Journal of Radiation Research, Vol. 59, No. 6, 2018, pp. 754–759
doi: 10.1093/jrr/rry065
Advance Access Publication: 14 August 2018

Radiation quality effects alteration in COX-2 pathway to trigger radiation-induced bystander response in A549 lung carcinoma cells

Alisa Kobayashi[1,2,3] and Teruaki Konishi[1,4,*]

[1]SPICE-BIO research core, International Open Laboratory, National Institute of Radiological Sciences (NIRS), National Institutes for Quantum and Radiological Science and Technology (QST), 4-9-1 Anagawa, Inageku, Chiba, 263-8555, Japan
[2]Department of Accelerator and Medical Physics, National Institute of Radiological Sciences (NIRS), National Institutes for Quantum and Radiological Science and Technology (QST), 4-9-1 Anagawa, Inageku, Chiba, 263-8555, Japan
[3]Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1, Tennodai, Tsukuba, Ibaraki 305-8575, Japan
[4]Department of Basic Medical Sciences for Radiation Damages, National Institute of Radiological Sciences (NIRS), National Institutes for Quantum and Radiological Science and Technology (QST), 4-9-1 Anagawa, Inageku, Chiba, 263-8555, Japan
*Corresponding Author. Department of Basic Medical Sciences for Radiation Damages, National Institute of Radiological Sciences (NIRS), National Institutes for Quantum and Radiological Science and Technology (QST), 4-9-1 Anagawa, Inageku, Chiba, 263-8555, Japan. Tel: +81-43-206-4695; Fax: +81-43-206-3514; Email: konishi.teruaki@qst.go.jp
(Received 22 April 2018; revised 12 June 2018; editorial decision 6 July 2018)

ABSTRACT

This study aimed to determine whether the radiation-induced bystander effect (RIBE) is affected by radiation quality. To mimic the different radiation qualities of the direct action (*D*)/indirect action (*ID*) ratio, A549 cells were exposed to X-rays, with either 100 mM of the radical scavenger, thio-urea (TU^+), or null (TU^-). Biological responses in irradiated and bystander cells were compared at equal lethal effects of a 6% survival dose, which was estimated from the survival curves to be 8 Gy and 5 Gy for TU^+ and TU^-, respectively. Cyclooxygenase-2 (COX-2) expression in TU^- irradiated cells increased up to 8 h post-irradiation, before decreasing towards 24 h. The concentration of prostaglandin E2 (PGE_2), a primary product of COX-2 and known as a secreted inducible factor in RIBE, increased over 3-fold compared with that in the control at 8 h post-irradiation. Conversely, COX-2 expression and PGE_2 production of TU^+ irradiated cells were drastically suppressed. These results show that the larger *D/ID* suppressed COX-2 expression and PGE_2 production in irradiated cells. However, in contrast to the case in the irradiated cells, COX-2 expression was equally observed in the TU^- and TU^+ co-cultured bystander cells, which showed the highest expression levels at 24 h post-irradiation. Taken together, these findings demonstrate that radiation quality, such as the *D/ID* ratio, may be an important factor in the alteration of signalling pathways involved in RIBE.

Keywords: radiation-induced bystander effect; COX-2; DNA damage complexity; indirect action; thio-urea; radiation quality, radical scavenger

INTRODUCTION

In classical radiation biology, DNA damage induced by the direct energy deposition of radiation is the main cause for the variety of radiobiological consequences. Hence, DNA is considered to be the primary target of radiation [1, 2]. Radiation-induced bystander effect (RIBE) [3] is a phenomenon observed in cells that have not been exposed to radiation, but have been affected through bidirectional intercellular signalling from irradiated cells [4, 5]. In the past several decades, multiple inter- and intracellular signalling pathways have been reported to be involved in RIBE, and the propagation of these signals involves gap junctional intercellular communication (GJIC) [6, 7] and media-mediated intercellular communication (MMIC) [8–12]. Several studies have clarified the major players in bystander signals, such as nitric oxides [9, 12, 13], cytokines [11, 14], exosomes [15], and inflammatory products, such as prostaglandin E2 (PGE_2) [16, 17]. In addition, evidence has accumulated showing that

RIBE exists within different types of radiation, such as photons of low linear energy transfer (LET) [6, 14, 18], neutrons [19], protons [5], α particles [3, 20], and high-LET heavy ions [6, 13, 14, 21]. However, it is still unclear whether RIBE is affected by radiation quality [6, 13, 14], or is only affected by the accumulated dose [21]. The biological effects of radiation are principally derived from DNA damage caused by both direct action (*D*) (in which atoms of the DNA are ionized or excited directly by the radiation) and indirect action (*ID*) (interaction between the radiation and atoms of other molecules to produce free radicals that are able to diffuse far enough to reach and damage the DNA) [22, 23]. The radiation qualities with larger *D/ID* ratios are known to induce a larger degree of complex and irreparable DNA damage [24]. These observations, whether in irradiated cells or RIBE cells, show that DNA damage can occur in various ways.

In this study, we mimicked differences in radiation quality in terms of the difference in *D/ID* ratio by suppressing the *ID*. This was achieved by using the hydroxyl (OH) radical scavenger, thio-urea (TU) [25] in the cell culture. We investigated the cyclooxygenase-2 (COX-2)/prostaglandin E_2 (PGE_2) pathway [17, 26–28] as an indicator for cellular responses in irradiated and bystander cells to clarify whether the RIBE and its signalling pathways would be affected by radiation quality.

MATERIALS AND METHODS

Cell cultures and treatments

Human lung adenocarcinoma cells, A549 (RCB0098), were obtained from the RIKEN Bioresource Center. The cells were maintained in Dulbecco's Modified Eagle's Medium (D-MEM; 041–30081, Wako Pure Chemical Industries, Ltd, Osaka, Japan) supplemented with 10% foetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin) at 37°C in a 5% CO_2 and humidified atmosphere.

X-ray irradiation

For irradiation, an X-ray generator (TITAN, Shimazu Co., Kyoto, Japan) set at 200 kVp and 20 mA was employed, and irradiation was made through a copper and aluminium filter with a thickness of 0.5 mm, producing an effective energy of ~83 keV. The samples received X-ray doses at a dose rate of ~1 Gy/min.

Measurement of OH radical production by X-ray irradiation

The production of OH radicals and other reactive oxygen species (ROS) by X-ray irradiation was measured by the oxidant-sensing fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) [27]. DCFH-DA solutions (100 µM DCFH-DA in PBS, 044–28241, WAKO, Osaka Japan) were prepared under two sets of conditions: null (TU^-), and with an OH radical scavenger, 100 mM thio-urea (TU^+) (33812–92, Nacalai Tesque Inc., Kyoto, Japan). A total of 1 ml of DCFH-DA solutions were irradiated with various doses (Fig. 1). After irradiation, 200 µl of DCFH-DA solutions were transferred per into each well of 96-well plate (164588, Thermo Fisher Scientific, MA), and the DCF fluorescence was quantified with a fluorescent image scanner (FLA-5100, FUJIFILM, Tokyo).

Colony formation assay

At 1 day prior to irradiation, 1×10^6 cells were seeded in T25 flasks (353014, Falcon, NY) with 5 ml of medium. Thirty minutes before irradiation, media were changed with freshly prepared media containing 100 mM thiourea or null. Immediately after irradiation, the irradiated cells and controls were harvested using 2.5 g/l trypsin solution (35555–54, Nakarai Tesque Inc., Kyoto, Japan), and plated in triplicate to obtain ~200 surviving cells per dish. After 12 days, the cells were fixed with 5% formalin in PBS, and stained with 1% methylene blue. Colonies that contained >50 cells were counted as survivors.

Measurement of prostaglandin E_2 in conditioned medium

The concentrations of prostaglandin E_2 were measured by a PGE_2 monoclonal ELISA kit (514010, Cayman Chemical, MI) according to the manufacturer's instructions. Briefly, 5×10^5 cells in 35 mm dishes were exposed to X-rays of 3, 5 or 8 Gy. At 8 h post-irradiation, the medium was collected and centrifuged, and an ELISA assay was performed. PGE_2 concentration within the samples was measured using a microplate reader (iMark™ plate reader, Bio-rad, CA).

Co-culture of irradiated and bystander cells

To investigate COX-2 expression through the RIBE, a cell co-culture insert dish (BD Falcon No. 3099, MA, USA) and companion TC plate well (BD Falcon No. 3052) were applied, as demonstrated by others [28]. The membrane bottom of the insert dish has 0.4 µm pores with a density of $1 \times 10^8/cm^2$ to allow for the passage of biomolecules. One day before irradiation, cells were seeded (5.0×10^5 cells/well in 2 ml medium) into 6-well companion plates to be 80–90% confluent at the time of irradiation. Simultaneously, the same number of cells was seeded on insert dishes. Prior to irradiation, the media of inserts were replaced with fresh media containing 100 mM thio-urea (TU^+) or null (TU^-). Cells were exposed to X-ray radiation at a 6% survival dose of 5 Gy for TU^- and 8 Gy for TU^+, which were estimated from survival curves (Fig. 2). Media of both inserts and the co-culture plates were changed to fresh medium and then they were co-cultured for up to 1, 4, 8 and 24 h post-irradiation.

Western blot analysis for COX-2 expression

Cells were washed with PBS, then collected into SDS sample buffer [4% (w/v) sodium dodecyl sulfate (SDS), 125 mM Tris-HCl (pH 6.8)] and heated at 95°C for 20 min. Each lysate (20 µg protein) was mixed with 4 × loading buffer solution (250 mM Tris-HCl, 0.02% bromo-phenol blue, 8% SDS 40% glycerol, 20% 2-mercaptoethanol pH 6.8; 191–13272, WAKO, Osaka Japan), loaded into wells of 10% SDS-PAGE gels for electrophoresis and then transferred to PVDF membranes. After blocking in 5% skim milk in Tris-buffered saline-Tween 20 (TBST), the PVDF membrane was incubated with the primary antibody at 4°C overnight. The antibodies for COX-2 (#12282), HRP-linked secondary antibody (#3683) and GAPDH (#7074) were purchased from Cell signalling

Technology, MA. Anti–COX-2 was labelled with HRP-linked secondary antibody, and anti-GAPDH was used as a loading control. All antibodies were diluted to 1/1000 in TBST and the membrane was washed three times with TBST between every step after incubation. Protein bands were visualized using a chemi-luminescence substrate (Pierce™ ECL Plus Western Blotting Substrate, Thermo Fisher Scientific, MA) and chemi-luminescence imager (EZ-capture MG, ATTO, Tokyo, Japan).

To measure COX-2 expression by PGE_2, cells were cultured in media supplemented with 0.1 ng/ml PGE_2 for 1, 4 or 8 h and lysates were collected as mentioned above.

Statistical analysis

A Student's *t*-test was performed by Sigma Plot 12 (Systat Software, Inc, CA) to analyse differences in data between TU^- and TU^+. Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

There is currently debate regarding the dependency of bystander effects on radiation quality. Generally, increasing LET would reduce OH radical production per dose, resulting in a larger *D/ID* ratio [23, 29, 30]. To mimic the differences in radiation quality, we set up a simplified model by reduced the *ID* by using TU to increase the *D/ID* ratio. We used a DCFH-DA fluorescent probe to estimate their production of ROS by X-rays as well as their reduction by 100 mM thio-urea (TU^+) to quantify the *ID*. As shown in Fig. 1, the slope of TU^+ was decreased to that of 35% of TU^-, indicating that 65% of the OH radicals per dose were scavenged by TU^+ compared with TU^-. The survival curves shown in Fig. 2 clearly show that TU^+ irradiated cells were protected compared with TU^- cells. Generally, the *D/ID* ratio for X-ray–induced cell killing is known to be at ~0.5. Hirayama *et al.* reported that 76% of X-ray–induced cell killing is from *ID* [23], resulting in a *D/ID* ratio of 0.315. The ID_{TU^+} was reduced to 35% of ID_{TU^-}, thus, TU^+ can be estimated to be $D_{TU^+}/ID_{TU^+} = 0.902$. Hence, our experimental system mimicked two radiation types of 2.86-fold different *D/ID* ratios.

Next, we measured the production of PGE_2, the primary product of COX-2, from the irradiated cells at 8 h post-irradiation (Fig. 3A). COX-2 is known as prostaglandin-endoperoxide synthase 2 and is a key regulatory enzyme not only responsible for multiple inflammatory mitogenic, and angiogenic activities, but also strongly involved in the development and progression of cancer [27]. PGE_2 is known to be a potent endogenous molecule, which binds to E-series prostanoid (EP) receptors [26, 27]. In other words, PGE_2 functions as a trigger signal to activate the expression of COX-2 in bystander cells [16, 17]. As shown in Fig. 3B, we have confirmed that PGE_2 activates COX-2 expression in A549 cells at concentrations as low as 0.1 ng/ml. The PGE_2 concentration of TU^- irradiated cells increased in a dose-dependent manner up to 5 Gy, which was the highest of all doses, and then decreased at 8 Gy. This trend was not observed with TU^+ cells, even at the iso-effect dose for cell killing of 8 Gy, in which the PGE_2 concentration was ~60% of the 5 Gy–exposed TU^- cells. The trend in the COX-2 expression was consistent with that of the PGE_2 expression (Fig. 4). COX-2 expression of TU^- cells was 2-fold higher than that of the control at 4 h post-irradiation, whereas TU^+ cells only showed a slight, insignificant increase. These results indicate that a higher *D/ID* ratio may reduce COX-2 expression, therefore suppressing PGE_2 production.

In contrast, COX-2 expression of TU^+ bystander cells increased to nearly 2-fold of the control toward 24 h post-irradiation (Fig. 4A-2

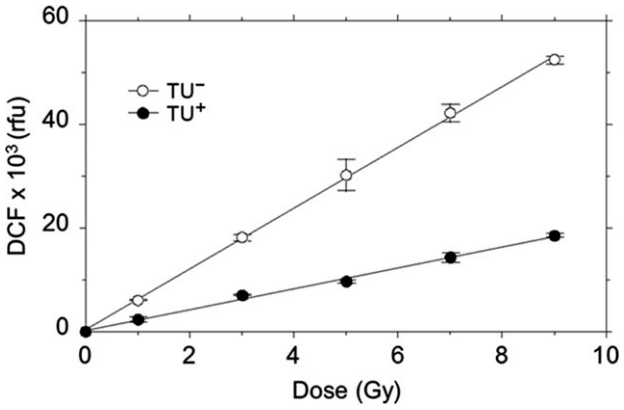

Fig. 1. Measurement of ROS production by X-ray exposure. Plots were fitted by linear regression after background subtraction. The slopes (rfu/Gy) of the graphs for TU^- and TU^+ were 5931 and 2059, respectively. DCF = dichlorofluorescein, rfu = relative fluorescence units.

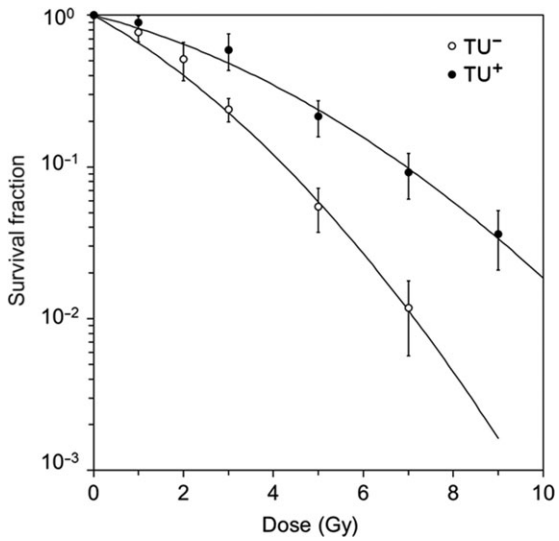

Fig. 2. Plots were fitted according to the linear quadratic model, $S = \exp(-\alpha \times D - \beta \times D^2)$, where *S* is the survival fraction, *D* is the dose in Gy, and α [Gy^{-1}] and β [Gy^{-2}] are the fitting parameters. The α, β, 10% survival dose (D_{10}) and 1% survival dose (D_1) for TU^- and TU^+ were calculated to be TU^-: 0.38, 0.037, 4.27 Gy and 7.14 Gy and TU^+: 0.175, 0.022, 6.96 Gy and 10.9 Gy, respectively.

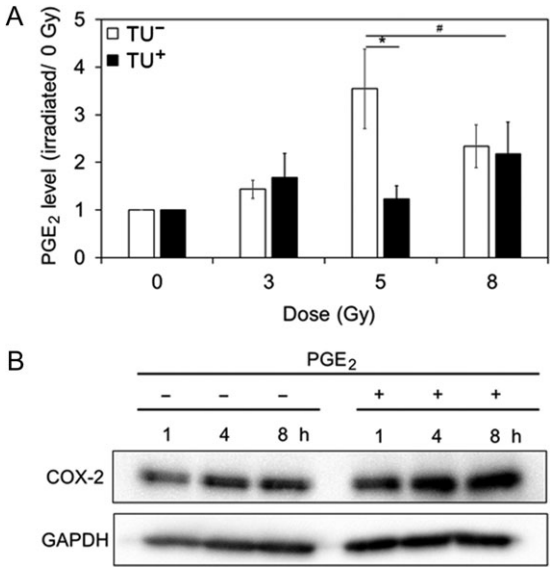

Fig. 3. Panel A shows the PGE_2 level of irradiated cells measured at 8 h post-irradiation with various X-ray doses. PGE_2 concentrations of 5 Gy–irradiated TU^- cells and TU^+ cells were 93.4 pg/ml and 38.1 pg/ml, respectively. PGE_2 concentration for 8 Gy TU^+ irradiated cells was 59.5 pg/ml, which was lower than 5 Gy TU^- irradiated cells ($^*P < 0.05$). Panel B shows COX-2 expression after the addition of 0.1 ng/ml PGE_2 ($\#P = 0.1$).

and B-2). Surprisingly, this trend was very similar to that in the TU^- bystander cells, and there was no significant difference between the two. COX-2 expression and PGE_2 concentration were greatly reduced in the TU^+ irradiated cells, indicating that the PGE_2 did not play a dominant role in inducing COX-2 signalling in TU^+ bystander cells. These results suggest that COX2 expression in TU^+ bystander cells was activated by pathways other than the COX-2/PGE_2 pathway, contrary to the situation in TU^- bystander cells. It is known that COX-2-derived PGE_2 can initiate a positive feedback loop to activate epidermal growth factor receptor (EFGR), resulting in enhanced expression of COX-2 and increased synthesis of prostaglandins. However, EGFR and its downstream effectors can be activated independently of COX-2/PGE_2 [27]. The NFκB/COX-2 pathway is also independent of the COX-2/PGE_2 pathway, and can be activated through hypoxia-inducible factor, HIF-1 [8]. In addition, other secreted diffusible factors, such as ROS [12, 13] and exosomes [15], which trigger medium mediated RIBE may contribute to COX-2 expression in bystander cells.

In conclusion, we demonstrated lower COX-2 expression and PGE_2 production in irradiated cells with a higher *D/ID* ratio. The pathway responsible for COX-2 expression as a bystander response could be an altered from the COX-2/PGE_2 pathway to others due to the radiation quality of higher *D/ID* ratios. Further investigation will be necessary in order to identify the alternative pathways for COX-2 expression and to understand the correlation between radiation quality and the pathways responsible for RIBE.

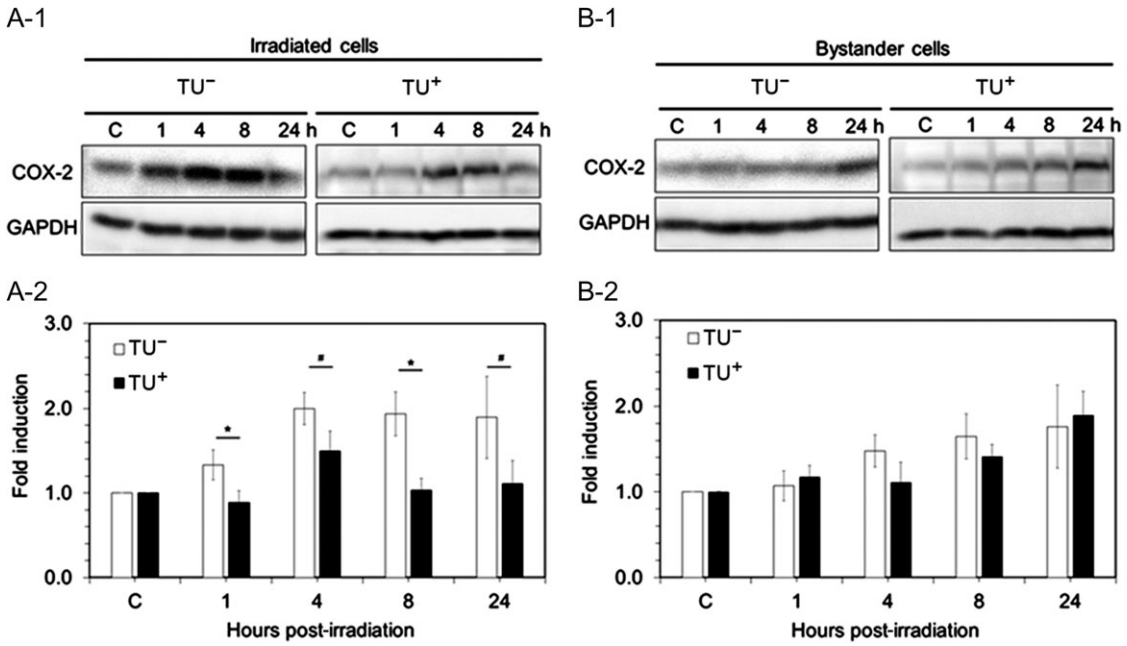

Fig. 4. Comparison of COX-2 expression in TU^- and TU^+ irradiated and bystander cells. Panels A-1 and B-1 are representative images of western blot analyses of irradiated cells and bystander cells, respectively. Panels A-2 and B-2 show the COX-2 expression for each hour post-irradiation of irradiated cells and bystander cells, respectively. $^*P < 0.05$.

SUPPLEMENT DATA

Supplementary data are available on *Journal of Radiation Research* online.

ACKNOWLEDGEMENTS

Part of results were presented at the 60th Japan Radiation Research Society meeting at Chiba in 2017.

CONFLICT OF INTEREST

The authors state that there are no conflicts of interest.

FUNDING

This study was supported in part by a Japan Society for the Promotion of Science (JSPS) KAKENHI Grant-in-Aid for Young Scientist B (17K16496) and a Grant-in-Aid for Scientific Research B (17H04268).

REFERENCES

1. Goodhead DT. Mechanisms for the biological effectiveness of high-LET radiations. *J Radiat Res* 1999;40:1–13.
2. Goodhead DT. New radiobiological, radiation risk and radiation protection paradigms. *Mutat Res* 2010;687:13–6.
3. Nagasawa H, Little JB. Induction of sister chromatid exchanges by extremely low doses of α-particles. *Cancer Res* 1992;52: 6394–6.
4. Liu Y, Kobayashi A, Fu Q et al. Rescue of targeted nonstem-like cells from bystander stem-like cells in human fibrosarcoma HT1080. *Radiat Res* 2015;184:334–40.
5. Kobayashi A, Tengku Ahmad TAF, Autsavapromporn N et al. Enhanced DNA double-strand break repair of microbeam targeted A549 lung carcinoma cells by adjacent WI38 normal lung fibroblast cells via bi-directional signaling. *Mutat Res* 2017;803–5: 1–8.
6. Autsavapromporn N, Suzuki M, Funayama T et al. Gap junction communication and the propagation of bystander effects induced by microbeam irradiation in human fibroblast cultures: the impact of radiation quality. *Radiat Res* 2013;180:367–75.
7. Autsavapromporn N, Plante I, Liu C et al. Genetic changes in progeny of bystander human fibroblasts after microbeam irradiation with X-rays, protons or carbon ions: the relevance to cancer risk. *Int J Radiat Biol* 2015;91:62–70.
8. Jung YJ, Isaacs JS, Lee S et al. IL-1beta–mediated up-regulation of HIF-1alpha via an NFkappaB/COX-2 pathway identifies HIF-1 as a critical link between inflammation and oncogenesis. *FASEB J* 2003;17:2115–7.
9. Matsumoto H, Hamada N, Takahashi A et al. Vanguards of paradigm shift in radiation biology: radiation-induced adaptive and bystander responses. *J Radiat Res* 2007;48:97–106.
10. Choi VW, Ng CY, Kobayashi A et al. Bystander effect between zebrafish embryos *in vivo* induced by high-dose X-rays. *Environ Sci Technol* 2013;47:6368–76.
11. Kong EY, Cheng SH, Yu KN. Induction of autophagy and interleukin 6 secretion in bystander cells: metabolic cooperation for radiation-induced rescue effect? *J Radiat Res* 2018;59:129–40.
12. Jella KK, Moriarty R, McClean B et al. Reactive oxygen species and nitric oxide signaling in bystander cells. *PLoS One* 2018;13: e0195371.
13. Tomita M, Matsumoto H, Funayama T et al. Nitric oxide–mediated bystander signal transduction induced by heavy-ion microbeam irradiation. *Life Sci Space Res (Amst)* 2015;6:36–43.
14. Dong C, He M, Tu W et al. The differential role of human macrophage in triggering secondary bystander effects after either gamma-ray or carbon beam irradiation. *Cancer Lett* 2015; 363:92–100.
15. Yuan D, Xu J, Wang J et al. Extracellular miR-1246 promotes lung cancer cell proliferation and enhances radioresistance by directly targeting DR5. *Oncotarget* 2016;7:32707–22.
16. Hei TK, Zhou H, Chai Y et al. Radiation induced non-targeted response: mechanism and potential clinical implications. *Curr Mol Pharmacol* 2011;4:96–105.
17. Chai Y, Calaf GM, Zhou H et al. Radiation induced COX-2 expression and mutagenesis at non-targeted lung tissues of *gpt* delta transgenic mice. *Br J Cancer* 2013;108:91–8.
18. Tomita M, Maeda M. Mechanisms and biological importance of photon-induced bystander responses: do they have an impact on low-dose radiation responses. *J Radiat Res* 2015;56:205–19.
19. Ng CYP, Kong EY, Kobayashi A et al. Neutron induced bystander effect among zebrafish embryos. *Radiat Phys Chem* 2015;117:153–9.
20. Little JB, Azzam EI, de Toledo SM et al. Bystander effects: intercellular transmission of radiation damage signals. *Radiat Prot Dosimetry* 2002;99:159–62.
21. Matsumoto Y, Hamada N, Aoki-Nakano M et al. Dependence of the bystander effect for micronucleus formation on dose of heavy-ion radiation in normal human fibroblasts. *Radiat Prot Dosimetry* 2015;166:152–6.
22. Hall EJ, Giaccia AJ. *Radiobiology for the Radiologist*. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins, 2012.
23. Hirayama R, Ito A, Tomita M et al. Contributions of direct and indirect actions in cell killing by high-LET radiations. *Radiat Res* 2009;171:212–8.
24. Tsuruoka C, Suzuki M, Hande MP et al. The difference in LET and ion species dependence for induction of initially measured and non-rejoined chromatin breaks in normal human fibroblasts. *Radiat Res* 2008;170:163–71.
25. Wang WF, Schuchmann MN, Schuchmann HP et al. Radical cations in the OH-radical–induced oxidation of thiourea acid tetramethylthiourea in aqueous solution. *J Am Chem Soc* 1999; 121:238–45.
26. Greenhough A, Smartt HJ, Moore AE et al. The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009;30:377–86.
27. Galati R. The role of cyclooxygenase-2, epidermal growth factor receptor and aromatase in malignant mesothelioma. In: Belli C and Anand S (eds). *Malignant Mesothelioma.* Rijeka: IntechOpen,

2012. https://www.intechopen.com/books/malignant-mesothelioma/the-role-of-cyclooxygenase-2-epidermal-growth-factor-receptor-and-aromatase-in-malignant-mesotheliom (21 April 2018, date last accessed).

28. Hsu HH, Lin YM, Shen CY et al. Prostaglandin E2-induced COX-2 expressions via EP2 and EP4 signaling pathways in human LoVo colon cancer cells. *Int J Mol Sci* 2017;18:pii: E1132.
29. Moritake T, Tsuboi K, Anzai K et al. ESR spin trapping of hydroxyl radicals in aqueous solution irradiated with high-LET carbon-ion beams. *Radiat Res* 2003;159:670–5.
30. Maeyama T, Yamashita S, Baldacchino G et al. Production of a fluorescence probe in ion-beam radiolysis of aqueous coumarin-3-carboxylic acid solution—1: beam quality and concentration dependences. *Radiat Phys Chem* 2011;80:535–9.