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MODULATION OF NF-κB IN RESCUED IRRADIATED CELLS

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Studies by different groups on the rescue effect, where unirradiated bystander cells mitigated the damages in the irradiated cells, since its discovery by the authors' group in 2011 were first reviewed. The properties of the rescue effect were then examined using a novel experimental set-up to physically separate the rescue signals from the bystander signals. The authors' results showed that the rescue effect was mediated through activation of the nuclear factor-κB (NF-κB) response pathway in the irradiated cells, and that the NF-κB activation inhibitor BAY-11-7082 did not affect the activation of this response pathway in the irradiated cells induced by direct irradiation.

INTRODUCTION

Radiation-induced bystander effect (RIBE) has been regarded as one of the most fascinating discoveries in radiobiology in the past decades. RIBE between cells refers to the scenario where unirradiated cells, upon receiving signals from irradiated cells, respond as if they have themselves been irradiated. Nagasawa and Little[1] first made use of the frequency of sister chromatid exchanges in cells to illustrate the presence of RIBE between cells. There have been numerous excellent reviews on RIBE (see e.g. refs.[2–7]). The two most widely accepted mechanisms underlying RIBE are (1) gap junction intercellular communication (GJIC) when there are physical contacts among the cells and (2) transfer of soluble factors from the irradiated cells to the unirradiated cells through the shared medium. Various soluble factors have been proposed to participate in RIBE, including tumour necrosis factor-alpha (TNF-α)[8], transforming growth factor-beta1 (TGF-β1)[9], interleukin-6 (IL-6)[10], interleukin-8 (IL-8)[11], nitric oxide[12–14] and reactive oxygen species (ROS)[15].

In 2011, the authors' group discovered a new phenomenon that was closely related to RIBE[16]. Briefly, the irradiated cells had smaller numbers of p53-binding protein 1 (53BP1) foci and micronucleus (MN) formation, and lower apoptosis levels after co-culturing with unirradiated bystander cells. This phenomenon was referred to as the 'rescue effect'. The effect was observed in both human primary fibroblast (NHLF) and cancer cells (HeLa).

Subsequent to the authors' discovery of the rescue effect, Widel *et al.*[17] also succeeded to observe the rescue effect by showing that the frequencies of MN formation and apoptosis in irradiated human melanoma (Me45) cells co-cultured with normal human dermal fibroblast (NHDF) cells were markedly decreased in comparison with those cultured alone. The authors also revealed that the rescue effect was accompanied by a significant reduction in the ROS level in the irradiated cells, but the mechanisms underlying the modulation of the ROS level remained unknown. More recently, Pereira *et al.*[18] irradiated embryonic zebrafish (ZF4) cells with gamma radiation for 4 h, then partnered them with unirradiated bystander ZF4 cells for 1 h and finally irradiated them with gamma radiation for another 20 h, and observed that these ZF4 cells developed significantly fewer γ-H2AX foci than control cells, which were irradiated for a total of 24 h from the beginning. As such, the authors claimed that they also successfully demonstrated the rescue effect between ZF4 cells. In another study, Desai *et al.*[19] used γ-H2AX foci as the biological endpoint to study the response of co-cultures of lung cancer (A549) cells irradiated with microbeam protons and normal (WI38) cells. The authors observed that the irradiated A549 cells did not affect the bystander WI38 cells, but the bystander WI38 cells had a rescue effect on the irradiated A549 cells, which was independent of GJIC.

Interestingly, the authors' group further confirmed the 'rescue effect' between irradiated and bystander organisms. Choi *et al.*[20] reported data demonstrating that zebrafish embryos irradiated by alpha particles were rescued by partnered unirradiated zebrafish embryos, in terms of a reduction in the number of apoptotic signals at 24 h post-fertilisation. Moreover, it was found that the strength of the rescue effect depended on the number of rescuing bystander unirradiated embryos.

Despite all these confirmations of the rescue effect, the mechanisms underlying the rescue effect are still

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unclear. Most recently, He *et al.*[21] explored the role played by the second messenger cyclic adenosine monophosphate (cAMP) in the rescue effect and the involved mechanisms. The authors first confirmed the presence of rescue effect between co-cultured irradiated human macrophage U937 cells and bystander unirradiated HL-7702 hepatocyte cells, by showing that the frequencies of MN formation in the irradiated cells were decreased when they were co-cultured with the bystander cells. With cell co-culture, the intracellular cAMP level in the irradiated cells increased after irradiation while the level in the bystander cells was reduced; without cell co-culture, the cAMP level in the irradiated cells did not increase. Based on these results, He *et al.*[21] claimed that the rescue effect was mediated by cAMP. The authors further proposed that cAMP was communicated through a membrane signalling pathway, and that cAMP was actually communicated from the bystander cells to the irradiated cells. While cAMP could work by activating the cAMP-dependent protein kinase (or protein kinase A, PKA), the particular function of the PKA in relation to the rescue effect, e.g. the decrease in the apoptosis or MN formation, was not yet identified. It is noted that cAMP binds to and activates PKA, which is normally inactive, to enable it to phosphorylate substrate proteins.

In the present work, the mechanism underlying the rescue effect was further studied. To be compatible with previous findings, it would be desirable if the mechanism could be cAMP-dependent and could modulate the intracellular ROS level in the irradiated cells. Activation of the nuclear factor-κB (NF-κB) response pathway in the irradiated cells by the bystander cells appeared to be a promising candidate. First, expression of NF-κB target genes in general promotes cellular survival, despite a few exceptions where NF-κB contributes to cell death. Second, the cAMP-dependent PKA phosphorylates serine 276 on p65 (a member of NF-κB family) once in the nucleus to increase its affinity for the transcriptional co-activators CBP/p300[22], where CBP is the CREB binding protein and CREB is the cAMP response element binding protein. Third, certain NF-κB-regulated genes play a major role in regulating the intracellular ROS levels[23]. As such, the authors studied the activation of the NF-κB response pathway in the irradiated cells by the bystander cells as a potential mechanism underlying the rescue effect.

MATERIALS AND METHODS

Cell culture

The cell line employed in this study was the human cervical cancer HeLa cells, which were routinely cultured in Dulbecco's modified eagle medium (Gibco, 10569) supplemented with 10 % fetal bovine serum (Gibco, 10270) and 1 % penicillin–streptomycin solution (Gibco, 15140-122), and maintained at 37°C in a humidified 5 % CO_2 incubator.

Alpha-particle irradiation

In the present experiments, alpha-particle irradiation of HeLa cells was always performed in a 'Mylar-film dish' as shown in Figure 1, which was a tissue culture dish with a 100 mm diameter, with a hole of 10 mm diameter drilled at the centre and covered by a Mylar film with a thickness of 3.5 μm glued to the bottom of the dish. The thin Mylar film allowed the alpha particles to pass through and hit the cells without significant energy loss.

Irradiation of the cells served two main purposes in the present investigation. The first one was to prepare irradiated cells on which the effects of different treatments would be studied. The second one was to prepare the medium (hereafter referred to as CM) having conditioned bystander cells partnered with irradiated cells. The deployment of the CM was important for studies on the potential activation pathways in the irradiated cells relevant to the rescue effect through the use of activation inhibitors, because this approach would physically separate the rescue signals from the bystander signals, in such a way that potential ambiguity of whether the activation inhibitors would have stopped the RIBE instead of stopping the rescue effect would be excluded.

A total of 4.5×10^4 cells were plated on the Mylar film and incubated overnight. Alpha-particle irradiation was carried out with an alpha particle dose of 5 cGy, using an ^{241}Am irradiation source (average alpha particle energy = 5.16 MeV, activity = 5.02 μCi, dose rate = 18 cGy min^{-1}).

In the experiments where only irradiated cells were needed and no CM was required, only one Mylar-film dish was used.

On the other hand, the experiments involving CM would be more complicated, which is schematically

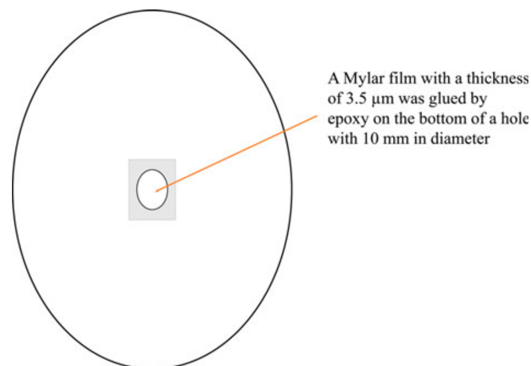

Figure 1. The top view of the Mylar-film dish used in the present work.

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depicted in Figure 2. In this protocol, a number of Mylar-film dishes were involved for different purposes, and these dishes would be referred to using different names described in the following. As shown in Figure 2, CM was prepared by first irradiating HeLa cells in a Mylar-film dish, and at the same time partnering these irradiated cells with bystander cells plated on 18 × 18 mm cover glasses. This irradiation-and-partnering set-up was a modification from the authors' previous set-up[24]. This Mylar-film dish hosting irradiated cells and partnered cells was hereafter referred to as the IR (irradiation) dish. After a specific time, the second step was to transfer the cover glasses into a new regular 100-mm tissue culture dish without a drilled hole and the covering Mylar film (hereafter referred to as the 'CM dish'), from which the CM was harvested and transferred into new Mylar-film dishes (hereafter referred to as the 'recipient dishes') for further experiments.

53BP1 immunofluorescent staining

In the present work, the authors employed the number of 53BP1 foci/cell as the biological endpoint to study the extent of rescue effect in the cells. 53BP1 is a p53-binding protein that binds to the central DNA-binding domain of the tumour suppressor protein p53. 53BP1 is required for the phosphorylation of numerous ataxia-telangiectasia-mutated (ATM) substrates during the double-strand break (DSB) response and is involved in repair and check point signal transduction[25, 26]. 53BP1 was also reported to be required for p53 accumulation in response to ionising radiation[27]. Shortly after irradiation, the cells would develop foci of 53BP1 protein, whose number was closely paralleling the number of DNA DSBs[28]. As such, fluorescent detection of these 53BP1 foci has been widely used as a marker of DNA damages[29–33].

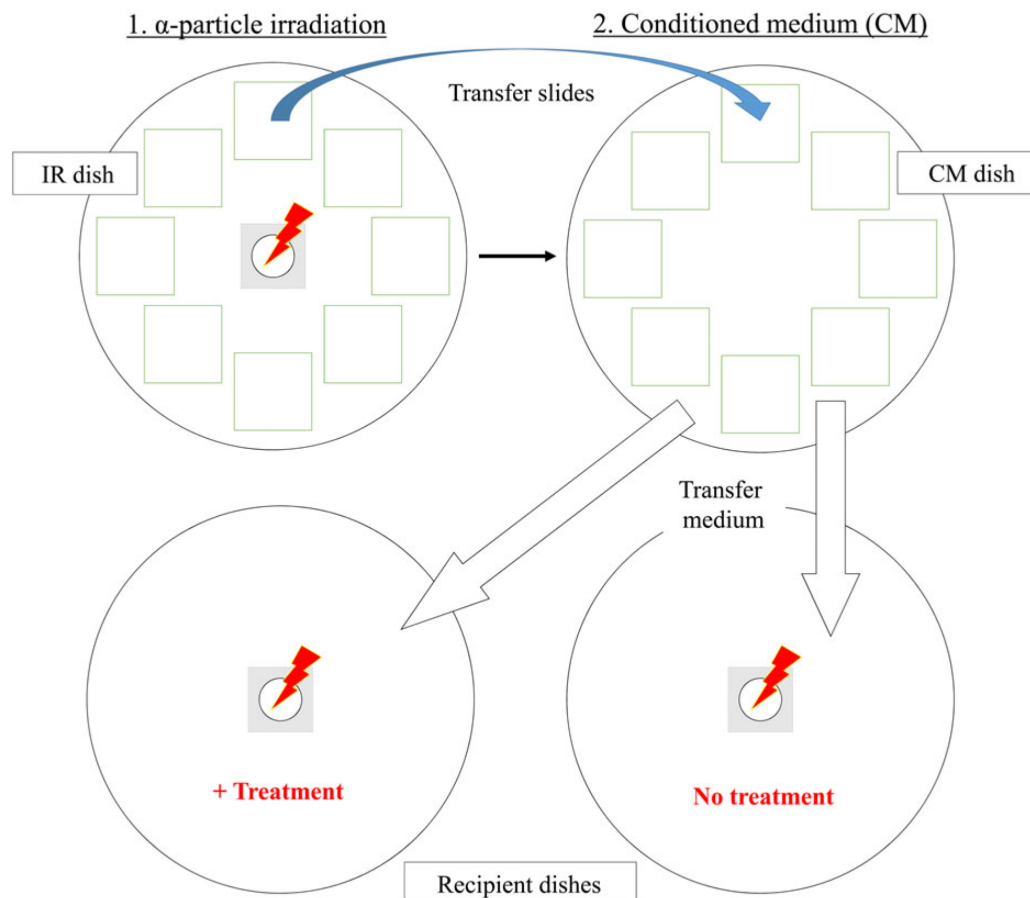

Figure 2. Schematic representation of the procedures. The bystander cells plated on 18 × 18 mm cover glasses were first partnered with the irradiated cells in the 'IR dish'. After a specific time, the cover glasses were transferred into the 'CM dish', from which the conditioned medium (CM) was harvested and transferred into 'recipient dishes' for further experiments.

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Briefly, the cells were fixed in 4 % paraformaldehyde at RT, and washed three times with phosphate-buffered saline (PBS). The cells were permeabilised in 0.5 % Triton X-100 at 37°C, then incubated with 1.5 % goat serum blocking solution and followed by primary anti-53BP1 antibody (Abcam, ab21083) at 37°C. After washing with PBS three times, secondary Alexa Fluor 488 goat anti-rabbit IgG antibody (Invitrogen, A11008) was added. The cells were counterstained with DAPI if needed and 53BP1 foci in the cell nuclei were scored under fluorescence microscopy. It is remarked here, for calculation purposes, that the number of foci would be counted as 10 for those nuclei with >10 foci.

Rescue effect and activation of NF-κB pathway in irradiated cells

In the present work, the rescue effect in HeLa cells induced by alpha-particle irradiation, and activation of the NF-κB pathway in irradiated cells by the bystander cells were studied.

To achieve the authors' objectives, HeLa cells in the recipient dishes as described in Figure 2 were first irradiated with 5 cGy of alpha particles and then subjected to different treatments, including (A) treatment with 15 ml fresh medium (FM); (B) treatment with 15 ml FM + BAY-11-7082 with a final concentration of 5 μM (hereafter referred to as BAY5); (C) treatment with 15 ml of the CM and (D) treatment with 15 ml of (CM + BAY5). After 30 min, the medium in the recipient dishes was replaced by FM and then incubated for a further 11.5 h (i.e. until 12 h post-irradiation) and fixed for the 53BP1 immunofluorescent staining. The authors had monitored the number of 53BP1 foci with time up to 24 h after treatment. The time point of 12 h after treatment was adopted since the fold change of the number of 53BP1 was the largest and statistically significant at 12 h after irradiation.

The NF-κB activation inhibitor BAY-11-7082 (Cayman, 10010266) blocked TNF-α-induced phosphorylation of IκBα with the half maximal inhibitory concentration (IC_{50}) of about 5–10 μM, but did not affect constitutive IκBα phosphorylation[34]. Details for preparing the CM were as follows. HeLa cells on the Mylar film in the IR dish with 15 ml medium were irradiated by alpha particles with a dose of 5 cGy, which were partnered with bystander cells plated on eight cover glasses. At the time of irradiation, both the cells on the Mylar film and the cover glasses reached ~80 % confluence. After co-culturing for 2 h, the cover glasses were transferred to the CM dish with 15 ml FM for another 2 h to prepare the CM.

The presence of rescue effect in HeLa cells induced by alpha-particle irradiation could be confirmed, or otherwise, by comparing the results from treatments (A) and (C). In the presence of rescue effect, the number of 53BP1 foci/nucleus would be significantly decreased with the treatment of CM. On the other hand, the importance of activation of the NF-κB pathway in irradiated cells by the bystander cells in the rescue effect was examined by comparing the results from treatments (C) and (D). In order to delineate the activation of the NF-κB pathway in irradiated cells induced by the bystander cells from that induced by direct irradiation alone of the irradiated cells, the results from treatments (A) and (B) were also compared.

Statistical analysis

All experiments had at least three replicates. The data were analysed to give means and standard deviations (SD). Comparisons between the means for the treated and control groups were made using Students' *t*-test. A *P*-value of 0.05 or smaller corresponds to significant differences between the means for the compared groups.

RESULTS

Representative images for immunofluorescence 53BP1 staining of HeLa cells after the four different treatments (A) to (D) described above are shown in Figure 3. The results are shown in Figure 4, where a total of 400–500 cells in each sample were analysed. For a reference, the numbers of 53BP1 foci/nucleus in the unirradiated cells showed no significant difference to the sham-irradiated cells.

As shown in Figure 4, the number of 53BP1 foci/nucleus was significantly lower after treatment (C) when compared with the treatment (A), which confirmed the presence of rescue effect in HeLa cells induced by alpha-particle irradiation.

Figure 4 also showed insignificant differences between the numbers of 53BP1 foci/nucleus in the irradiated HeLa cells treated (A) with FM and (B) with BAY5 for 30 min. This suggested that BAY-11-7082 did not affect the activation of NF-κB response pathway in the irradiated cells induced by direct irradiation alone. On the other hand, there were significant differences between the numbers of 53BP1 foci/nucleus in irradiated Hela cells treated (C) with CM for 30 min and (D) with (CM + BAY5) for 30 min. As it was shown above that BAY-11-7082 did not affect the activation of NF-κB response pathway in the irradiated cells induced by direct irradiation, the significant differences here proved the presence of a rescue signal in the CM, which could activate the NF-κB response pathway in the irradiated cells. The authors' results suggested that inactivation of NF-κB response pathway in the irradiated cells could abrogate the rescue effect on the irradiated cells.

DISCUSSION

The present results regarding the NF-κB activation inhibitor

BAY-11-7082 first showed that it did not affect the activation of the NF-κB response pathway in the

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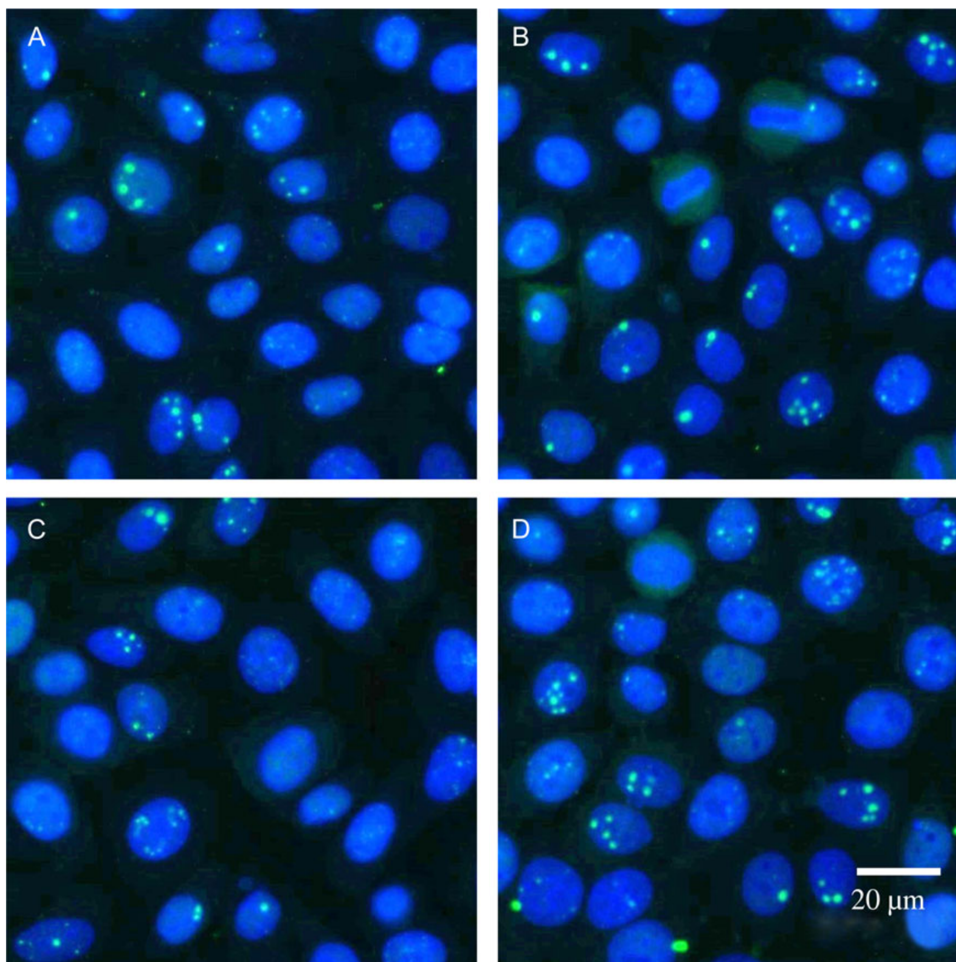

Figure 3. Representative images for immunofluorescence 53BP1 staining results of HeLa cells after first irradiated with 5 cGy of alpha particles and then subjected to different treatments for 30 min: (**A**) treatment with FM; (**B**) treatment with BAY5; (**C**) treatment with CM and (**D**) treatment with (CM + BAY5), after which all media were replaced with FM. At 12 h post-treatment, the irradiated cells were fixed for the 53BP1 immunofluorescent staining.

irradiated cells induced by direct irradiation alone, and then provided evidence that inactivation of the NF-κB response pathway in the irradiated cells could abrogate the rescue effect from the bystander cells. These proved the presence of a rescue signal in the CM, and that the rescue effect (in terms of mitigating the 53BP1 foci) from the bystander cells was mediated through activation of the NF-κB response pathway in the irradiated cells.

The NF-κB activation in the irradiated cells was verified using a different experimental set-up to examine the phosphorylated NF-κB (p-NF-κB) expression in the irradiated cells through the anti-NF-κB p65 (phospho S536) antibody (Abcam, ab86299). The intensity of p-NF-κB expression in irradiated HeLa cells (with an alpha-particle dose of 5 cGy) in the presence of bystander HeLa cells (39 times more than the irradiated cells) was increased by 5.5 % when compared with that in the absence of bystander HeLa cells, and was increased by 18.2 % when compared with that in sham-irradiated cells (unpublished data).

NF-κB is a family of transcription factors with five members comprising RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2), from which various homo- or hetero-dimers are formed[35]. A prototypical example is the heterodimer with p65 and p50. While both p65 and p50 contribute to DNA binding, only p65 possesses transactivation domains which can initiate transcription. For most cell types, while in the inactivated state, these dimers are

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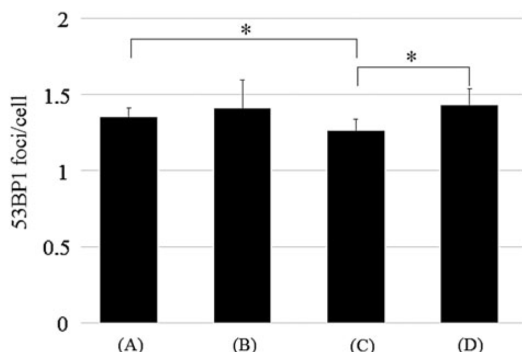

Figure 4. Effects of different treatments on HeLa cells, which had been first irradiated with 5 cGy of alpha particles in terms of the number of 53BP1 foci/cell in nuclei at 12 h post-treatment. Treatments (for 30 min): (**A**) treatment with FM; (**B**) treatment with BAY5; (**C**) treatment with CM and (**D**) treatment with (CM + BAY5). After 30 min, all media were replaced with FM. At 12 h post-treatment, the irradiated cells were fixed for the 53BP1 immunofluorescent staining. Experiments were repeated at least three times. $*P < 0.05$, and bars represent $\pm$SD.

localised to the cytoplasm by the inhibitory IκB-family proteins such as IκBα, IκBβ and IκBε. A variety of extracellular signals can activate NF-κB through first activating the multiprotein IκB-kinase (IKK) complex, which phosphorylates IκB leading to its ubiquitination and then rapid degradation by the proteasome, to free up NF-κB. The activated NF-κB is then translocated into the nucleus to bind to specific sequences of DNA called response elements. The DNA/NF-κB complex then recruits other proteins such as co-activators and RNA polymerase to perform the transcription.

The rescue effect would depend on cellular survival promoted by expression of NF-κB target genes. The anti-apoptotic proteins regulated by NF-κB were reviewed by Magné *et al.*[36], including (i) inhibitors of apoptosis protein (IAP) family, c-IAP-1 and c-IAP-2, which directly blocked caspase functions or indirectly induced their ubiquitination and proteasome-dependent degradation[37], (ii) TRAF (tumour necrosis factor receptor (TNFR)-associated factor) proteins, which amplified NF-κB activation and interfered with the caspase cascade at the TNFR1 receptor[38], (iii) A20 zinc finger protein, which perturbed caspase activation at the TNF receptor[39], (iv) c-FLIP (Cellular FLICE (FADD-like IL-1β-converting enzyme)-like inhibitory protein), which competed with and blocked caspase 8 activation[40] and (v) anti-apoptotic proteins of the bcl-2 family, which prevented cytochrome c release from the mitochondria and the subsequent caspase 9 activation.

The results reported in the present article were the first to demonstrate that the rescue effect was mediated through activation of the NF-κB response pathway in the irradiated cells by the bystander cells. The rescue effect is still a relatively new phenomenon[41], and a lot more research work will be necessary before the authors can have a better comprehension of the phenomenon.

In the present work, the study was restricted to only one cell line (HeLa cells), one irradiation dose (5 cGy) and one type of radiation (alpha particles). It would be pertinent to confirm the presence or absence of the rescue effect in different cell lines, under different irradiation doses and using different types of radiation (in terms of linear energy transfer), and to explore whether the rescue effect is similarly mediated through the activation of the NF-κB response pathway in the irradiated cells. The effects of the relative abundance between the bystander cells and the irradiated cells on the rescue effect would also be interesting. In particular, the strength of the rescue effect between irradiated and bystander zebrafish embryos depended on the number of rescuing bystander unirradiated embryos[20].

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