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Manuela Buonanno, Geraldine Gonon, Badri N. Pandey, Edouard I. Azzam. The intercellular communications mediating radiation-induced bystander effects and their relevance to environmental, occupational, and therapeutic exposures. *International Journal of Radiation Biology*, 2023, 99 (6), pp.964 - 982. 10.1080/09553002.2022.2078006 . hal-03718548

HAL Id: hal-03718548

<https://hal.science/hal-03718548>

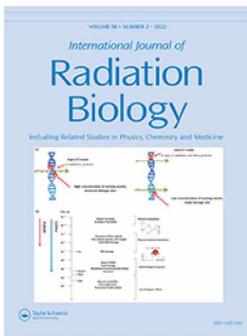
Submitted on 8 Jul 2022

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To cite this article: Manuela Buonanno, Géraldine Gonon, Badri N. Pandey & Edouard I. Azzam (2022): The intercellular communications mediating radiation-induced bystander effects and their relevance to environmental, occupational, and therapeutic exposures, International Journal of Radiation Biology, DOI: [10.1080/09553002.2022.2078006](https://doi.org/10.1080/09553002.2022.2078006)

To link to this article: <https://doi.org/10.1080/09553002.2022.2078006>



Accepted author version posted online: 13 May 2022.



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The intercellular communications mediating radiation-induced bystander effects and their relevance to environmental, occupational, and therapeutic exposures

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Running title: Radiation bystander responses and human exposures

Key words: out-of-field radiation effects; radiation protection; radiotherapy; radiation dose; linear energy transfer; radiation-induced abscopal effects

Biographical Sketches

Manuela Buonanno received her B.Sc. in Physics from the University of Naples (Italy), and Ph.D. in Radiobiology from Rutgers University (USA). She is currently an Associate Research Scientist at the Center for Radiological Research at Columbia University, USA.

Géraldine Gonon received her engineering diploma in Biosciences from the Université Paris-Est Créteil in France, then her Ph.D. in radiobiology from Rutgers University (USA) and University of Franche-Comté, (France). She is currently a researcher at Institut de Radioprotection et de Sûreté Nucléaire (IRSN), France.

Badri Pandey completed his M.Sc. at Gorakhpur University (India) and his Ph.D. at Mumbai University (India). He pursued post-Doctoral research at Rutgers University (USA). Currently he is Head of Radiation and Cancer Biology Section, Radiation Biology and Health Sciences Division, Bhabha Atomic Research Centre, Mumbai, India.

Edouard Azzam completed his post-doctoral studies under the mentorship of the late Professor John B. Little. He is Professor Emeritus at Rutgers New Jersey Medical School (USA), and currently works as Research Scientist at Canadian Nuclear Laboratories, Chalk River, Canada.

Abstract

Purpose: The assumption that traversal of the cell nucleus by ionizing radiation is a prerequisite to induce genetic damage, or other important biological responses, has been challenged by studies showing that oxidative alterations extend beyond the irradiated cells and occur also in neighboring bystander cells. Cells and tissues outside the radiation field experience significant biochemical and phenotypic changes

that are often similar to those observed in the irradiated cells and tissues. With relevance to the assessment of long-term health risks of occupational, environmental and clinical exposures, measurable genetic, epigenetic, and metabolic changes have been also detected in the progeny of bystander cells. How the oxidative damage spreads from the irradiated cells to their neighboring bystander cells has been under intense investigation. Following a brief summary of the trends in radiobiology leading to this paradigm shift in the field, we review key findings of bystander effects induced by low and high doses of various types of radiation that differ in their biophysical characteristics. While notable mechanistic insights continue to emerge, here the focus is on the many means of intercellular communication that mediate these effects, namely junctional channels, secreted molecules and extracellular vesicles, and immune pathways.

Conclusions: The insights gained by studying radiation bystander effects are leading to a basic understanding of the intercellular communications that occur under mild and severe oxidative stress in both normal and cancerous tissues. Understanding the mechanisms underlying these communications will likely contribute to reducing the uncertainty of predicting adverse health effects following exposure to low dose/low fluence ionizing radiation, guide novel interventions that mitigate adverse out-of-field effects, and contribute to better outcomes of radiotherapeutic treatments of cancer. In this review, we highlight novel routes of intercellular communication for investigation, and raise the rationale for reconsidering classification of bystander responses, abscopal effects, and expression of genomic instability as non-targeted effects of radiation.

Introduction

A historical perspective

Since the discovery of X rays in 1895, physics has had a large impact on the advances in radiation biology and radiation oncology: the physical characteristics of the radiation and the size of the dose absorbed by irradiated cells and tissues determine the nature and magnitude of the biological response to the exposure. Using first generation X ray tubes, major radiobiological discoveries were made shortly after Roentgen's discovery of X rays. In 1906, Bergonié and Tribondeau determined that rapidly proliferating cells are more radiosensitive than specialized cells (Bergonié and Tribondeau 2003), and in the early 1920's, Regaud showed that fractionation of the radiation dose mitigates damage to normal tissue (Regaud and Ferroux 1927), a finding that has had an enduring impact on the mode of delivery of radiotherapy. In the decades that followed, advances in the development of techniques and instruments capable of detecting and quantifying subtle biochemical changes in specific cells within tissues greatly advanced the field of radiobiology. Simultaneously, novel radiation delivery equipment, advanced imaging techniques, and 3-dimensional treatment planning systems enhanced the efficacy of therapeutic treatments. The rapid pace of these technological developments and scientific discoveries enriched our basic knowledge of the molecular, cellular, and tissue responses to radiation exposure. However, even up to the 1990's, it remained generally assumed that radiation traversal through the nucleus is necessary to produce genetic damage or an important biological response (Little 2003), although a prominent role for membranes had been advocated (Alper 1977; Petkau 1980). The assumption was mainly influenced by the target theory, which evolved during a period spanning the mid-1920's to mid-1940's to explain the adverse biological effects of ionizing radiation, mainly killing of unicellular organisms. The classical target theory was based on a physical model and assumed that cell killing is directly related to the amount of energy deposition (number of hits) in radiosensitive targets with biological function (Lea 1947). Through seminal work by Zirkle and co-workers, we came to understand that these targets are contained within the nucleus (Zirkle and Bloom 1953), which coincided with the discovery of the 3-dimensional

structure of the DNA molecule. Notably, the early work of Zirkle and colleagues highlighted the importance of the biophysical characteristics (specifically linear energy transfer, LET) of the impacting radiation in inducing chromosomal damage (Zirkle and Tobias 1953). Follow-up work using sparsely (low LET) and densely (high LET) ionizing radiations showed that the DNA double strand break was the central lesion inducing cell death (reviewed in (Ward 1988)).

At the onset of the pioneering work of the radiation sciences founders (reviewed in (Bedford and Dewey 2002)), the concept of radiation absorbed dose and its importance in the biological response of irradiated cells was proposed by Gioacchino Failla (Failla 1921) to allow comparisons between irradiations at different laboratories. A 100 years ago, Failla observed that the same physical dose of the two radiation sources available at that time (radiation emitted from radium or 140 kV X rays) produced different levels of biological effects (Failla 1926). While radiation dose was originally conceived to measure the amount of energy deposited per unit volume, Failla suggested that “doses of radiation be reckoned according to the amount of energy absorbed by the radiated tissue”, or in other words the amount of energy deposited per unit mass. However, biological effects vary greatly whether the mass in consideration is cellular (on the order of nanogram) or nuclear (on the order of picogram), or in the case of a densely ionizing particle whether the diameter of the radiation track (core vs penumbra) (Goodhead and Nikjoo 1989) is considered. Yet, numerous observations made during the past century showed that the biological response is not solely affected by energy deposition in the irradiated cells or tissues. In a landmark paper, Hatsumi Nagasawa and John B. Little (Nagasawa and Little 1992) showed that the target for genetic damage following exposure to α particles extends significantly beyond the traversed cells and occurs in neighboring cells that do not experience energy deposition (*i.e.*, *bystander cells*): recombination events leading to sister chromatid exchanges were increased in $\sim 30\%$ of cells in monolayer cultures exposed to α particles where less than 1 % of nuclei were estimated, by dosimetric calculations, to be traversed by a particle track (note that the range of δ rays produced by the 3.6 MeV α particles used in these studies is less than 0.1 μm and cross-irradiation is unlikely). These findings led to a paradigm shift

in radiation biology, and implied that assessment of the collective response of a cell population to ionizing radiation is more informative of potential health outcomes than the responses of individual cells wherein the physical dose is deposited.

In the early work, the expression of bystander effects, measured by cytogenetic alterations and changes in gene expression, was thought to be independent of the dose absorbed by the irradiated cells and saturated at relatively low mean absorbed doses (Nagasawa and Little 1992). However, later studies have shown that the amount of dose absorbed by irradiated cells affects the extent of spread of the induced stress and the magnitude of biochemical changes in bystander cells (*e.g.*, (Shao, Furusawa, et al. 2003; Persaud et al. 2005; Buonanno, de Toledo, and Azzam 2011; Buonanno et al. 2011; Gonon et al. 2013; Shao, Aoki, and Furusawa 2003; Mitchell et al. 2004; Maguire et al. 2005)). These observations led to a fundamental change in radiation biology and have been considered of importance in the assessment of health risks associated with radiation exposure, whether from environmental sources or during occupational activities and in clinical settings. Moreover, they advanced the thinking of exploiting the mechanisms underlying bystander effects for beneficial therapeutic outcomes, such as amplifying toxic effects among irradiated tumor cells ('cohort effects') (Autsavapromporn et al. 2011) and leading to regression of tumors at distant sites, a phenomenon known as 'abscopal effect' (Demaria et al. 2004). In the following, we review the field of bystander responses, and briefly that of genomic instability, two radiobiological aspects that have been impacted by pioneering work of Professor John B. Little.

Paradigm shift in radiation biology: evidence for out-of-field effects

Since the beginning of the twentieth century intriguing biological phenomena were reported describing biological changes in unirradiated cells and tissues present in irradiated living specimens. For example, as early as 1915, Murphy and Morton observed that tumors implanted in irradiated syngeneic animals shrank by 50% (Murphy and Morton 1915) (for historical review of early observations see (Mothersill et al. 2018)). Over subsequent decades, several studies showed that lymphocytes cultured in

the presence of plasma from individuals exposed to radiation several years earlier, whether accidentally, during the A-bombing in Hiroshima and Nagasaki or as a result of the Chernobyl nuclear accident, underwent genetic damage (Goh and Sumner 1968; Hollowell and Littlefield 1968; Pant and Kamada 1977; Emerit et al. 1995). Notably, Brooks and co-workers showed in animal experiments performed in the 1970s that α particles emitted by plutonium dioxide ($^{239}\text{PuO}_2$) concentrated in a small fraction of the total liver cell population resulted in chromosomal damage in the majority of cells in the liver (Brooks, Retherford, and McClellan 1974). These early observations, together with the seminal work of Nagasawa and Little in the 1990s with α particles from an external plutonium source, cemented the challenge to the assumption that biological effects are limited to cells receiving energy deposition in their nuclei. Collectively, the observed effects have been classified as radiation-induced non-targeted effects and include bystander responses and the expression of genomic instability. However, as the field has evolved, the term ‘non-targeted effect’ is ripe for redefinition as it can be argued that a ‘bystander cell’ or the ‘progeny of irradiated or affected bystander cells’ are also targets of the radiation exposure. Hence, here we use the term ‘out-of-field’ effect.

Genomic instability, a hallmark of cancer (Hanahan and Weinberg 2011), has been the subject of extensive research in radiobiology (reviewed in (Kronenberg 1994; Baulch 2019)). Briefly, studies have demonstrated evidence for non-clonal mutations and chromosome aberrations appearing in the descendants of irradiated mammalian cells several generations of replication after exposure (*e.g.*, (Pampfer and Streffer 1989; Little, Gorgojo, and Vetrovs 1990; Watson et al. 2001; Chang and Little 1992; Holmberg et al. 1995; Grosovsky et al. 1996; Harper, Lorimore, and Wright 1997; Little et al. 1997; Sudo et al. 2008)). Notably, hematopoietic cells derived from murine stem cells exposed to moderate mean absorbed doses of ^{238}Pu α particles (LET ~ 121 keV/ μm) harbored a high frequency of non-clonal aberrations in their clonal descendants (Kadhim et al. 1992). Ensuing papers have also reported an enhanced rate of non-clonal mutations and other changes as a result of genome destabilization of the descendants of mammalian cells exposed to radiations with a wide range of high LET (386-13,600

keV/ μm) (Sabatier, Dutrillaux, and Martin 1992) (reviewed in (Morgan 2003a, 2003b)), and generated evidence for factors that contribute to genomic stability (Wiese et al. 2002). This evidence was preceded by seminal observations made decades earlier. In 1949, Newcombe and Scott reported on the delayed appearance of radiation-induced mutants in bacteria and speculated that the delayed effect resulted from induced ‘gene instability causing an increase in the spontaneous mutation rate over a number of cell generations’ (Newcombe and Scott 1949), a phenomenon reminiscent of the induction, by mustard gas of chromosomal instabilities in *Drosophila melanogaster* (Auerbach 1947). In the 1970s, Terzaghi and Little (Terzaghi and Little 1976) also showed that expression of the transformed state of a single irradiated mammalian cell requires several cell replications, depending on the cell type. They suggested that exposure to radiation induces a genetic instability in irradiated cells, which enhances the rate of mutations and transformation in their progeny. Here, we highlight evidence for genomic instability in progeny of bystander cells, a response with significant implication in assessment of the health risks of exposure to radiation.

Bystander effects: *in vitro* evidence using broadbeam and microbeam irradiators, and internally incorporated radionuclides

The scientific interest in studying bystander effects was particularly stimulated by the concern about lung cancer caused by α particles emitted by environmental radon gas and its decay products. At exposures similar to those from indoor radon, the majority of cells in the bronchial epithelium would not be traversed by an α particle track, and those whose nuclei are in the path of the tracks are likely to experience a large energy deposition that can lead to their death (Barendsen 1994). Therefore, the study of bystander responses became an attractive approach to investigate biological changes, including neoplastic transformation, in non-irradiated cells adjoining α particle-irradiated cells (Sawant et al. 2001; Buonanno, de Toledo, and Azzam 2011).

Using α -particle broadbeams, energetic helium ion microbeams, and X ray-, electron- and heavy ion broadbeams and microbeams, numerous studies rapidly confirmed Nagasawa and Little's discoveries (Nagasawa and Little 1992). Using the text 'radiation bystander', a March 2022 PubMed search reveals over ~ 1600 papers describing such findings. A selection of key results describing *in vitro* manifestation of bystander effects is outlined in Table 1.

< Table 1 >

Using a variety of mammalian cells maintained under confluent or sparse culture conditions and a multitude of biological endpoints, bystander effects were firmly shown to occur following exposure of a small fraction of cells in a culture to low fluences of α particles delivered from broadbeam irradiators. They were also observed in protocols involving co-culture experiments, and transfer of growth medium from cell cultures exposed to moderate and high doses of X and γ rays, or particulate radiations to unirradiated cultures. A few studies involving medium transfer have also shown that low doses of X and γ rays, similar to those encountered in clinical and occupational settings, induce cytotoxic and clastogenic effects (Mothersill and Seymour 1997; Lyng, Seymour, and Mothersill 2002; Yang, Asaad, and Held 2005).

Using both broadbeam and microbeam irradiators and various cell culture techniques, bystander effects were also shown to be elicited by low fluences of densely ionizing energetic heavy ions (Kobayashi et al. 2009), which is of concern for prolonged human deep space exploration missions (Shao, Furusawa, et al. 2003; Yang, Anzenberg, and Held 2007; Held 2009; Gonon et al. 2013; Autsavapromporn et al. 2015). Bystander effects were also detected in unirradiated cells co-cultured with cells exposed to high doses of external beams of densely or sparsely ionizing radiations (*e.g.*, (Gerashchenko and Howell 2003; Yang, Anzenberg, and Held 2007)). Likewise, they were detected in experiments involving co-culture of cells labelled with short range α -emitters (5-[125]iodo-2'-

deoxyuridine, ^3H -thymidine) with unlabeled cells. In these experiments the labelled cells imparted cytotoxic effects to adjoining non-labeled cells (Bishayee, Rao, and Howell 1999; Xue et al. 2002; Persaud et al. 2005).

In the initial experiments with α particle broadbeam irradiators, the concept of bystander responses provided a suitable explanation for the fact that the number of adversely affected cells greatly exceeded that expected on the basis of an effect due solely to traversal by radiation of an estimated small fraction of nuclei (Nagasawa and Little 1992; Deshpande et al. 1996; Azzam et al. 1998). The development of precision microbeam irradiators allowed to unequivocally identify the cellular organelle that is irradiated and quantifying biological changes in traversed cells and in the bystanders (Prise et al. 1998; Folkard et al. 2001; Randers-Pehrson et al. 2001). Refinements in microbeam technology now permit delivery of a specified number of particles to selected cell compartments with sub-micrometer precision. Mimicking original experiments using a polonium tipped needle positioned at different distances from cell nuclei (Munro 1970), microbeam irradiation has been employed to study the effect of either nuclear or cytoplasmic irradiation on biochemical changes in both the irradiated and bystander cells (Wu et al. 1999; Shao et al. 2004). Recent studies of cells exposed to microbeams of heavier ions (*e.g.*, carbon, neon, and argon) have shown that, for any particle, the induced DNA-damaging bystander effect is dose-dependent and is facilitated by junctional intercellular communication (Suzuki and Tsuruoka 2004; Autsavapromporn et al. 2013). Using a staining technique to distinguish between irradiated and bystander cells in an exposed monolayer culture, Ponnaiya *et al.* (Ponnaiya et al. 2004) have shown that traversal of a cell by even a single energetic helium ion can induce micronucleus formation and cell cycle delays in bystander cells. Such results visually supported the initial work based on microdosimetric consideration that non-irradiated cells in the population contribute to the risk of exposure to low fluences of α particles. When exposed to low fluences of helium nuclei from microbeam, significantly more cells than those that were irradiated harbored genetic damage leading to enhanced mutations (Zhou et al. 2000)

and neoplastic transformation (Sawant et al. 2001), implying that the risk of adverse health outcomes at these levels of exposure can be higher than expected (Brenner, Little, and Sachs 2001).

Overall, a multitude of *in vitro* studies in various cell types challenged the paradigm that radiation traversal through a cell nucleus is the only prerequisite to produce genetic damage or other biological changes. They indicate that bystander cells in the vicinity of irradiated cells or those incubated in growth media harvested from irradiated cultures may undergo measurable biochemical modifications that are often similar in nature to those induced in the irradiated cells.

***In vivo* evidence of bystander effects**

In addition to observations in cell culture experiments, radiation-induced bystander effects (RIBE) were detected in tissue models and in partial body-irradiated animals. In tissue explants, or reconstituted 3-dimensional tissue models, α particle microbeam irradiation of a thin plane of cells through the tissue induced a variety of biological changes in bystander cells as a function of the distance away from the irradiated cell plane (Belyakov et al. 2002). Further, tissue culture media conditioned by placentae irradiated *in utero* induced DNA damage in neonatal bone marrow cells cultured *ex vivo*, supporting the two-hit model of childhood leukemogenesis through bystander signalling (Mansell et al. 2019). Out-of-field effects were also reported in tissues distant from the irradiated area (*e.g.*, (Khan, Hill, and Van Dyk 1998; Kovalchuk et al. 2016; Chai et al. 2013)). Investigation of bystander effects in complex models not only contributed to a better understanding of the phenomenon but also in characterizing the extent of its propagation and its relevance in therapeutic treatments and assessment of health risks (Prise and O'Sullivan 2009; Azzam et al. 2013).

Early *in situ* investigations using a broadbeam irradiator and cell culture dishes with a solid-state nuclear track detector grafted to the cell growth surface, indicated that low fluence α particles induced the stress responsive protein CDKN1A (p21^{Waf1}) within a 100 μ m radius from a targeted cell (Gaillard et al. 2009). Similarly, in the simple living organism *Caenorhabditis elegans* (Sugimoto et al. 2006;

Bertucci et al. 2009) a 1 μm diameter 3 MeV proton microbeam induced bystander stress responses up to $\sim 150 \mu\text{m}$ away from the irradiated region of the worm. Comparable results were obtained in a mouse ear model with induction of $\gamma\text{-H2AX}$ foci formation as endpoint (Buonanno, Randers-Pehrson, et al. 2015). Models of complex living organisms in which multicellular architecture and physiological condition are preserved have contributed to a better understanding of the various implications of bystander effects *in vivo*; they informed on the molecular pathways mediating the effects, and showed that redox modulated metabolic events as well as genetic and epigenetic changes occur in bystander tissues (Koturbash et al. 2008; Jain et al. 2011; Chai et al. 2013; Lorimore et al. 2001; Tamminga and Kovalchuk 2011). With significance to cancer risk, oncogenic effects were observed in the cerebellum of radiosensitive mice when only their lower body was X irradiated (Mancuso et al. 2008). Although the radiation-induced bystander/abscopal effect has been linked to deregulation of cellular energetics, oxidative stress, and inflammatory responses (Heeran, Berrigan, and O'Sullivan 2019; Azzam, de Toledo, and Little 2003b) intriguing mechanistic insights continue to emerge.

Mechanistic aspects

Several mechanisms have been shown to mediate RIBE with intercellular communications and oxidative metabolism being extensively investigated (reviewed in (Azzam, de Toledo, and Little 2003b; Spitz et al. 2004; Pouget, Georgakilas, and Ravanat 2018) (Figure 1 with focus on intercellular communications). Often, a crosstalk between the pathways mediating these mechanisms has been described (Little 2003). DNA repair and cell cycle checkpoints were also found to be involved (Huo, Nagasawa, and Little 2001). Moreover, inflammatory responses are emerging as critical pathways in mediating both beneficial and adverse abscopal effects. Here, we review mainly the role of direct and indirect modes of intercellular communications.

As described in Figure 1, RIBE occur in cells that are contiguous to irradiated cells or in their vicinity, and in distant (abscopal) cells/organs (Figure 2). When bystander cells are adjacent to irradiated

cells, transmissible factors may be exchanged intercellularly through junctional channels (*e.g.*, gap junctions) and/or conduits (*e.g.*, tunneling nanotubes, TNTs). On the other hand, vesicles, cytokines, and other molecules secreted by irradiated cells/tissues can lead to biochemical changes in bystander cells in their vicinity or in cells/tissues that are distant. These effects occur at lethal or sub-lethal doses of radiation, and when they occur *in vivo* may involve participation of the immune system (Rodriguez-Ruiz et al. 2018). When lethal doses of radiation are applied, different profiles of secreted factors result in bystander or abscopal effects. It is thought that the responses depend on the magnitude of injury and the induced mode of cell death and occur with or without involvement of the immune system (Heeran, Berrigan, and O'Sullivan 2019; Grass, Krishna, and Kim 2016). Hence, in purview of mechanistic aspects, inter- and extracellular modalities of communication are discussed below (Figure 2 with focus on various forms of *in vivo* intercellular communications: cell-to-cell contact, paracrine and non-paracrine signaling).

A. Intercellular communications

A.1 Gap junctions: Under normal physiological conditions, gap junctional intercellular communication (GJIC) is required for maintenance of tissue homeostasis. It is also a major pathway for cell-to-cell transmission of the toxic effects of radiation and chemotherapeutic agents (reviewed in (Aasen et al. 2019)). Its role has been studied at the onset to understand the means of propagation of radiation-induced stressful effects under both *in vitro* and *in vivo* conditions. Gap junctions are integral membrane channels composed of diverse isoforms of connexin (Goodenough, Goliger, and Paul 1996). They link the cytoplasm of contiguous cells through the exchange of small molecules (<1 kDa), such as atomic ions (K^+ and Ca^{2+}), second messengers (*e.g.*, cAMP, cGMP, IP_3), glutathione, glucose and other molecules that permeate through their aqueous pores. In the process, they couple contiguous cells not only metabolically but also electrically (Harris 2018). A gap junction is composed of connexin (Cx) proteins, with Cx43 being the most abundant and well-studied form in the Cx family (21 isoforms in humans). Notably,

different Cxs form channels with distinctive selectivity for molecular permeants (*e.g.*, second messengers (Niessen et al. 2000; Harris 2007)) leading to transmission of either damage or rescue signals between irradiated cells and their neighboring bystanders (de Toledo et al. 2017; Zhao et al. 2014). The phosphorylation of Cx proteins governing their gating and trafficking properties is a post-translational modification that was found to be exquisitely sensitive to low fluence α particle irradiation (Azzam, de Toledo, and Little 2003a). Under stress conditions, gap junctions facilitate rapid intercellular communication of shared biochemical signals, which contributes to attenuating dilution or degradation effects that may occur when these signals are secreted extracellularly.

Initial evidence for the involvement of gap junctions in RIBE was generated after exposure of cell cultures to low fluences of broadbeam α particles. In studies involving confluent, density inhibited human diploid fibroblasts, a higher fraction of cells than predicted upregulated the level of stress inducible CDKN1A (p21^{Waf1}). The effect was abrogated by treatment with the gap junction inhibitor lindane (Azzam et al. 1998). Direct evidence of the involvement of Cx43 was subsequently generated through genetic approaches revealing DNA damage and other stress responses in a higher fraction of cells than the nuclei traversed by α particles. The effect was observed only in *CX43* wild-type and gap junction-competent cells (Azzam, de Toledo, and Little 2001). The role of gap junctions in transmitting lethal and mutagenic effects to bystander cells was studied in a variety of other cell types exposed to low fluences of high LET particulate radiations (Zhou et al. 2001; Suzuki and Tsuruoka 2004; Suzuki, Yasuda, and Kitamura 2020) or photon radiation (Hoorelbeke et al. 2020). Together, the studies showed that radiation-induced elevation of reactive oxygen species (ROS) generation is associated with gap junctional permeation of signaling molecules between irradiated and bystander cells. For example, due to its size and charge, Ca²⁺ easily permeates through gap junctions (Harris 2018), and previous studies have described the contribution of Ca²⁺ to bystander effects (Lyng et al. 2006).

The role of GJIC in propagating radiation induced effects to distant bystander sites has been also reported *in vivo*. The oncogenic bystander effect in the shielded cerebellum of radiosensitive Patched-1

(Ptch1) heterozygous (Ptch1^{+/-}) mice exposed to X rays shown by Mancuso *et al.* (Mancuso et al. 2008) was mitigated after treatment with 12-O-tetradecanoylphorbol-13-acetate, a potent protein kinase C activator and cell-to-cell communication inhibitor. Although numerous studies have addressed the role of connexin phosphorylation in assembly/disassembly, degradation, and gating of the channels they form (Lampe and Lau 2004), the role of Cx phosphorylation in RIBE effects warrants further investigation. Interestingly, in γ particle-irradiated non-tumorigenic HepG2 cells, activation of Src kinase leading to a Cx43 phosphorylation (Tyr265) responsible for RIBE was regulated by ROS-mediated autophagy (Yang et al. 2018). On the other hand, exposure of human A549 lung cancer cell cultures to a low fluence of γ particles resulted in enhanced proliferation and a greater tumor volume upon implantation in SCID mice. The effect was also associated with increased phosphorylation of Cx43, but a decrease in GJIC (Rajan and Pandey 2021). Studies examining phosphorylation changes in specific amino acids of Cx proteins in irradiated cells and whether these changes enable or inhibit functional junctional communication would shed light on the underlying biochemical events.

It has been proposed that in addition to extracellular release, long-lived reactive oxygen and nitrogen species permeate among cells through gap junctions (Upham and Trosko 2009). Notably, membrane bound NAD(P)H oxidase, which has been implicated in γ particle-induced adverse bystander effects (Narayanan, Goodwin, and Lehnert 1997; Azzam et al. 2002), is located in cholesterol-rich domains of the plasma membrane where junctional channels are also localized. In addition, recent reports have shown that gap junctions are permeable to microRNAs (miRNAs) (Xu et al. 2015; Hong et al. 2015). The latter are ~22 nucleotide long single-stranded non-coding RNAs that form linear molecules with a diameter of ~1 nm in the order of gap junction channel pore size. Their highly efficient transfer between irradiated and bystander cells (Zong et al. 2016; Peng et al. 2019) would control the expression of target genes, leading to significant phenotypic and behavioral changes. There is evidence that different Cx channels display differential permeability to miRNAs (Cx43 > Cx26/30 > Cx26 > Cx31 > Cx30) (Zong et al. 2016). Whereas Cx mutations and gap junction blockers eliminate miRNA transfer, gap

junction enhancers and overexpression of Cx increase it (Zong et al. 2016). Therefore, gap junction mediated transfer of miRNA may play a crucial role in the mechanism(s) underlying RIBE and is a significant avenue for further investigation, particularly in the search for mitigators of adverse effects and in treatment of gap junction competent tumors.

A.2 Bidirectional bystander effects and the role of communication channels

Contrary to canonical unidirectional bystander signals propagated from irradiated cells, bidirectional transmission of signals has been observed under various conditions, showing that transmission of signals from bystander to irradiated cells may attenuate or enhance the stress induced in irradiated cells, at least transiently. Notably, a rescue effect leading to attenuation of DNA damage in proton-irradiated cancer cells was detected only when the cells were co-cultured with normal bystander cells, and not with other cancer cells (Desai et al. 2014). The communication of protective bystander effects has been also reported after exposure to other radiation types (photons, α particles) (Yu 2019) but with limited mechanistic insights. Compensation for cyclic adenosine monophosphate (cAMP) levels in irradiated cells by bystander cells was suggested to contribute to the rescue mechanism (He et al. 2014). Other studies have shown that rescue effects by bystander cells involve nitric oxide (*NO) and the NF- κ B pathway (Yu 2019), as well as autophagy (Kong, Cheng, and Yu 2018).

The role of the selective permeability of junctional channels in propagating damaging or protective effect was also investigated in studies of long-term effects in progeny of bystander cells. Whereas rescue effects were communicated at early times through junctional channels consisting of Cx32 (Autsavapromporn et al. 2013), several generation of replication later, the progeny of bystander cells that communicated with irradiated cells through Cx32 channels expressed elevated levels of spontaneous DNA damage (de Toledo et al. 2017). In contrast when irradiated and bystander cells communicated through junctions consisting of Cx26 or Cx43, the harmful effects detected in bystander cells within hours after co-culture decayed in their progeny, perhaps as a result of death or permanent proliferative arrest of

the affected bystander cells (de Toledo et al. 2017). These results suggest that intercellular communications play different roles under stress conditions, and permeability of the junctional channels contribute to both early effects in bystander cells and delayed effects in their progeny. The permeability is likely impacted by the redox state of the cell and radiation-induced post-translational modifications of the connexins forming the channel (Zhao et al. 2014). Clearly, identification of the modified amino acids (phosphorylated, nitrosylated, ubiquitinated) sites and the responsible enzymes, together with the communicated molecules, may help development of countermeasures to harmful effects and may enhance the efficacy of radiotherapeutic treatments.

A.3 Pannexin channels and tunneling nanotubes

Like Cx hemichannels, pannexin (Panx) channels are rapidly activated following exposure to stressful agents and mediate the controlled release of small signaling molecules such as ATP, which has been associated with expression of radiation bystander/abscopal effects (Ohshima et al. 2012). Along with recruiting inflammatory cells to the site of insult, extracellular ATP activates cellular receptors, such as P2 receptors, in an autocrine and paracrine fashion to activate caspases (Aguirre et al. 2013), thus triggering death of out-of-field cells. While Panxs have no sequence homology to Cxs, both proteins have similar membrane topology (Penuela, Gehi, and Laird 2013) and both are regulated by redox potential, thus examining their role in bystander effects is warranted.

Tunneling nanotubes (TNTs, also referred to as tumor microtubules in tumors) are intercellular bridges that form *in vitro* and *in vivo*. TNTs act as conduits with variable diameter (~ 50-800 nm) connecting cells situated up to 300 μm apart. They enable the bidirectional transfer of proteins, RNAs, organelles, pathogens *etc.* (Rustom et al. 2004). They are formed during normal developmental processes in immune cells, are present among tumor cells, and between tumor and immune cells residing in their micro-environment (Patheja et al. 2015; Patheja and Sahu 2017). They have been reported to cause chemo-resistance and promote metastasis of cancer cells (reviewed in (Ariazi et al. 2017)). Interestingly,

TNTs possess gap junctional components (*e.g.*, Cx43) and enable the transfer of electrical signals between remote cells in a manner dependent on the presence of connexons interposed at the membrane interface between TNT and the connected cell (Wang et al. 2010), which leads to speculate that these two intercellular communication systems have evolved to complement each other in multicellular organisms. Even though numerous publications have described the role of TNTs in cancer and normal cells, only one recent study reports about their effect in the response to irradiation. In this study, faster and greater TNT network formation was observed in γ particle-irradiated human glioblastoma cells (Matejka and Reindl 2020). Since TNTs provide an efficient conduit for the transfer of molecules/organelles among distant cells, investigating their role in communication of RIBE would further enhance our understanding of the underlying mechanisms and range of propagation of the effect.

B. Extracellular vesicles

Under normal physiological conditions and during stress, cells release a variety of extracellular vesicles (EVs). Whereas these EVs were initially considered as cell debris, with advances in characterizing their content (miRNA, mRNA, DNA, non-coding RNA, proteins, lipids, mitochondria, *etc.*) and their association with pathological conditions, they have emerged as key modalities for intercellular communication (Devhare and Ray 2018; Mallocci et al. 2019). Depending on their origin, EVs are classified as exosomes, microvesicles (or microparticles), apoptotic bodies, large oncosomes, and microplasts. While exosomes (50-130 nm) are created from an involution of endosomes, microvesicles are larger in size (100-1000 nm) and are produced by direct outward budding of the plasma membrane. EVs released by apoptotic cells (apoptotic bodies) are larger (100-5000 nm), and oncosomes are even bigger (1-10 μ m).

The ability to deliver their varied content to recipient cells makes EVs ideal candidates for an active role in radiation bystander and abscopal effects. However among the EVs, it is primarily exosomes that have been studied in the context of RIBE (reviewed in (Al-Mayah et al. 2015)). Both the magnitude

of release and the composition of exosomes' cargo are altered following irradiation (Jelonek, Widlak, and Pietrowska 2016; Colangelo and Azzam 2020), which renders them efficient 'extracellular messengers' able to modulate signalling pathways that control survival and physiological functions in recipient cells. *In vivo*, exosomes are stable and are continuously produced in the human body. Analyses of their content revealed increases in the levels of proteins involved in important functions, including ROS metabolism, DNA repair, chromatin packaging, protein folding, and metastasis, among others (Abramowicz et al. 2019; Colangelo and Azzam 2020). Together, the studies provided insight into the mechanism underlying the responses to irradiation (increased killing, invasiveness of tumor cells, regression of metastatic foci, *etc.*), and informed on how the tumor microenvironment modulates radiosensitivity (He et al. 2021).

With respect to bystander signaling, exosomes purified from growth media of irradiated cells or sera of irradiated mice were found to contain mitochondrial DNA that increased γ -H2AX and 53BP1 foci in recipient cells (Ariyoshi et al. 2019). In other studies, exosomes shed from irradiated glioma cells decreased proliferation of bystander neural stem cells, and when injected in the hippocampi of mice, caused inhibition of neurogenesis and cognitive impairment (Yang et al. 2021). Furthermore, exosomes derived from the organs of whole or partial body irradiated mice led to changes in viability, DNA damage, and redox changes in recipient mouse embryonic fibroblasts (Tuncay Cagatay et al. 2020). Even though exosomes harbour a varied cargo, it is their role as miRNA carrier that has been mainly studied in RIBE. Significantly, exosomes released from irradiated human pancreatic cancer cells showed higher internalization than exosomes from non-irradiated cells, and resulted in miR-6823-5p mediated inhibition of antioxidant defense and increase of DNA damage in bystander cells (Nakaoka et al. 2021). In other studies, exosomes containing miR-1246, when secreted from γ -irradiated human bronchial epithelial cells affected the efficiency of non-homologous end joining (NHEJ) pathway of DNA repair in bystander cells (Mo et al. 2018). Moreover, γ -irradiation of mouse brain caused the release of exosomal miR-7 triggering autophagy in shielded lung (Cai et al. 2017) by mechanisms likely involving disruption of the blood-brain-barrier (reviewed in (Gao et al. 2021)). Exosomal miR-7-5p from γ -irradiated epithelial

bronchial cells enhanced autophagy in recipient cells through EGFR/Akt/mTOR signaling (Song et al. 2016).

In addition to exosomes, it was found that microparticles derived from irradiated tumor cells control tumor growth through ferroptosis and immune reprogramming by repolarizing pro-tumoral M2 macrophages to anti-tumoral M1 macrophages (Wan et al. 2020). A limited literature exists about other EVs (microvesicles, microplasts, large oncosomes) in the cellular response to radiation, and to our knowledge, their role in the mechanism of bystander/abscopal effects has not been reported. In sum, the various EVs and their content constitute promising biomarkers of radiation exposure and provide new avenues for therapeutic interventions, including the modulation of amount and function of candidate molecules through selective enrichment or inactivation.

C. Secreted molecules

Several studies examined alterations in the nature and level of factors secreted by cultured cells exposed to radiation (Liu et al. 2006; Balduzzi et al. 2010), including proteomic profiling of supernatant from γ -irradiated cells revealing increased levels of certain proteins (*e.g.*, annexin A1, actin (cytoplasmic 1/2)), but decreased levels of others (*e.g.*, annexin A2, cofilin (Desai et al. 2016)). Analyses of the secretome of X-irradiated reconstituted 3-dimensional skin tissue model showed that release of certain proteins (*e.g.*, carboxypeptidase E and ubiquitin carboxyl-terminal hydrolase isozyme L1) is independent of the exposure dose, but activation of others (*e.g.*, the proteasome activator complex subunit 2 protein) is dose-dependent (Zhang et al. 2014). In other studies, peroxidase was increased when a small proportion of low dose X-irradiated cells were co-cultured with a large fraction of bystander cells (Abdelrazzak et al. 2016). Notably, it is the retrospective analysis of cytokines and chemokines in the sera of patients receiving radiotherapy that alerted on the role of inflammatory/immune responses in beneficial abscopal effects (Ohba et al. 1998).

Among the secreted extracellular factors/molecules, cytokines and chemokines orchestrate the interactions among epithelial, mesenchymal and immune cells under physiological and stress conditions. Radiation dose, dose rate, and type, as well as the origin of the irradiated tissue govern the nature and magnitude of cytokines release. Characterization of analytes in the secretome of human cancer cell lines (HT1080, U373MG, HT29, A549 and MCF-7) under normal culture conditions and after acute single bolus or fractionated doses of γ radiation revealed the presence of cytokines/growth factors, namely TNF- α , IL-1 β , PDGF-AA, TGF- β 1, fractalkine, IL-8, VEGF and GCSF. A dose-dependent increase in the levels of most of these molecules was observed, with fold changes being lower after fractionated compared to acute single bolus doses, and A549 showing the largest changes. Functionally, clonogenic survival of bystander A549 cells treated with medium harvested from irradiated cells was decreased (Desai, Kumar et al. 2013). More recently, fractionation of an 8 Gy X ray dose delivered to the thoracic region of mice was shown to induce DNA damage and apoptosis in distant bone marrow cells, which was attributed to an increase in systemic TNF- α and serum amyloid A (Song et al. 2021). In other studies with high LET radiations, secreted IL-33 and prostaglandin-2 (PGE-2) activated the NF-kappa B and MAPK pathways through autocrine and paracrine types of communication (Ivanov et al. 2010), and IL-6 and IL-8 enhanced generation of ROS/RNS in bystander cells (Mariotti et al. 2012).

Melanoma differentiation associated gene-7 (*mda-7*), a member of the expanding interleukin (IL)-10 gene family named *mda-7/IL-24*, exhibits selective killing and radio-sensitization effects in cancer cells and not in normal cells, rendering it unique in its function. MDA-7/IL-24 binds to its receptor dimers (IL-20R1 and IL-20R2)/(IL-22R1 and IL-20R2), which in turn activates JAK/STAT3 downstream signaling to control proliferation and differentiation of target cells (Menezes et al. 2014). Mda-7/IL-24 secreted by irradiated normal cells was cytotoxic to bystander tumor cells with functional IL-20/IL-22 receptors and those overexpressing anti-apoptotic bcl-2/bcl-xL (Su et al. 2005); such results encourage characterizing the role of this protein in abscopal effects.

Factors such as nitric oxide are also initiators/mediators of intercellular signalling. Irradiation stimulates inducible nitric oxide synthases, thereby generating large amounts of $\cdot\text{NO}$, which upon reacting with $\text{O}_2^{\cdot-}$ forms highly reactive peroxynitrite anion capable of attacking a wide range of cellular targets (Azzam, Jay-Gerin, and Pain 2012). Being hydrophobic in nature, $\cdot\text{NO}$ is not dependent on gap junctions for transmission to bystander cells as it can diffuse through the plasma membrane. $\cdot\text{NO}$ release can cause post-translation modifications of target proteins through S-nitrosylation and tyrosine nitration (Yakovlev 2015), events that greatly impact the function of antioxidants such as thioredoxin and peroxiredin (Wu et al. 2010). $\cdot\text{NO}$ mediated bystander signaling in high LET irradiated cell cultures was shown to involve downstream Cox-2 and Nrf-2 pathways (Tomita et al. 2015; Han et al. 2010) with increase in nitrile concentrations in human lung fibroblasts (Yokota et al. 2015). Notably, microbeam irradiation with energetic helium ions showed that inhibition of inducible nitric oxide synthase prevents the increase in DNA damage in bystander cells (Shao, Folkard, and Prise 2008).

The release of damage associated molecular patterns (DAMPs) from dying irradiated cells is a prominent feature of immunogenic cell death (ICD), which was reported to cause in few instances regression/tumor control at distant sites (Golden and Apetoh 2015). In the process, the released DAMPs trigger the engulfment of released tumor antigens by dendritic cells, thereby improving antigen presentation to cytotoxic immune cells whose migration and function is facilitated by secreted cytokines (Golden et al. 2014). Clearly, thorough elucidation of the underlying mechanism may be exploited in controlling the growth of micrometastases and tumors at distant sites (Reynders et al. 2015). Within this framework, HMGB1 released from irradiated breast cancer cells was shown to promote bystander macrophages to secrete $\text{TNF-}\alpha$ through TLR-4 pathway responsible for inhibition of proliferation and migration of bystander cancer cells (Zhu et al. 2021).

< Table 2 >

Relevance of radiation-induced bystander effects

Radiation protection

Residential radon: The manifestation of adverse bystander effects in cell populations exposed to low fluences of high LET radiation such as α particles imply an impact on the assessment of cancer and other health risks (Figure 3). Because health effects at low level radiation exposures are uncertain and require large human cohort size to achieve statistical significance, mechanistic laboratory studies are essential to understanding biological effects and characterizing the extent of health risks. Currently, assessment of lung cancer risk from exposure to environmental radon (*e.g.*, in poorly ventilated homes and workplace) is based on extrapolation of data from studies of uranium mine workers who are typically exposed to inhaled radon at rather higher doses while simultaneously inhaling/ingesting uranium ore dust (Brenner and Sachs 2003, 2002). However, during exposure in residential situations, the majority of cells in the bronchial epithelium are not traversed by an α particle track from radon gas and its decay products. Based on experimental studies, these cells may show deleterious bystander responses. In addition, the progeny of such bystander cells may be prone to increased genomic instability (de Toledo et al. 2017; Hu et al. 2012).

Space exploration: Quantification of health risks from occupational radiation exposures also concerns space exploration. During mission, only parts of the astronaut body are irradiated at any one time (Cucinotta, Nikjoo, and Goodhead 1998), and radiation traversals are separated in both tissue location and time (Held 2009). It has been estimated that during transit beyond low Earth orbit, every cell nucleus within an astronaut's body is traversed, on average, by an energetic proton every few days, by a helium nucleus once every few weeks, and by heavier high-charge particles (HZE) every few months (Blakely 2000; Cucinotta, Nikjoo, and Goodhead 1998) while the rest of the cells would be bystanders. Besides highly localized energy deposition, HZE particles give rise to secondary radiation along the track due to heavy ion fragments and energetic electrons (*i.e.*, δ rays). Whereas the radial spread of dose due to secondary heavy ion fragments extends up to 10-20 μm (Gonon et al. 2013), the range of δ rays can extend up to several cell diameters (Metting et al. 1988; Cucinotta, Nikjoo, and Goodhead 1998). In cell

cultures exposed to doses wherein a small fraction of the cells are targeted with HZE particles, markers of damage were increased in more cells than expected based on the number of cells traversed by HZE particles (Gonon et al. 2013). Using an insert co-culture system that allowed investigating HZE-particle-induced bystander effects in the absence of δ rays and secondary fragmentation products, bystander effects were shown to persist in progeny of bystander cells many generations after co-culture with HZE particle-irradiated cells (Buonanno et al. 2011), and depended on the type of junctional channels that connected the irradiated donor cells with the bystander cells (de Toledo et al. 2017). The propagation of such detrimental effects was not observed in progeny of bystander cells present in cultures where less than 1% of the cells were exposed to microbeam protons, suggesting LET-dependence (Autsavapromporn et al. 2015). The possibility of increased risk of carcinogenesis caused by exposure to space radiation during prolonged space travel has been considered a limiting factor for human exploratory missions (National Research Council 2008). Work in mouse embryo fibroblasts has supported this premise by showing increased frequency of spontaneous neoplastic transformation in progeny of bystander cells from cultures exposed to densely ionizing radiations, including HZE or α particles (Buonanno, de Toledo, and Azzam 2011). Notably, gap junctional communication contributed to mediating the effect. Hence, whether in the case of residential radon or space exploration, studies of bystander effects have the potential to inform the available limited epidemiological surveys.

Normal tissue damage and second cancer formation: The development of genomic instability in progeny of bystander cells (Hu et al. 2012; de Toledo et al. 2017), in particular the work showing that these progeny are at a greater risk of developing spontaneous neoplastic transformation (Buonanno, de Toledo, and Azzam 2011) is a fitting explanation for the emergence of degenerative conditions and second cancers at sites distant from the irradiated primary tumor, in particular in survivors of childhood cancer (Friedman et al. 2010). In this context, there is need for studies of bystander effects in stem cells: when harmed, tissue homeostasis may be perturbed as they differentiate, which would lead to the development of pathologic conditions and impaired response to therapy. Thorough characterization of the intercellular communications and the signaling pathways they affect would enhance understanding the

basis of secondary effects of radiotherapy, and guiding novel interventions that mitigate adverse out-of-field delayed outcomes.

Out-of-field effects and enhancement of radiotherapy benefits

Evidence for the relevance of bystander effects in therapeutic treatments with radionuclides was generated when tumor cells labeled with 5-[¹²⁵I]iodo-2-deoxyuridine (DNA bound I-125 emits electrons with subcellular range) were mixed with non-labeled tumor cells and administered subcutaneously into nude mice. Compared to control, growth of the tumor formed by the cell mixture was significantly slowed (Xue et al. 2002). Similar results were obtained with studies involving γ rays where slow growth of the formed tumor was associated with increased senescence and poor angiogenesis (Desai et al. 2016). With relevance to treatment of cancer disseminated to bone, studies with a mouse model of metastatic breast cancer have shown that the bone seeking α particle-emitting radiopharmaceutical radium-223 dichloride delayed, in a bystander manner, the growth of tumor cells disseminated to the marrow (Leung et al. 2020; Canter et al. 2021). These studies highlight the role of bystander effects in enhancing the effectiveness of targeted radionuclide therapy where uptake of radioactivity is often non-uniform.

In the past two decades there has been a marked interest in harnessing the power of the intercellular communications mediating abscopal responses for beneficial outcomes. Compared to bystander effects that are mostly facilitated by intercellular communications involving cell-to-cell contact and signaling pathways triggered by secreted factors, abscopal effects promoting regression of distant unirradiated tumors are thought to be mostly immune-mediated (Demaria et al. 2004). In the process, the irradiated tumor becomes an *in situ* vaccine whereby DNA fragments released by irradiated cells activate the cGAS/STING [cyclic GMP-AMP synthase (cGAS)/stimulator of Interferon genes (STING)] pathway, which triggers release of a variety of inflammatory cytokines and chemokines that help recruit innate and adaptive immune cells to the tumor (Constanzo et al. 2021). Immature dendritic cells become active and mature, and cytotoxic CD8⁺ T lymphocytes get activated (Golden et al. 2014).

By building on pioneering work showing the power of the host's immune capability in radiocurability (Stone, Peters, and Milas 1979), research on the abscopal effect is translating in promising proof-of-principle clinical trials (Golden et al. 2015). Whether the mechanisms underlying the abscopal effect can be also combined with PD-1/PD-L1 checkpoint inhibitors is attractive to investigate. Likewise, elucidating how cytosolic DNA is transported from irradiated to bystander cells, and whether charged particles are more effective than photons at inducing clinical abscopal effects would be enlightening.

In addition to abscopal effects, immune system activation is assumed to contribute to the benefits of microbeam/spatially fractionated radiotherapy (Kanagavelu et al. 2014). In this modality, cells receiving very large doses are contiguous to cells receiving lower doses. The latter mimic bystander cells and are thought to receive signals from neighboring cells in the high dose region (Desai et al. 2014; Dilmanian et al. 2007; Asur et al. 2012). Remarkably, while the volume of tissue that is irradiated in this modality is reduced, yet tumor regression is observed with reduced normal tissue toxicity and the spared healthy tissue acting as center of regeneration ((Prezado et al. 2018) and reviewed in (Yan et al. 2020)). Several *in vitro* and *in vivo* studies (Suchowerska et al. 2005; Asur et al. 2012; Butterworth et al. 2011) have implicated radiation-induced abscopal effects as potential mediators of biological responses following advanced radiotherapy modalities such as fractionated radiation therapy (GRID) and intensity modulated radiotherapy (IMRT) (Asur et al., 2015; reviewed in Griffin et al. 2020). In sum, while the contribution of out-of-field effects to the balance between tumor eradication and normal tissue complications in the context of radiotherapeutic regimens remains ripe for further investigations (Suit et al. 2007; Newhauser and Durante 2011; Durante, Brenner, and Formenti 2016), examination of the intercellular communication mediated by immune responses promises to result in translational clinical applications.

Conclusions and future directions

Though research in the past three decades has revealed how out-of-field radiation effects contribute to adverse outcomes, more recent work is investigating how the underlying mechanisms can be

harnessed to help achieve beneficial effects. In either case, the investigations have shown that both direct and indirect modes of intercellular communications, with and without involvement of the immune system, are pivotal in mediating these effects. These intercellular communications were found to be exquisitely sensitive to redox modulation (Azzam et al. 2002; Narayanan, Goodwin, and Lehnert 1997), indicating the participation of ROS regulated pathways with significant support from work involving the role of perturbations in oxidative metabolism whether through disruption of mitochondrial function (Zhou et al. 2008) or treatment with antioxidant enzymes (Azzam et al. 2002; Lehnert, Goodwin, and Deshpande 1997). Elucidating the sequence of the early molecular signaling events underlying the propagation of oxidative stress and induction of DNA damage in bystander cells would enhance our understating of the phenomenon, in particular when the irradiated cells are targeted with either high or low LET radiations. Since the landmark report of Nagasawa and Little (Nagasawa and Little 1992), there has been ample evidence indicating that in presence of functional gap junctions between affected cells, the induced oxidative stress is rapidly amplified (Zhao et al. 2014). Furthermore, the progeny of those cells, and those adjacent but not directly damaged, exhibit propagated DNA damage and elevated ROS even though they are many generations removed from the radiation exposure. On the other hand, signaling events propagated from cells exposed to low dose/low fluence radiation were also shown to protect bystander cells, at least transiently, against oxidative damage from normal metabolism or exposure to a challenge dose of radiation (Portess et al. 2007; Rajan and Pandey 2021; Buonanno, De Toledo, et al. 2015). Follow up studies in progeny cells have informed on whether early harmful effects decay and whether early apparent protective effects are followed by an effect on intracellular metabolic redox reactions that contribute to genomic instability. Although abundant evidence described adverse abscopal effects (Mancuso et al. 2008; Mancuso et al. 2011; Chai et al. 2013; Koturbash et al. 2008; Jain et al. 2011), proteomic studies in affected abscopal organs revealed modulation of pathways leading to increased stress, and also increased defense against the induced oxidative stress (Jain et al. 2011). Therefore, the net effect likely involves a role for the tissue microenvironment and immune reprogramming (Ochoa de Olza et al. 2020).

Studies of radiation-induced bystander responses are yet another instance where the radiation sciences have contributed to basic knowledge. They have led to new understanding of the intercellular communications that occur under mild and severe radiation stress, in both normal and cancerous tissues. They have highlighted the critical role that the plasma membrane exercises as initially emphasized by Tikvah Alper (Alper 1977). It is in the plasma membrane where connexin and pannexin channels, cyclooxygenase-2, sphingomyelinase, NAD(P)H oxidase, cytokine and chemokine receptors, and key kinases that regulate gating of communicating channels are localized, with many residing in its cholesterol rich domains. Therefore, studies of lipid mediators and changes in cholesterol levels in the plasma membrane would inform not only on the microenvironment of communicating channels, but also on events associated with inflammatory responses (Laiakis et al. 2014). Together the findings promise to translate in enhancing the efficacy of cancer treatments, whether by radiation or other treatment modalities. Enhancement of intercellular communication by a mild treatment with one modality (*e.g.*, gap junctional communication by mild hyperthermia (Azzam, de Toledo, and Little 2003b), may increase the efficacy of radiotherapy through bystander/cohort effects. Conversely, elucidation of the intercellular communications between normal bystander cells and tumor cells treated with novel techniques such as SBRT (Stereotactic Body Radiation Therapy), SFRT (Spatially Fractionated Radiation Therapy) or hadron therapy (Tubin et al. 2020) may inform on adverse effects that may develop long after the treatment. Knowledge of these communications and how they are regulated will likely refine the delivery of treatments and advise on measures that mitigate harm. The growing interest within the Radiation Science community to elucidate the mechanisms by which FLASH radiotherapy (*i.e.*, irradiation at ultra-high dose rate) achieves tumor control while sparing healthy surrounding organs, may shed light on the relationship between dose-rate and the manifestation of bystander/abscopal effects when compared with outcomes observed following conventional treatments. Long term follow up studies in experimental animals will inform on whether delayed adverse out-of-field emerge following FLASH radiotherapy, which is currently unknown.

To reiterate, rapid advances in the past three decades in detecting and quantifying biological changes strongly show that when specific cells or tissues are traversed by ionizing radiation, a significant proportion of non-irradiated cells and tissues adjacent to the irradiated zone, or distant from it, experience measurable biological changes that are transient, persistent, or whose expression emerge at later time. Important biological changes may also manifest in their progeny. Hence, bystander and abscopal responses, as well as the expression of genomic instability in progeny of surviving bystander or irradiated cells cannot be considered as non-targeted effects. The cells and tissues that were not themselves irradiated are also targets of the radiation exposure and experience biochemical changes that are often similar to the cells traversed by radiation. Therefore, classification of these responses as non-targeted effects may be inappropriate. Here, it is proposed that these effects be classified as out-of-field effects of ionizing radiation.

While further work is needed to elucidate the role of the redox environment and sensitivity of the irradiated tissue in propagation of bystander/abscopal effects, studies of the modulating effect of age and sex of the organism would be enlightening. The use of human organoids will help compare findings obtained in animal models. Together, the results will contribute to generation of biologically based dose response models in which out-of-field effects will help reduce the uncertainty in predicting the risk of adverse health outcomes following low dose/low fluence environmental or occupational radiation exposures (NCRP 2020) and enhance understanding of secondary outcomes of radiotherapy (Friedman et al. 2010).

Acknowledgements

We thank Sonia M. de Toledo for her instrumental participation in our research on the radiation bystander effect. We also thank Roger W. Howell and members of the Division of Radiation Research at Rutgers New Jersey Medical School for the many helpful and instrumental discussions. EA thanks Walter Schimmerling for insightful conversation on classification of bystander effects. This work received

support from the National Institutes of Health (Grants CA92262 and CA049062), the National Aeronautics and Space Administration (grants NNJ06HD91G and NNX15AD62G), the US department of Energy Low Dose Radiation Research program (DE-FG02-02ER63447), the New Jersey Commission on Cancer Research (Grant No. 02-1081-CCR-S2), and the Federal Nuclear Science and Technology program at Canadian Nuclear Laboratories (project FST-51320.50.19.06).

While best effort was made to include all the relevant references, the authors express regret if a significant reference had not been included in this review.

Disclosure of interest

The authors report no conflict of interest

Accepted Manuscript

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Figure legends

Figure 1. Schematic illustrating the propagation of radiation-induced bystander effects *in vitro*.

Cells traversed by ionizing radiation propagate molecular signals to non-irradiated cells in the population i) directly via gap junctional channels connecting contiguous bystander/irradiated cells, or tunneling nanotubes connecting cells situated up to 300 μm apart, and/or ii) indirectly via released factors, including soluble molecules and vesicles (contiguous, in vicinity, and distant bystander cells). The propagated signals may induce transient, persistent or delayed effects in the bystander cells. Biological changes may also occur in progeny of both bystander and surviving irradiated cells. The image elements are not to scale with each other. Images were modified using Servier Medical Art by Servier which is licensed under a Creative Commons Attribution 3.0 Unported License <https://smart.servier.com/>.

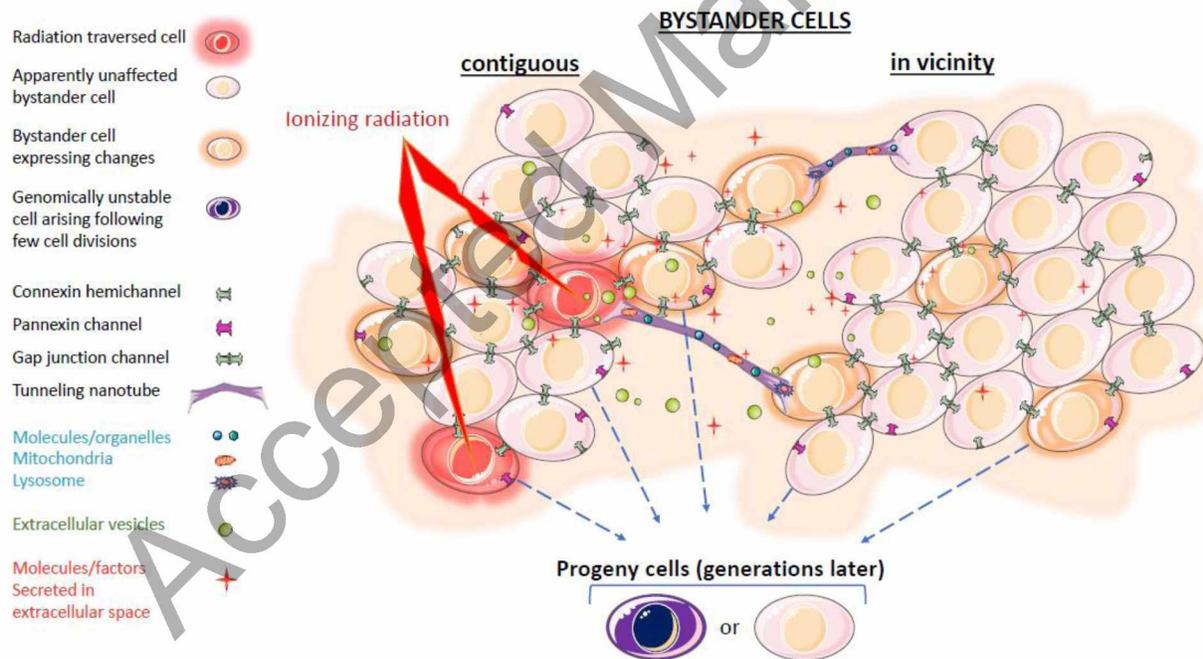
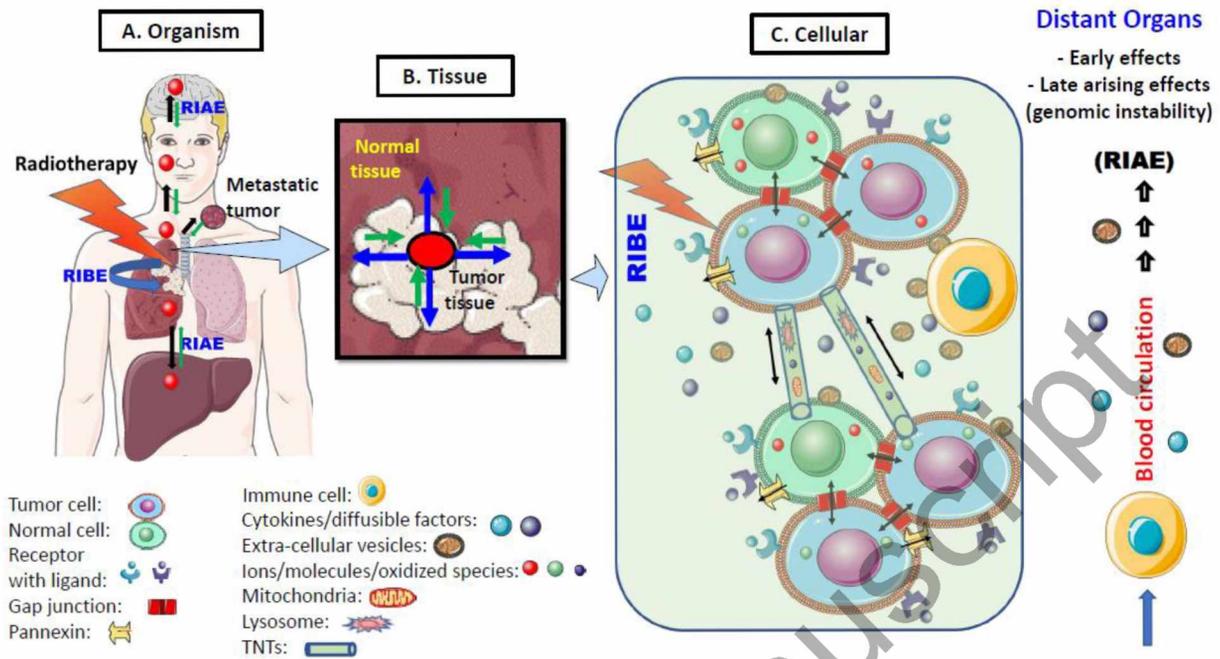


Figure 2. Schematic illustration of radiation-induced bystander effect (RIBE) and radiation-induced abscopal effect (RIAE) at the cellular, tissue and organism levels in the context of cancer radiotherapy. At the organism level (A), when a tumor (for example lung tumor here) is irradiated, two scenarios may arise. In the first, an effect may be propagated from the irradiated to unirradiated tumor or normal regions (RIBE). In the second situation, irradiation of the tumor may induce systemic changes affecting distant organs (*e.g.*, brain and liver as described here) or metastatic tumor sites. The effect would not be only unidirectional (*i.e.*, from irradiated to unirradiated organs/tissues), but also bidirectional (as illustrated by the arrows), and the magnitude of the effects may vary depending on several factors (magnitude of radiation damage, redox environment, type of tumor/normal tissue, *etc.*). At the tissue level (B), induced effects may be transmitted from irradiated tumor tissue to unirradiated tumor or normal tissue regions. At the cellular level (C), irradiated cells transmit the bystander signals to contiguous cells directly through gap junctions, or to distantly located cells through tunneling nanotubes (TNTs), which may involve transfer of cell organelles (like mitochondria, lysosomes, ions/molecules) to bystander cells. Irradiated cells may also secrete soluble factors (*e.g.*, cytokines/chemokines, ions, ATP), and extracellular vesicles among others, which are transferred to distant locations through circulating blood. Secretion of soluble factors affecting close or distant cells may involve pannexin channels. The RIAE at distant organs or metastatic sites may occur with or without involvement of the immune system. These effects may manifest early or may be expressed in the progeny of bystander or irradiated cells through mechanisms of genomic instability. The image elements are not to scale with each other. Images were modified using Servier Medical Art by Servier which is licensed under a Creative Commons Attribution 3.0 Unported License <https://smart.servier.com/>.



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Figure 3: Relevance of radiation-induced bystander effects (RIBE). RIBE are relevant for radiation protection and therapeutic applications. The manifestation of RIBE during environmental exposures such as residential radon or occupational exposures (*e.g.*, mining, energy generation, clinical work, space exploration) may contribute to biologically based dose response models that seek to reduce the uncertainty in estimating health risks associated with low doses/low fluence exposures. With regard to radiotherapy, out-of-field/abscopal effects may contribute to improve outcomes, but may also contribute to emergence of degenerative conditions and second cancers.

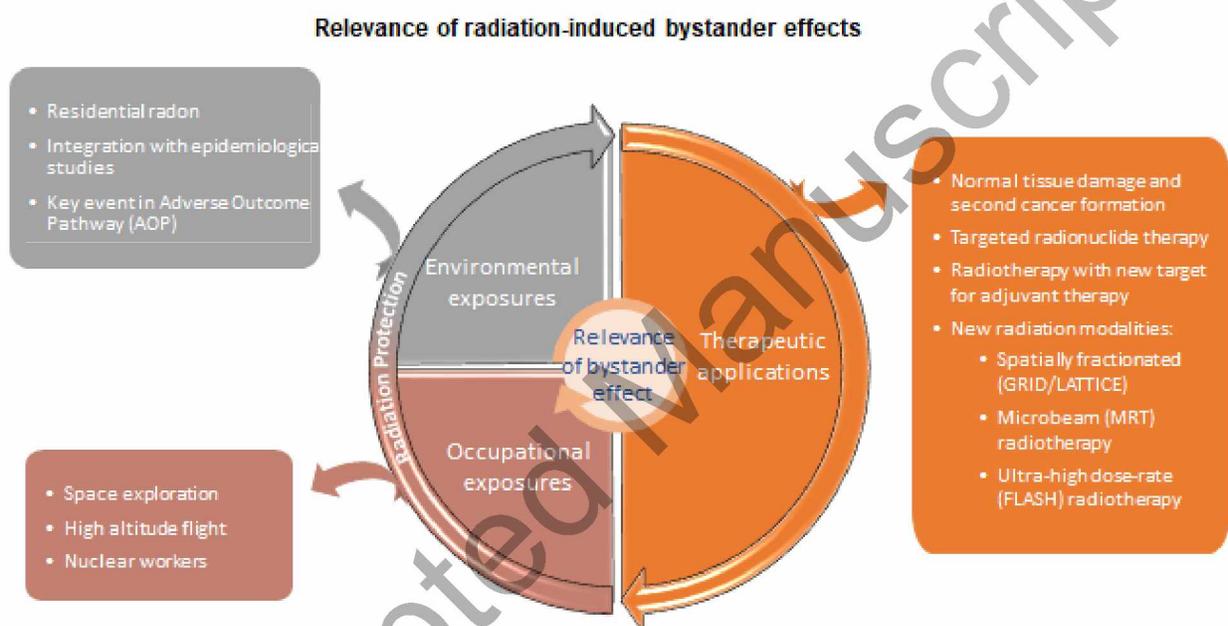


Table 1: Key findings describing the bystander effect phenomenon *in vitro*

Radiation type		Irradiation method	Main finding	References
<i>High LET</i>	┐ particles	Broadbeam	Sister chromatid exchanges are induced in a greater fraction of cells than those whose nuclei are traversed by ┐ particles.	(Nagasawa and Little 1992, Deshpande, Goodwin et al. 1996)
		Broadbeam	Evidence that gap-junction intercellular communication is involved in transmission of detrimental signals to non-irradiated bystander cells.	(Azzam, de Toledo et al. 1998, Azzam, de Toledo et al. 2001, Zhou, Suzuki et al. 2001)
		Broadbeam	Bystander effects are induced by factors secreted by irradiated cells.	(Lehnert, Goodwin et al. 1997)
		Microbeam	Nuclear irradiation induces bystander effects in non-hit cells.	(Prise, Belyakov et al. 1998, Zhou, Randers-Pehrson et al. 2000)
		Microbeam	Bystander effects were elicited by cytoplasmic irradiation.	(Shao, Folkard et al. 2004)
		Microbeam	Cytoplasmic irradiation induces nuclear mutations.	(Zhou, Suzuki et al. 2001)
<i>High LET other than ┐ particles</i>	Heavy ions	Microbeam	Micronuclei are induced in bystander cells after irradiation of a single cell within a cell monolayer with a single energetic ⁴⁰ Ar or ²⁰ Ne ion.	(Shao, Furusawa et al. 2003)
		Microbeam & Broadbeam	Heavy ions reduce the clonogenic potential of bystander cells; the timescale of the response to heavy ions differed between irradiated and bystander cells.	(Hamada, Ni et al. 2008)
<i>Moderate LET</i>	Soft X rays	Microprobe	Bystander responses are detected in hamster fibroblasts when only a single cell is irradiated.	(Folkard, Schettino et al. 2001, Schettino, Folkard et al. 2002)
<i>Low LET</i>	┐ rays	Broadbeam	Medium from ┐ irradiated epithelial cells reduces the clonogenic survival of recipient unirradiated cells.	(Mothersill and Seymour 1997)
	X rays	Microbeam	DNA foci are induced by a synchrotron X ray microbeam.	(Usami, Maeda et al. 2006)
	High energy electrons	Broadbeam	Medium from cells exposed to high-energy electrons reduces the clonogenic survival of recipient unirradiated cells.	(Gow, Seymour et al. 2010)
	Protons	Microbeam	Bystander effects are induced by proton microbeam.	(Frankenberg, Greif et al. 2006, Desai,

				Kobayashi et al. 2014)
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Table 2: Major intercellular routes and signaling events in the mechanisms of radiation-induced bystander effects

		Main references
Routes and Pathways	GJIC mediated by Connexin 43	(Azzam, de Toledo et al. 1998, Azzam, de Toledo et al. 2001, Zhou, Suzuki et al. 2001)
	Exosomes	(Al-Mayah, Irons et al. 2012, Jella, Rani et al. 2014)
	TNTs	(Matejka and Reindl 2020)
	DNA damage and repair	(Little, Nagasawa et al. 2003, Nagasawa, Huo et al. 2003)
	Oxidative metabolism	(Narayanan, Goodwin et al. 1997, Azzam, De Toledo et al. 2002)
	Double stranded DNA (cGAS/Sting pathway)	(Jesenko, Bosnjak et al. 2020)
Signals/Molecules Transmitted to Bystander Cells	Soluble factors cocktail secreted in medium	(Mothersill and Seymour 1997, Iyer, Lehnert et al. 2000, Desai, Kumar et al. 2013)
	Cox 2	(Zhou, Ivanov et al. 2005)
	Rad 9	(Zhu, Zhou et al. 2005)
	TGF- β	(Hu, Xu et al. 2014)
	TNF- α and serum amyloid albumin	(Song, Hu et al. 2021)
	IL-6 and IL-8	(Mariotti, Bertolotti et al. 2012)
	Nitric oxide and superoxide	(Shao, Stewart et al. 2003)
	Prostaglandin-2 (PGE-2)	(Ivanov, Zhou et al. 2010)
	HMGB1	(Zhu, Hu et al. 2021)
	Ions/small molecule transmission by GJIC	(Meşe, Richard et al. 2007)
	Circulating miRNAs	(Xu, Wang et al. 2015)
	ATP	(Burdak-Rothkamm, Rothkamm et al. 2008, Mancuso, Pasquali et al. 2011, Ohshima, Tsukimoto et al. 2012)