro*. It is only with proof of bystander signalling, *in vivo*, under conditions relevant to human radiation exposures that the bystander effect will mature from a curious phenomenon into an assessable risk. The adoptive transfer method described here is one such model. The experimental data shown here demonstrate that an irradiation scenario applicable to radiation exposure received by the public, can be constructed *in vivo*, in a normal, living, functioning tissue. By altering the experimental parameters, the effects of time, dose, dose-rate, radiation quality, genetic background/strain, age and sex can be explored for a variety of biological endpoints. Given a large enough study, the method even has the potential to study the induction of cancer itself. The bystander cells can be examined *in situ*, in two- or three-dimensions to give highly precise measurements of any fluorescent-endpoint that can be examined in the cryopreserved tissue. The method is sensitive enough to detect meaningful changes in bystander endpoints using as few as five mice per group, and by increasing the number of animals examined, the method has proven statistical power to exclude changes of >10% from the control level.

The preservation of spatial information in the tissues allows the examination of local or tissue-wide effects and for radiolabelled donor cell experiments, the irradiation status and level can be confirmed *in situ* using autoradiography. In this study, spleen

was chosen as the first tissue to be analysed, however, the lodging of donor cells in the lung, liver, gastrointestinal tract and bone marrow might allow this versatile model to be used to analyse bystander responses *in vivo* in a range of relevant tissues. Further, the ability to change the genotype/phenotype of the donor or recipient mice, including transgenic mice, adds the possibility of examining any candidate mechanisms by the knockout or addition of proteins of interest.

The results from the first series of experiments conducted using the adoptive transfer method showed no evidence for bystander apoptosis or proliferation *in vivo*. By no means, do these results prove that bystander effects do not occur; there are still a range of times, doses, equivalent tissue dose-rates and endpoints that need to be examined. However, the failure to see the same effects as repeatedly demonstrated by others *in vitro* should prompt caution in the interpretation of bystander observations in cell culture experiments. Knowledge of how our bodies respond to the radiation exposure constantly received from natural background sources, not only will inform radiation risk assessment, but could provide key insights into potential avenues for modifying the response, or harnessing the body's own natural defences, for future cancer prevention and treatment strategies. If bystander signalling does occur *in vivo*, such intercellular regulation of homeostasis may also be employed after other stressors and damage; understanding the intercellular signalling following sparse irradiation may also provide information on other ways in which tissues can co-ordinate the response to a variety of perturbations. This adoptive transfer method provides a unique opportunity to monitor communication between cells *in vivo*.

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