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<th>Radiation Induced Bystander Effect in vivo</th>
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<td>Chai, Yunfei; Hei K. Tom</td>
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Radiation Induced Bystander Effect \textit{in vivo}

Yunfei HAI,\textsuperscript{1} Tom K. HEI\textsuperscript{1,2}

\textsuperscript{1}Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, USA
\textsuperscript{2}Center for Radiological Research, Department of Radiation Oncology, College of Physicians and Surgeons, Columbia University, New York, USA

Radiation-induced bystander effect is defined as the induction of biological effects in cells that are not directly traversed by radiation, but merely in the presence of cells that are. Although radiation induced bystander effects have been well defined in a variety of \textit{in vitro} models using a range of endpoints including clonogenic survival, mutations, neoplastic transformation, apoptosis, micronucleus, chromosomal aberrations and DNA double strand breaks, the mechanism(s) as well as the presence of such an effect \textit{in vivo} are not well described. In this review, we summarize the evidence of radiation induced bystander effect in various \textit{in vivo} systems including rodents, fish and plants. Many biological endpoints such as epigenetic changes, DNA damage, miRNA, apoptosis, cell proliferation, gene expression and tumorgenesis have been demonstrated in the non-targeted regions \textit{in vivo}. Although the bystander effect is evolutionarily conserved in rodent systems, the bystander response depends on gender, tissue and strain. However, the studies about mechanism of radiation induced bystander effect \textit{in vivo} are still limited.

\textbf{Keywords:} Radiation; Bystander effect; \textit{In vivo}; Cox-2

\section*{Introduction}

Ionizing radiation is a well-established human carcinogen and induces cancer in a stochastic fashion. The risk of cancer after high and moderate doses of radiation is relatively well understood from epidemiological studies of the Japanese atomic bomb survivors.\textsuperscript{1} Studies concerning life span of Hiroshima and Nagasaki survivors show a linear relationship between cancer mortality and high doses of radiation.\textsuperscript{2} The United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) proposed the linear non-threshold (LNT) theory in 1958 (UNSCEAR 1958). Cancer risk is also assumed to be proportional to the dose of radiation even at low doses and without a threshold. Comparing with the majority of laboratory studies on high dose radiation and clinical exposure situations, where irradiation is usually acute, high dose, high-dose-rate exposure, evaluating risk in low dose radiation is further complicated because environmental exposures are predominantly protracted low-dose, low-dose-rate exposures or high-dose-rate exposures to smaller fractions. At low doses, the deleterious effects of radiation are expected to decline because fewer cells are likely to be directly damaged according to currently well-accepted dogma. Unlike the inference in many radiation models in which only the cells or tissues actually being irradiated are burdened by the legacy of radiation, the biological effects of low-dose radiation are considerably more complex than predicted by the linear non-threshold model.\textsuperscript{2} Evidence accumulated over the past decade has indicated that both extranuclear targets and extracellular events may play an important role in determining the biological responses to low dose ionizing radiation.\textsuperscript{3-4}

In cultured cells, Nagasawa \textit{et al} observed sister chromatid exchange in >30% of the total cells, whereas only 1% of the cells' nuclei are hit by particles.\textsuperscript{5} This phenomenon was named as "bystander effect" to describe the ability of affected cells to transfer damages to other cells not directly being targeted.\textsuperscript{6} There are many detectable biological endpoints of the radiation-induced bystander effects, including genetic instability, signal transduction, altered gene expression, radioadaptive response, apoptosis, production of ROS, and neoplastic transformation.\textsuperscript{7-9} These effects can be induced by different types of irradiation, including $\alpha$-particle, X-ray, $\gamma$-ray etc.\textsuperscript{5,10,11} Meanwhile, the carcinogenic risk increased as a result of chromosomal imbalance and loss of heterozygosity, which is critical for silencing of tumor suppressor gene and result in genomic instability.\textsuperscript{12} Until recently, most of the current knowledge on radiation-induced bystander effects has been derived from \textit{in vitro} studies. Although \textit{in vitro} assays have a long history of providing quantitative and mechanistic data, they have many limitations. Conventional cell cul-
Bystander studies in rodent models

The bystander effects were found in the unirradiated region of the same organ after localized exposure to irradiation. Khan et al. showed that irradiation of partial lung can induce bystander responses in the unirradiated part of the lung.22 With irradiation of the lower region of the lung, either 30% or 70% of whole lung, the frequency of micronuclei increased in the out-of-field upper lung relative to the sham group. The induction of DNA damage in the non-targeted lung tissues were inhibited by superoxide dismutase (SOD) and L-N\(^-\)Nitroarginine methyl ester (L-NAME), which suggested that production of reactive oxygen species and nitric oxide resulted in indirect DNA damage and induce bystander effect in the neighboring tissue. The protection of DNA damage by Eukarion-189, a SOD mimetic protected DNA damages in the in and out-of-field lungs also supports the point that DNA damage in the non-targeted lung may be caused by chronically produced ROS by a radiation induced inflammatory response.24 Calvey et al. showed that DNA damage, the activation of macrophages and the expression of inflammatory cytokines all fluctuated in a cyclic pattern in the directly irradiated and bystander regions of the same lung tissues.25 RNA levels of cytokines including IL-1α, IL-1 IL-6, TNF-α and TGF-β and activated macrophages were elevated to a similar degree both in targeted and non-targeted lung tissues whereas there were more micronuclei in the directly irradiated tissues. In the skin model, Koturbash et al. showed that partial body irradiation exposure to X-ray lead to the induction of DNA damage in distant (>0.7 cm from irradiated tissues), lead-shielded, bystander skin tissue of mice in vivo as early as 6 hours after irradiation.26 Rad51, a gene involved in the DNA damage repair, was upregulated in both irradiated and bystander tissues even 4 days after irradiation. DNA methylation plays an important role in safeguarding genome stability, regulating gene expression and chromatin structure. Along with the increased levels of DNA (cytosine-5-)methyltransferase 1 (DNMT1) expression in bystander tissue, the levels of methyl Cpg binding protein 2 (MeCP2) and methyl-CpG binding domain protein 2 (MBD2), proteins involved in transcriptional silencing, increased in bystander but not irradiated skin. Interestingly, the expression changes in bystander tissue were not symmetric as the response was more pronounced when the left side of the body was irradiated. The bystander effect in this model may be, in part, linked to internal organ exposure especially heart and liver.

The bystander effects can be induced in an unirradiated organ distant from irradiated organs in an animal. For the first, Mancuso et al. reported cancer induction in the unirradiated tissues after radiation.27 In radiosensitive Patched-1\(^{+/–}\) (Pch\(^{1+/-}\)) mouse model, genetic damage in non-targeted brain caused by distant irradiation contributed to cancer risk in mouse central neuron system, with drastic acceleration of medulloblastoma in the mice irradiated with skull shielded. Neonatal mice were partially irradiated with 3 Gy dose of X rays in the lower half of the body while upper half including the head was protected by individual cylindrical lead shields. A significant increase in medulloblastoma rate (39%) occurred in the partial body irradiated heterozygous mice comparing to sham treated group. The study also showed the induction of gH2AX, a marker of DSBs and apoptosis in bystander cerebellum. The reduction of the gH2AX formation and apoptosis in bystander cerebellum by TPA, a gap junction inhibitor suggest that gap junctions involve in bystander signal transmission. Although these short-term bystander responses could be detected in different strains after similar treatment, the carcinogenesis in cerebellum was specific for the heterozygous animals and suggested that the endpoints are dependent on the genotype of animals. This can be an explanation why the changes of various short term endpoints can be found in the unirradiated tissues of many patients but the incidence of secondary tumors after radiotherapy is very low.

Localized cranial irradiation can induce epigenetic changes and regulate the related gene expression in distant organs such as spleen, sperm, testes and skin. Long-term bystander effects are demonstrated in radiosensitive hematopoietic organs such as spleen, distant from radiation exposure region.28 Localized cranial exposure to 20 Gy of X-rays leads to a profound epigenetic dysregulation in the bystander spleen tissue that manifested as a significant loss of global DNA methylation, alterations in methylation of long interspersed nucleotide element-1 (LINE-1) retrotransposable elements and down-regulation of DNA methyltransferases and MeCP2 24 h after radiation and sustained for at least 7 months. Similar to high dose exposure, cranial exposure to a 1Gy dose of X-ray also resulted in persistently altered levels of cellular proliferation, apoptosis, and expression of p53 protein in the bystander spleen tissue in two different strains of mice; C57BL/6 and BALB/c. Tamminga et al. showed that cranial X-ray irradiation also induced bystander effect in the reproduction organs of rats.28 DNA damage and g-H2AX foci were accumulated in bystander testes while g-H2AX foci were not detected in spermatozoa.
The bystander-induced DNA damage was not formed but not repaired in the mature sperm cells because of the low expression of ATM, ATR and DNA-PK in the bystander mature sperms. The bystander effect can be inherited from partial body irradiated parents to unirradiated offspring, which is similar as transgenerational epigenetic changes after whole body irradiation of animals. The loss of DNA methylation in bystander testes and mature sperms may regulate the epigenetic reprogramming after fertilization, and lead to the altered DNA methylation in various organs of the offspring including bone marrow, thymus, spleen and liver. DNA methylation, cell proliferation and apoptosis appeared to be sex-specific patterns in the exposed and bystander spleen tissues of male and female mice. Compared with female mice, cranial irradiation induced more profound global genome loss of DNA methylation in the exposed and bystander spleen of male mice after only skull exposure to irradiation.\(^\text{11}\) Gonadectomy of animals leads to significantly diminished sex differences in bystander spleen tissue.

Another bystander endpoint concerning gene expression regulation is the maturation of miRNAs. miRNAs are single-stranded RNA molecules approximately 21–23 nucleotides in length. miRNAs are evolutionally conserved from plants to animals, including the plant Arabidopsis thaliana, C. elegans, mouse and human and are recognized to regulate gene expression. Pri-miRNA, a primary transcript of miRNAs is processed into a short stem-loop structure. Dicer, a member of the RNAse III superfamily, cleaves the pri-miRNA in the cytoplasm to form a mature miRNA, which associate with the RNA induced silencing complex (RISC) protein\(^\text{27}\) to regulate target miRNAs expression. Cranial exposure also influences miRNAs in shielded bystander spleen of the male and female mice.\(^\text{12}\) These sex associated differences were probably due to different time course of Dicer increase in male and female mice. Whole body and cranial radiation exposure led to a significant (p < 0.05) upregulation of Dicer expression in the spleen of male and female mice 6 and 96 hours after exposure. Dicer expression patterns in gonadotomy-castrated (CAST) or ovariectomized (OVX) animals were different from that of intact mice. A small but significant Dicer induction was seen only 96 hours after irradiation. Similarly, a small but statistically significant change is also noted in the OVX spleen. Besides the sex-specific pattern, the regulation of miRNAs also showed tissue-dependence. Cranial irradiation led to very different patterns of miRNA expression in skin and spleen. Acute and fractional cranial exposures induced distinct epigenetic bystander effects in the lead-shielded skin and spleen which were same distance from the irradiated targets.\(^\text{13}\) Fractionated radiation exposure also induced pronounced and persistent epigenetic bystander effects in spleen but not in skin. Fractionated irradiation led to hypomethylation in the bystander spleen 6 hr, 96 hr, and 14 days after cranial only exposure whereas similar changes in skin of the same animals were seen only in head exposure. MeCP2 was significantly reduced in mouse spleen 6 hr, 96 hr, and 14 days after acute and fractionated head irradiation but MeCP2 changes were only noted in skin 6 hrs after the acute head exposure.

The bone marrow transplantation system has been used to study the impacts of ionizing radiation on bone marrow stem cells. Using the system, Watson et al. have demonstrated that the descendants of irradiated stem cells, but not irradiated recipient stroma, are able to induce genetic instability in the descendants of unirradiated stem cells.\(^\text{34}\) A cytogenetic marker was used to distinguish irradiated and unirradiated donor bone marrow cells for transplantation. After transplantation with neutron-irradiated or g-irradiated bone marrow cells into nude mice, chromosomal instability especially translocations and deletions in the progeny of unirradiated hemopoietic stem cells were observed; however, in addition, there was a significantly greater frequency of unstable aberrations in the recipient bone marrow.\(^\text{35,36}\) The findings support a bystander mechanism in which the descendants of irradiated stem cells are able to induce genetic instability in the descendants of unirradiated stem cells. Further studies revealed that the ongoing production of inflammatory-type damaging signals play a role in maintaining the long-term consequence of the initial radiation exposure. The macrophages may be an important intermediate in this process. CBA/Ca mice are more susceptible to radiation induced AML and delayed radiation-induced instability than C57BL/6 mice. Hemopoietic tissues of irradiated CBA/Ca mice exhibited increased levels of p53, p21, and apoptosis which were directly correlated with increased 3-nitrotyrosine, a marker of damaging nitrogen/oxygen species in macrophages.\(^\text{37}\) Macrophages derived from CBA/Ca mice characterized as M1-like (pro-inflammatory) with persistent productions of ongoing damaging signals such as cytokines TNF-a which would ultimately cause ROS stress in the non-targeted tissue. As a contrast, macrophages from C57BL/6 mice characterized as M2-like (anti-inflammatory). After irradiation in vivo, but not in vitro, C57BL/6 macrophages showed a reduction in NOS2 and an increase in arginase activities, indicating a further M2 response, whereas CBA/Ca macrophages retained an M1 phenotype. The data indicate that macrophage activation is not a direct effect of radiation but a tissue response, secondary to the initial radiation exposure.\(^\text{38}\)

Normal hemopoietic clonogenic stem cells exhibited chromosomal instability unlike the descendants of directly g-irradiated cells after being exposed to g-irradiated bone marrow or bone marrow derived macrophage conditioned medium.\(^\text{39}\) Crossgenetic experiments showed that the induction of the instability phenotype requires both the producer and responder cells to be of the susceptible CBA/Ca genotype. These results reflect that the risk of macrophage mediated bystander effects are also dependent on genome background. It is interesting to note that in the in vitro system the frequency of cells expressing chromosomal instability is greater than that found in vivo. These differences could be explained by the more effective recognition and removal of abnormal cells in vivo than in cell culture. Such differences highlight the importance of in vivo studies about radiation induced bystander effect especially when considering the potential health effects of genomic instability.

### Bystander studies in fish model

The radiation induced bystander response is demonstrated in not only rodent systems but also in fish systems, suggesting that it may
be evolutionally conserved in animals. Mothersill et al. showed that radiation induced bystander effects could transmit from an irradiated fish to a unirradiated one.\textsuperscript{41} 2 days after combining them in the same container. The harvested culture medium from most of the examined organs, such as skin, fin, kidney and spleen of unirradiated fish, which partnered with irradiated fish, resulted in reduction of clonogenic survival of HPV-G reporter cells. The effect of growth medium from zebrafish gill and skin explants on HPV-G survival was broadly similar to that seen in rainbow trout.\textsuperscript{41} The ratio of bcr-2/e-myc, previously shown to correlate with radiation sensitivity, was similar in X-ray irradiated and unirradiated groups.\textsuperscript{41} These results suggest that similar to those found in mice, genetic differences determine the outcome at the cellular level, following radiation exposure. The balance between bcr-2 and e-myc appears to be a major regulating mechanism.

Bystander studies in plant model

\textit{Arabidopsis thaliana} is a widely used research model with several advantages such as short generation period (about 5-6 weeks), small size and a sequenced small genome. The embryo of \textit{Arabidopsis} represents simple cellular patterns comprised of few basic tissues especially shoot apical meristem (SAM), tightly controlling the orientation and frequency of cell division as well as cell morphology and differentiation in development. The size of the \textit{Arabidopsis} embryo, in the range of tens to hundreds of micrometers, allows accurate microbeam targeting at designated locations. Qin et al. found high LET radiation induced DNA damage in \textit{Arabidopsis} seeds.\textsuperscript{42} Different fluence-response curves have shown that water-imbedded seeds were more sensitive to proton irradiation than dry seeds. According to the TRIM calculations and seed structure, the 6.5 MeV protons can pass through the entire seed and the 2.6 MeV protons can penetrate only half of the seed. However, 1.1 MeV protons and 30 keV N\textsuperscript{+} stop in the shallow region of the seed and cannot reach the SAM. Multi-SAM malformations were observed after irradiation of 1.1 MeV protons or 30 keV N\textsuperscript{+} which ion range in a seed is too short to reach the SAM. It implied the possibility of transmission of damage effects by signal transduction from the secondary target to the primary target. Yang et al. reported that the low-energy heavy ions irradiation also induced bystander effect in \textit{Arabidopsis} seeds by.\textsuperscript{43} Since 30 keV Ar\textsuperscript{+} ions generally stop in the proximal surface of the seeds, shoot apical meristem and root apical meristem cells were not damaged directly by radiation. Long distance bystander response exist in the intact seed by which damage signals were transferred from the irradiated cells to the unirradiated shoot apical meristem and root apical meristem cells leading to long term developmental alteration. Short-term and long-term postembryonic development was significantly inhibited including germination, root hair differentiation, primary root elongation and lateral root initiation. Similarly as by animal models, ROS played essential roles in the low-energy heavy-ion radiation induced long-distance bystander effects in the intact organism.

Summary

Radiation induced bystander effect can be found in different tissues and organs of plants and animals after either short term or long term post irradiation. Various biological endpoints in non-targeted region \textit{in vivo} can be affected by different types of irradiation; acute or fractionated, low dose or high dose, low LET or high LET. The signals can transferred from irradiated region to unirradiated region of same organ, from irradiated organ to another unirradiated organ in same animal, from one generation to another generation of same species, even from one irradiated individual animal to another unirradiated animal. Although radiation induced bystander effect indicated by different biological endpoints are confirmed in many \textit{in vivo} systems, few studies illustrated the signal pathway(s) involved in the bystander effect. A multiple signal cascade model based on the \textit{in vitro} data is proposed to demonstrate an initiating event and downstream signaling steps, necessary to mediate the bystander process.\textsuperscript{44} Radiation induced cytokines including transforming growth factor beta (TGF-b), tumor necrosis factor alpha (TNF-a), interleukin-1 beta (IL-1b) and different stress factors bind to their respective receptors on the bystander cells and potentially initiate the bystander effect in the non-targeted cells.\textsuperscript{44} The activation of Ras-Raf-MEK-ERK-AP-1 and IKK-NF-kB pathways elevated cyclooxygenase 2 (COX-2) gene transcription. COX-2 is a key enzyme catalyzing the metabolism of arachidonic acid into prostaglandins and finally involved in cellular inflammation, carcinogenesis and genomic instability.\textsuperscript{44} However, it is still not clear if the proposed \textit{in vitro} model is applicable \textit{in vivo}. Many important questions remain such as: What is the initiating event of the bystander effect \textit{in vivo}? Is the bystander effect \textit{in vivo} induced by cytokines released from targeted tissue into the blood or by irradiated cells circulating to non-targeted tissues? Do circulating irradiated cells release the cytokines to affected regions or contact the cells in the non-targeted region via gap junction? Are the potential cytokines similar \textit{in vivo} and \textit{in vitro}? Is MAPK or NF-kB path way involved \textit{in vivo}? Does COX-2 serve as a role for mutagenesis and genomic instability? More \textit{in vivo} studies in depth are needed to make a clear understanding about the mechanism of radiation induced bystander effect, which will contribute to the application of this theory, especially on the induction of secondary cancers after radiotherapy.

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69