Cytochrome-c mediated a bystander response dependent on inducible nitric oxide synthase in irradiated hepatoma cells

M He¹, S Ye¹, R Ren¹, C Dong¹, Y Xie¹, D Yuan¹ and C Shao*¹
¹Institute of Radiation Medicine, Fudan University, No.2094 Xie-Tu Road, Shanghai 200032, China

BACKGROUND: Radiation-induced bystander effect (RIBE) has important implication in tumour radiotherapy, but the bystander signals are still not well known.

METHODS: The role of cytochrome-c (cyt-c) and free radicals in RIBE on human hepatoma cells HepG2 was investigated by detecting the formation of bystander micronuclei (MN) and the generation of endogenous cyt-c, inducible nitric oxide (NO) synthase (iNOS), NO, and reactive oxygen species (ROS) molecules.

RESULTS: When HepG2 cells were cocultured with an equal number of irradiated HepG2 cells, the yield of MN in the nonirradiated bystander cells was increased in a manner depended on radiation dose and cell coculture time, but it was diminished when the cells were treated with cyclosporin A (CsA), an inhibitor of cyt-c release. Meanwhile the CsA treatment inhibited radiation-induced NO but not ROS. Both of the depressed bystander effect and NO generation in the CsA-treated cells were reversed when 5 μM cyt-c was added in the cell coculture medium. But these exogenous cyt-c-mediated overproductions of NO and bystander MN were abolished when the cells were pretreated with s-methylisothiourea sulphate, an iNOS inhibitor.

CONCLUSION: Radiation-induced cyt-c has a profound role in regulating bystander response through an iNOS-triggered NO signal but not ROS in HepG2 cells.

Keywords: ionising radiation; bystander effect; cytochrome-c; iNOS; ROS

Ionising radiation induces DNA damage not only in the directly exposed cells but also in their neighbouring nonirradiated cells, termed as radiation-induced bystander effect (RIBE) that was first reported by Nagasawa and Little (1992). Since then, the research advance in RIBE has significantly impacted on the radiobiological studies and cancer risk evaluation. It has been known that a series of bystander responses, including cell killing (Lewis et al., 2001; Schettino et al., 2003), genomic instability (Seymour and Mothersill, 1997; Morgan and Sowa, 2007), neoplastic transformation (Sawant et al., 2001; Mancuso et al., 2008), changes in gene expression (Azzam et al., 1998), and DNA methylation (Ilnytskyy et al., 2009), can be triggered by the soluble molecules that are released from irradiated cells and affect neighbouring cells via gap junction (Azzam et al., 2000; Shao et al., 2003b) and/or culture medium (Baskar et al., 2007; Dickey et al., 2009). Because RIBE has an important implication in radiotherapy, tumour cells have been widely applied for the studies on this phenomenon (Shao et al., 2003a; Harada et al., 2009). Multiple RIBEs, including cell growth stimulation, DNA damage, and cell death, have been observed in tumour cells in vitro (Shao et al., 2003a; c; Gow et al., 2010). Using mouse model, the bystander responses of internal tumour cells or tissues were also confirmed in vivo, and cancer-associated events, such as p53 alteration, MMPs activity, and epigenetic change, were proved to be involved in the RIBE (Camphausen et al., 2003; Koturbash et al., 2007; Lemay et al., 2011).

Several bystander signalling molecules, such as free radicals (Narayanan et al., 1997; Shao et al., 2002; Han et al., 2009), proteins (Narayanan et al., 1999; Shao et al., 2008b), calcium flux (Lyng et al., 2002; Shao et al., 2006), and hormones (Shao et al., 2008a) have been disclosed. Recent studies have shown that cytochrome-c (cyt-c) is also involved in the RIBE either as a sensor of bystander response (Yang et al., 2009) or as a signalling factor transmittable through gap junction (Peixoto et al., 2009). Our previous study demonstrated that a p53-dependent cyt-c release from the mitochondria of irradiated cells had an important role in the regulation of RIBE (He et al., 2011). cyt-c is an electron transporting protein and belongs to a part of the respiratory chain localised in the inner mitochondrial membrane (Schagger, 2002). Release of cyt-c from mitochondria into cytoplasm is a key event of radiation-induced apoptosis (Ogawa et al., 2002).

However, most of the previous works have paid close attention in the role of cyt-c itself in RIBE but not its relationship with other bystander signals. To clarify the signalling pathways underlying the cyt-c-mediated bystander response, we detected two principal reactive species, that is, reactive oxygen species (ROS) and nitric oxide (NO), and attempted to determine which could be a downstream signal factor modulated by cyt-c in RIBE.
**MATERIALS AND METHODS**

**Tumour Cell Line**

Human hepatoma HepG2 cells (Shanghai Cell Bank, Chinese Academy of Science, Shanghai, China) were cultured in Dulbecco's modified Eagle's medium (HyClone Co., Beijing, China) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Cölbe, Germany), 100 U ml⁻¹ penicillin, 100 U ml⁻¹ streptomycin, and 2 mM glutamate. All culture conditions were maintained in a humidified atmosphere of 5% CO₂ in air at 37 °C.

**Cell irradiation, coculture, and drug treatment**

HepG2 cells were seeded onto a 26 × 21 mm² coverslip (1.5 × 10⁵ cells) within a 35-mm dish and allowed to grow overnight for cell attachment. Cells were then irradiated with γ-rays generated by a ¹³⁷Cs irradiator (Gammacell-40, Nordion International Inc., Kanata, ON, Canada) at a dose rate of 0.83 Gy min⁻¹. Immediately after irradiation, the irradiated cells and nonirradiated cells growing on different coverslips were placed face-to-face with a 3-mm gap and cocultured in a 35-mm dish with fresh medium for 24 h before irradiation. 12 h after irradiation, HepG2 cells were irradiated with 3 Gy. MN, was calculated with the cytokinesis block micronucleus (CNB-MN) assay (Kanata, ON, Canada) at a dose rate of 0.83 Gy min⁻¹. Immediately after irradiation, the irradiated cells and nonirradiated cells growing on different coverslips were placed face-to-face with a 3-mm gap and cocultured in a 35-mm dish with fresh medium until further assay.

In some experiments, HepG2 cells were pretreated with 5 μM cyclosporin A (Cyclosporin A, Sigma Co., St Louis, MO, USA) for 1 h and/or 500 μM m-sulphoraphane (SMRT, Sigma Co.) for 10 h before irradiation. CsA is an inhibitor of mitochondrial permeability transition pore (MPTP) and SMRT is a selective inhibitor of inducible NO synthase (iNOS) (Szabo et al., 1994). After irradiation, both drugs were immediately washed with PBS triply. Because CsA was stored (1000 mg ml⁻¹) in 12 000 rpm at 4 °C, the membrane was incubated for 16 h at 4 °C with 1:1000 dilution of primary antibodies for iNOS (Cell Signaling Technology, Danvers, MA, USA) and α-Tubulin (Beyotime Biotechnology). Blots were washed three times with PBS at 5 min intervals followed by incubation with 1:2500 dilution of secondary antibody (Cell Signaling Technology) for 1 h at room temperature. The transferred proteins were visualised with the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) using an ECL detection kit (Millipore) and analysed with the Quantity One software (Bio-Rad Laboratories).

**MN assay**

Formation of MN were measured with the cytokinesis block technique that has been widely used to estimate genotoxic damage (Fenech, 2007). Briefly, after irradiation and cell coculture, HepG2 cells were treated with 2 μg ml⁻¹ cyclochalasin-B (Sigma Co.) for 28 h followed by 0.075 M KCl hypotonic treatment for 3 min and then fixed in situ with methanol-acetic acid (9:1 v/v) for 20 min. Air-dried cells were stained with 20 μg ml⁻¹ acridine orange (Sigma Co.) for 3 min. MN were scored in at least 1000 binucleated cells each sample under a fluorescence microscope (Olympus, Tokyo, Japan). The MN yield, Y_MN, was calculated as the ratio of the number of MN to the number of scored binucleated cells.

**Immunofluorescence localisation of cyt-c**

Intracellular cyt-c was immunocytochemically detected in situ. Briefly, HepG2 cells (5 × 10⁴ cells) were grown on a glass coverslip for 24 h before irradiation. 12 h after irradiation, HepG2 cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min then incubated with 3% BSA plus 0.5% Triton X-100 in PBS for 1 h to permeabilise the cells and block nonspecific protein interaction. Subsequently, these cells were incubated overnight with the sheep polyclonal cyt-c antibody (Abcam, Cambridge, MA, USA) at a 1:100 dilution at 4 °C. After removing the unbound antibody by rinsing with PBS, the cells were incubated with FITC-labeled rabbit anti-sheep IgG (H+L) conjugate (Invitrogen, Carlsbad, CA, USA) at a 1:200 dilution for 1 h in dark. Cell nuclei were stained with 100 ng ml⁻¹ DAPI (Sigma Co.) for 2 min. The stained cells were then secured with coverslip and sealed with mounting medium (Vector Laboratories, Burlingame, CA, USA). The cell fluorescence image was captured with the MicroPublisher digital camera (QImaging, Surrey, BC, Canada) mounted on a fluorescence microscope (Olympus) and analysed with the Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA). Cyt-c released cells (cyt-c was diffusely expressed in cytosol) and cyt-c concentrated cells (cyt-c was concentrated relatively in mitochondria around the nucleus) were respectively counted in 10 randomly chosen fields. Approximately, 100 cells from each sample were analysed for the cyt-c distribution assay.

**Western Blot Analysis**

After the treatments described above, the culture cells (2 × 10⁶) were harvested and treated with the RIPA lysis (Beyotime Biotechnology, Shanghai, China) containing phoshatase inhibitor cocktail (1:100) (Sigma Co.) and phenylmethanesulfonyl fluoride (1 mM) (Sigma Co.) for 5 min on ice. Cell lysate was centrifuged at 12 000 rpm at 4 °C for 5 min. Supernatant was collected and total protein concentration was quantified by the bichinchoninic acid protein assay kit (Beyotime Biotechnology). Cell lysate (40 μg protein) was boiled in sodium dodecyl sulphate (SDS) buffer for 10 min before electrophoresis on 10% SDS-polyacrylamide gel. After transfer to polyvinilidene fluoride membrane (Millipore, Bedford, MA, USA), the blots were blocked with 5% fat-free dry milk-PBST (PBS containing 0.1% Tween-20) for 1 h at room temperature. The membrane was incubated for 16 h at 4 °C with 1:1000 dilution of primary antibodies for iNOS (Cell Signaling Technology, Danvers, MA, USA) and α-Tubulin (Beyotime Biotechnology). Blots were washed three times with PBS at 5 min intervals followed by incubation with 1:2500 dilution of horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature. The transferred proteins were visualised with the ChemiDoc XRS system (Bio-Rad Laboratories, Hercules, CA, USA) using an ECL detection kit (Millipore) and analysed with the Quantity One software (Bio-Rad Laboratories).

**Measurement of intracellular ROS and NO**

The intracellular ROS and NO were measured in situ by using fluorescence probes of 2',7'-dichlorofluorescin diacetate (DCFH-DA) and 2-aminomethyl-2',7'-dichlorofluorescin diacetate (DAF-FM-DA) (Molecular Probes, Eugene, OR, USA), respectively. Briefly, after irradiation, HepG2 cells seeded on 24-well plates (1 × 10⁵ cells) were treated with 3 μM DCFH-DA or 5 μM DAF-FM-DA for 30 min at 37 °C in dark, then additional dye was washed with PBS and the cells were incubated for an additional 30 min at 37 °C in order for a complete de-esterification of the intracellular diacetate. The fluorescence intensity was then recorded by a microplate reader (Synergy HT, BioTek, Winooski, VT, USA) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm for DCFH or an excitation wavelength of 495 nm and an emission wavelength of 515 nm for DAF-FM. The relative levels of ROS and NO were calculated as the mean fluorescence intensity of irradiated cells compared with the mean intensity of control cells without irradiation.

**Statistical analyses**

The data presented as mean ± s.e. were obtained from two or three independent experiments with three replicates in each. Comparison is performed by the Student’s two-tailed t-test, whereas multiple comparisons are carried out using one-way analysis of variance. Statistical significance is acceptable at the level of P < 0.05. Data are analysed with the software SPSS11.5 (SPSS Inc., Chicago, IL, USA).
RESULTS

Radiation-induced direct and bystander damage

Figure 1A illustrates that, as a consequence of DNA strand breaks, MN were induced in the irradiated HepG2 cells and its yield increased with dose. When nonirradiated HepG2 cells were cocultured with the irradiated cells for 12 h, MN were induced in the bystander cells. The yield of bystander MN increased when the radiation dose increased to 3 Gy, but then had a tendency of decrease at higher doses even nonsignificantly. The bystander response also had a relationship with the cell coculture time. When the target cells were irradiated with 3 Gy of γ-rays, the maximum bystander damage appeared at 12 h during the coculture period from 4 to 24 h (Figure 1B). Accordingly, 12 h post 3 Gy irradiation was an ideal condition for the induction of a bystander response in HepG2 cells and hence was chosen as the representative point for the following mechanistic investigations.

Influence of cyclosporin-A and cyt-c on radiation responses

To explore the potential role of mitochondria in radiation damage, 1 h before irradiation, HepG2 cells were treated with CsA to close the mitochondrial membrane pores and inhibit cyt-c release. It was found that this treatment not only decreased the yield of radiation-induced MN by about 70% but also significantly diminished the mitochondrial function may be involved in the RIBE. To know the situation of cyt-c release, we measured the distribution of cyt-c inside the cytoplasm of HepG2 cells with and without irradiation by the method of immunochemistry in situ. As shown in Figure 3A, for most of the nonirradiated HepG2 cells, the cellular cyt-c protein was concentrated inside mitochondria. After 3 Gy exposure, the cyt-c was released from mitochondria and diffused in the whole cytosol so that the percentage of cyt-c-released cells was increased from 35% of control to 60%. However, this cyt-c release was effectively inhibited by 5 μM CsA so that the percentage of the cyt-c-released cells significantly decreased to a level near control (Figure 3B), which is in parallel with the result in Figure 2 that CsA diminishes RIBE on MN induction.

Generation of radiation-induced NO and ROS

Two established bystander signalling molecules of NO and ROS were assayed after 3 Gy irradiation. Figure 4A illustrates that the fluorescence intensity of DAF-FM corresponding to the intracellular NO was accumulated in the irradiated HepG2 cells over the time from 2 to 12 h postirradiation and it was significantly increased to about 1.5-fold of the control at 12 h postirradiation. Normally, the intracellular NO is a reaction product of L-arginine catalysed by iNOS. Figure 4B shows that the level of iNOS expression was increased gradually after irradiation and it approached to 1.4-fold of the nonirradiated control at 6 h postirradiation and then became relatively stable up to 12 h after irradiation. Figure 4A also revealed the time response of radiation-induced ROS that was represented by the relative fluorescence intensity of DCFH in the irradiated cells. It was seen that the kinetics of the intracellular ROS was different from the generation of intracellular measured. The results showed that although CsA blocked the induction of bystander MN, when the cells were treated with 5 μM cyt-c in substitution for intrinsic cyt-c in the CsA-treated cells, the yield of MN in bystander cells was partly recovered from 0.056 of CsA-treated cells to 0.068, but this exogenous cyt-c failed to increase the MN yield in the directly irradiated cells under CsA treatment (Figure 2). In addition, this exogenous cyt-c treatment itself had no influence on the MN induction of both irradiated and bystander cells. These data suggest that, as a critical event, cyt-c could be involved in RIBE rather than direct radiation damage.

CsA inhibited cyt-c release in the irradiated cells

To know the situation of cyt-c release, we measured the distribution of cyt-c inside the cytoplasm of HepG2 cells with and without irradiation by the method of immunochemistry in situ. As shown in Figure 3A, for most of the nonirradiated HepG2 cells, the cellular cyt-c protein was concentrated inside mitochondria. After 3 Gy exposure, the cyt-c was released from mitochondria and diffused in the whole cytosol so that the percentage of cyt-c-released cells was increased from 35% of control to 60%. However, this cyt-c release was effectively inhibited by 5 μM CsA so that the percentage of the cyt-c-released cells significantly decreased to a level near control (Figure 3B), which is in parallel with the result in Figure 2 that CsA diminishes RIBE on MN induction.

Generation of radiation-induced NO and ROS

Two established bystander signalling molecules of NO and ROS were assayed after 3 Gy irradiation. Figure 4A illustrates that the fluorescence intensity of DAF-FM corresponding to the intracellular NO was accumulated in the irradiated HepG2 cells over the time from 2 to 12 h postirradiation and it was significantly increased to about 1.5-fold of the control at 12 h postirradiation. Normally, the intracellular NO is a reaction product of L-arginine catalysed by iNOS. Figure 4B shows that the level of iNOS expression was increased gradually after irradiation and it approached to 1.4-fold of the nonirradiated control at 6 h postirradiation and then became relatively stable up to 12 h after irradiation. Figure 4A also revealed the time response of radiation-induced ROS that was represented by the relative fluorescence intensity of DCFH in the irradiated cells. It was seen that the kinetics of the intracellular ROS was different from the generation of intracellular

![Figure 1](https://example.com/f1.png)

**Figure 1** (A) Dose response of the yield of MN in the irradiated HepG2 cells and bystander HepG2 cells that were cocultured with irradiated cells for 12 h. (B) Time response of bystander MN formation in HepG2 cells that were cocultured with 3 Gy γ-irradiated HepG2 cells. **P < 0.01, ***P < 0.001 compared with the control without irradiation.

![Figure 2](https://example.com/f2.png)

**Figure 2** Influence of CsA and exogenous cyt-c on the MN formation of 3 Gy γ-irradiated HepG2 cells and bystander HepG2 cells, which was cocultured with 3 Gy γ-irradiated cells for 12 h. **P < 0.01, ***P < 0.001 compared with the control without irradiation or to the indicated group with drug treatment.
NO. ROS was induced immediately after irradiation and its yield increased rapidly to a maximum level at 2 h after irradiation and then decreased gradually to the control level at 4–12 h postirradiation.

**Relationship between radiation-induced cyt-c and NO, ROS**

To know the relationship between cyt-c and radiation-induced free radicals, we treated cells with a MPTP inhibitor before irradiation. It was found that when the cells were treated with 5 \( \mu M \) CsA before irradiation, the induction of intracellular NO decreased significantly but still higher than that of nonirradiated control. When the culture medium contained 5 \( \mu M \) cyt-c together with CsA, the intracellular level of NO in the irradiated cells was recovered to the level of directly irradiated cells without any drug treatment (Figure 5A). These results disclose that the radiation-induced NO is regulated by the release of mitochondrial cyt-c.

More interestingly, although the fluorescence of DCFH in the irradiated cells was effectively reduced when the cells were treated with CsA alone, at the representative time points of 2 and 12 h postirradiation, it was uneventful in the CsA-treated HepG2 and had no relationship to the exogenous cyt-c treatment (Figure 5B). Thus, the generation of ROS was not a downstream event of cyt-c release. Taken together, our results indicated that NO, rather than ROS, was a downstream product of radiation-induced cyt-c release. This finding is coincident with our previous report that NO and ROS were involved in the bystander responses triggered by irradiated tumour cells and normal cells, respectively (Shao et al., 2005).
that neither CsA nor exogenous cyt-c had any effect on the INOS expression at 12 h postirradiation, indicating that INOS was not a downstream molecule of the intracellular cyt-c. Considering from the opposite side, we pretreated HepG2 cell with an INOS inhibitor SMT and then reexamined the cyt-c/INOS-mediated RIBE. The results showed that although CsA could still somewhat attenuate the radiation-induced NO production, there were no any increases of NO (Figure 6B) and attendant bystander MN in the irradiated HepG2 cells in the presence of exogenous cyt-c and INOS inhibitor (Figure 6A). All of the above results indicated that, as an initial source of NO, the INOS expression was essential to cyt-c/NO-mediated bystander response.

DISCUSSION

The present study found that the bystander MN could be induced in the nonirradiated HepG2 cells after coculturing with irradiated cells and its yield was dependent on both radiation dose and coculture time, which was consistent with some of the other reports (Harada et al., 2008; Asur et al., 2009). It can be assumed that the level of bystander response might correspond to radiation-induced cellular damage, but once the cell damage was too serious to be insufficient in generating more bystander signals, the RIBE would reach to a platform and even then descent as that shown in Figure 1A.

RIBE is also related to the cell situation. It was found here that, even at an optimum condition of RIBE, the bystander MN could be nearly eliminated by the treatment of cells with CsA, indicating that mitochondria-dependent intracellular factor(s) may be involved in the stimulation of bystander response. The result of immunocytochemical assay provided a clue that cyt-c has a possibility to have a key role in the generation of bystander effect. These conjecture was confirmed by the data that supplement of exogenous cyt-c into cell coculture medium partly recovered the bystander MN induction, which had been blocked by CsA, that is, the exogenous cyt-c could substitute the function of endogenous cyt-c and then stimulates irradiated cells to generate some unknown bystander signalling factors and further induce cellular damage in adjacent cells. However, for the directly irradiated cells, the yield of MN in the CsA-treated HepG2 cells could not be recovered by exogenous cyt-c. Accordingly, the cyt-c release may be essential for RIBE but is not a key point for direct radiation damage.

Mitochondria is the main pool of radiation-induced cyt-c associated with the generation of free radical signals of NO and...
ROS (Chen et al., 2003; Aykin-Burns et al., 2011) that could act as important mediators of RIBE (Azzam et al., 2004; Shao et al., 2008c). NO is postulated to be produced from l-arginine catalysed by iNOS (Nathan, 1992) after irradiation (Matsumoto et al., 2001), and it can act as an intercellular signalling molecule, cause DNA damage, and disturb DNA repair in bystander cells (Han et al., 2007). Our previous study showed that the amount of NO released from irradiated tumour cells was related to the dose and LET of irradiation, and suggested that radiation-induced NO might be modulated by some unknown factors during cell-programmed death (Shao et al., 2001). The present study observed that the generation of NO in the irradiated cells could be significantly inhibited by the treatment of cells with a MPTP inhibitor CsA, but this inhibition could be fully recovered when the exogenous cyt-c was supplied to the CsA-treated cells, which confirms that the NO generation is associated with mitochondrial cyt-c release in the process of apoptosis.

So far, there are a great deal of interests in the possibility that NO might be generated by a source other than NO synthase, such as the release of S-nitrosothiols during deoxygenation (Stamler et al., 1997) or the reduction of NO2- to NO by haemoglobin (Cosby et al., 2003). The precise mechanisms of NOS-regulated intracellular NO and its redox state operated by mitochondrial cyt-c were also widely discussed (Torres et al., 2000; Palacios-Callender et al., 2007). Therefore, it is quite essential and interesting to know the role of iNOS in cyt-c-mediated NO generation and bystander effect. We found here that the inhibition of iNOS could suppress the cyt-c-mediated RIBE by blocking the source of NO, thus the cyt-c NO-mediated bystander effect was in an iNOS-dependent manner. Based on the present findings we hypothesise that cyt-c has an important role in an efficient cascade amplification of the overproduction of NO generated from an iNOS-catalysed reaction.

ACKNOWLEDGEMENTS

This study was supported by the National Nature Science Foundation of China (Grant Nos. 31070758, 11179002).

REFERENCES

Asur RS, Thomas RA, Tucker JD (2009) Chemical induction of the bystander effect in normal human lymphoblastoid cells. Mutat Res 676: 11 – 16

Aykın-Burns N, Slane BG, Liu AT, Owens KM, O’Malley MS, Smith BJ, Domann FE, Spitz DR (2011) Sensitivity to low-dose/low-LET ionizing radiation in mammalian cells harboring mutations in succinate dehydrogenase subunit C is governed by mitochondria-derived reactive oxygen species. Radiat Res 175: 150 – 158

Azzam EI, de Toledo SM, Gooding T, Little JB (1998) Intercellular communication is involved in the bystander regulation of gene expression in human cells exposed to very low fluences of alpha particles. Radiat Res 150: 497 – 504

Azzam EI, de Toledo SM, Little JB (2004) Stress signaling from irradiated to non-irradiated cells. Curr Cancer Drug Targets 4: 53 – 64

Azzam EI, de Toledo SM, Waker AJ, Little JB (2000) High and low fluences of alpha-particles induce a G1 checkpoint in human diploid fibroblasts. Cancer Res 60: 2623 – 2631

Baskar R, Balajee AS, Geard CR (2007) Effects of low and high LET radiations on bystander human lung fibroblast cell survival. Int J Radiat Biol 83: 551 – 559

Camp Hansen K, Moses MA, Menard C, Spreul M, Beecken WD, Folkman J, O’Reilly MS (2003) Radiation abscopal antitumor effect is mediated through p53. Cancer Res 63: 1990 – 1993

Chen Q, Chai YC, Mazumder S, Jiang C, Macklis RM, Chisolm GM, Almasan A (2003) The late increase in intracellular free radical oxygen species during apoptosis is associated with cytochrome c release, caspase activation, and mitochondrial dysfunction. Cell Death Differ 10: 323 – 334

Chen S, Zhao Y, Zhao G, Han W, Bao L, Yu KN, Wu L (2009) Up-regulation of ROS by mitochondria-dependent bystander signalling contributes to genotoxicity of bystander effects. Mutat Res 666: 68 – 73

Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter GD, Martyr S, Yang BK, Wadlaw MA, Zalos G, Xu X, Huang KT, Shields H, Kim-Shapiro DB, Schechter AN, Cannon 3rd RO, Gladwin MT (2003) Nitrite reduction to nitric oxide by deoxyhaemoglobin vasodilates the human circulation. Nat Med 9: 1498 – 1505

Dickey JS, Baird BJ, Redon CE, Sokolov MV, Sedelnikova OA, Bonner WM (2009) Intercellular communication of cellular stress monitored by gamma-H2AX induction. Carcinogenesis 30: 1686 – 1695

Fenech M (2007) Cytokinesis-block micronucleus cytose assay. Nat Protoc 2: 1084 – 1104

Gow MD, Seymour CB, Ryan LA, Mothersill CE (2010) Induction of bystander response in human glioma cells using high-energy electrons: a role for TGF-beta1. Radiat Res 173: 769 – 778

Han W, Wu L, Chen S, Bao L, Zhang L, Jiang E, Zhao Y, Xu A, Hei TK, Yu Z (2007) Constitutive nitric oxide acting as a possible intercellular signalling molecule in the initiation of radiation-induced DNA double strand breaks in non-irradiated bystander cells. Oncogene 26: 2330 – 2339

Han W, Wu L, Chen S, Yu KN (2009) Exogenous carbon monoxide protects the bystander Chinese hamster ovary cells in mixed coculture system after alpha-particle irradiation. Carcinogenesis 31: 275 – 280

Harada K, Nonaka T, Hamada N, Sakurai H, Hasegawa M, Funayama T, Kakizaki T, Kobayashi Y, Nakano T (2009) Heavy-ion-induced bystander killing of human lung cancer cells: role of gap junctional intercellular communication. Cancer Sci 100: 684 – 688

Harada T, Kashino G, Suzuki K, Matsuda N, Kodama S, Watanabe M (2008) Different involvement of radical species in irradiated and bystander cells. Int J Radiat Biol 84: 809 – 814

He M, Zhao M, Shen B, Prise KM, Shao C (2011) Radiation-induced intercellular signaling mediated by cytochrome-c via a p53-dependent pathway in hepatoma cells. Oncogene 26: 1947 – 1955

Ilnytskyy Y, Koturbash I, Kovalchuk O (2009) Radiation-induced bystander effects in vivo are epigenetically regulated in a tissue-specific manner. Environ Mol Mutagen 50: 105 – 113

Koturbash I, Boyko A, Rodriguez-Juarez R, McDonald RJ, Tryndyak VP, Kovalchuk I, Pogribny IP, Kovalchuk O (2007) Role of epigenetic effectors in maintenance of the long-term persistent bystander effect in spleen in vivo. Carcinogenesis 28: 1831 – 1838
Lehnert BE, Goodvin EH (1997) A new mechanism for DNA alterations induced by alpha particles such as those emitted by radon and radon progeny. *Environ Health Perspect* 105(Suppl 5): 1095 – 1101

Lemay R, Archambault M, Tremblay L, Bujoil R, Lepage M, Paquette B (2011) Irradiation of normal mouse tissue increases the invasiveness of mammary cancer cells. *Int J Radiat Biol* 87: 472 – 482

Lewis DA, Mayhugh BM, Qin Y, Trott K, Mendonca MS (2001) Production of delayed death and neoplastic transformation in CGL1 cells by radiation-induced bystander effects. *Radiat Res* 156: 251 – 258

Lyng FM, Seymour CB, Mothersill C (2002) Initiation of apoptosis in cells exposed to medium from the progeny of irradiated cells: a possible mechanism for bystander-induced genomic instability? *Radiat Res* 157: 365 – 370

Mancuso M, Pasquali E, Leonardi S, Rebessi S, Di Majo V, Pazzaglia S, Toni MP, Pimpanella M, Covelli V, Saran A (2008) Oncogenic bystander radiation effects in Patched heterozygous mouse cerebellum. *Proc Natl Acad Sci USA* 105: 12445 – 12450

Matsumoto H, Hayashi S, Hataashita M, Ohnishi K, Sh_ioura H, Ohtsubo T, Kitai R, Ohnishi T, Kano E (2001) Induction of radioresistance by a nitric oxide-mediated bystander effect. *Radiat Res* 155: 387 – 396

Morgan WF, Sowa MB (2007) Non-targeted bystander effects induced by ionizing radiation. *Mutat Res* 616: 159 – 164

Nagasawa H, Little JB (1992) Induction of sister chromatid exchanges by extremely low doses of alpha-particles. *Cancer Res* 52: 6394 – 6396

Narayanan PK, Goodwin EH, Lehnert BE (1997) Alpha particles initiate biological production of superoxide anions and hydrogen peroxide in human cells. *Cancer Res* 57: 3963 – 3971

Narayanan PK, LaRue KE, Goodwin EH, Lehnert BE (1999) Alpha particles induce the production of interleukin-8 by human cells. *Radiat Res* 152: 57 – 63

Nathan C (1992) Nitric oxide as a secretory product of mammalian cells. *FASEB J* 6: 3051 – 3064

Ogawa Y, Nishioka A, Kobayashi T, Kariya S, HamaSato S, Saibara T, Nakayama K, Seguchi H, Yoshida S (2002) Mitochondrial cytochrome c release in radiation-induced apoptosis of human peripheral T cells. *Int J Mol Med* 10: 263 – 268

Ogura A, Oowada S, Kon Y, Hirayama A, Yasui H, Meike N, Kobayashi S, Kuwahara M, Inanami O (2009) Redox regulation in radiation-induced cytochrome c release from mitochondria of human lung carcinoma A549 cells. *Cancer Lett* 277: 64 – 71

Palacios-Callender M, Hollis V, Mitchison M, Frakhich N, Untitt D, Moncada S (2007) Cytochrome c oxidase regulates endogenous nitric oxide availability in respiring cells: a possible explanation for hypoxic vasodilation. *Proc Natl Acad Sci USA* 104: 18508 – 18513

Peixoto PM, Ryu SY, Pruzanski DP, Kuriakose M, Gilmore A, Kinnally KW (2009) Mitochondrial apoptosis is amplified through gap junctions. *Biochim Biophys Acta* 1786: 38 – 43

Riley PA (1994) Free radicals in biology: oxidative stress and the effects of ionizing radiation. *Int J Radiat Biol* 6: 27 – 33

Sawant SG, Randers-Pehrson G, Geard CR, Brenner DJ, Hall EF (2001) The bystander effect in radiation oncogenesis I: Transformation in C3H 10T1/2 cells in vitro can be initiated in the unirradiated neighbors of irradiated cells. *Radiat Res* 155: 397 – 401

Schagger H (2002) Respiratory chain supercomplexes of mitochondria and bacteria. *Biochim Biophys Acta* 1555: 154 – 159

Schettino G, Folkard M, Prise KM, Vojnovic B, Held KD, Michael BD (2003) Low-dose studies of bystander cell killing with targeted soft X rays. *Radiat Res* 160: 505 – 511

Seymour CB, Mothersill C (1997) Delayed expression of lethal mutations and genomic instability in the progeny of human epithelial cells that survived in a bystander-killing environment. *Radiat Oncol Investig* 5: 106 – 110

Shao C, Aoki M, Furusawa Y (2001) Medium-mediated bystander effects on HSG cells co-cultivated with cells irradiated by X-rays or a 290 MeV/u carbon beam. *J Radiat Res (Tokyo)* 42: 305 – 316

Shao C, Aoki M, Furusawa Y (2003a) Bystander effect on cell growth stimulation in neoplastic HSGc cells induced by heavy-ion irradiation. *Radiat Environ Biophys* 42: 183 – 187

Shao C, Aoki M, Furusawa Y (2004) Bystander effect in lymphoma cells vicinal to irradiated neoplastic epithelial cells: nitric oxide is involved. *J Radiat Res (Tokyo)* 45: 97 – 103

Shao C, Folkard M, Held KD, Prise KM (2008a) Estrogen enhanced cell-signalling in breast cancer cells exposed to targeted irradiation. *BMC Cancer* 8: 184

Shao C, Folkard M, Michael BD, Prise KM (2005) Bystander signaling between glioma cells and fibroblasts targeted with counted particles. *Int J Cancer* 116: 45 – 51

Shao C, Folkard M, Prise KM (2008b) Role of TGF-beta1 and nitric oxide in the bystander response of irradiated glioma cells. *Oncogene* 27: 434 – 440

Shao C, Furusawa Y, Aoki M, Matsumoto H, Ando K (2002) Nitric oxide-mediated bystander effect induced by heavy-ions in human salivary gland tumour cells. *Int J Radiat Biol* 78: 837 – 844

Shao C, Furusawa Y, Kobayashi Y, Funayama T, Wada S (2003b) Bystander effect induced by counted high-LET particles in confluent human fibroblasts: a mechanistic study. *FASEB J* 17: 1422 – 1427

Shao C, Lyng FM, Folkard M, Prise KM (2006) Calcium fluxes modulate the radiation-induced bystander responses in targeted glioma and fibroblast cells. *Radiat Res* 167: 479 – 487

Shao C, Prise KM, Folkard M (2008c) Signaling factors for irradiated glioma cells induced bystander responses in fibroblasts. *Mutat Res* 638: 139 – 145

Shao C, Stewart V, Folkard M, Michael BD, Prise KM (2003c) Nitric oxide-mediated signaling in the bystander response of individually targeted glioma cells. *Cancer Res* 63: 8437 – 8442

Stamler JS, Jia L, Eu JP, McMahon TJ, Bonaventura J, Gennert K, Plantadisso CA (1997) Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. *Science* 276: 2034 – 2037

Szabo C, Southan GJ, Thiemermann C (1994) Beneficial effects and improved survival in rodent models of septic shock with S-methylisosothiourea sulfate, a potent and selective inhibitor of inducible nitric oxide synthase. *Proc Natl Acad Sci USA* 91: 12472 – 12476

Torres J, Sharpe MA, Rosquist A, Cooper CE, Wilson MT (2000) Cytochrome c oxidase rapidly metabolises nitric oxide to nitrite. *FEBS Lett* 475: 263 – 266

Yang G, Wu L, Chen S, Zhu L, Huang P, Tong L, Zhao Y, Zhao G, Wang J, Mei T, Xu A, Wang Y (2009) Mitochondrial dysfunction resulting from loss of cytochrome c impairs radiation-induced bystander effect. *Br J Cancer* 100: 1912 – 1916

Yang H, Assad N, Held KD (2005) Medium-mediated intercellular communication is involved in bystander responses of X-ray-irradiated normal human fibroblasts. *Oncogene* 24: 2096 – 2103

This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution-NonCommercial-Share Alike 3.0 Unported License.