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Radiation induces apoptosis primarily through the intrinsic pathway in mammalian cells

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ABSTRACT

Radiation-induced tumor cells death is the theoretical basis of tumor radiotherapy. Death signaling disorder is the most important factor for radioresistance. However, the signaling pathway(s) leading to radiation-triggered cell death is (are) still not completely known. To better understand the cell death signaling induced by radiation, the immortalized mouse embryonic fibroblast (MEF) deficient in "initiator" caspases, "effector" caspases or different Bcl-2 family proteins together with human colon carcinoma cell HCT116 were used. Our data indicated that radiation selectively induced the activation of caspase-9 and caspase-3/7 but not caspase-8 by triggering mitochondrial outer membrane permeabilization (MOMP). Importantly, the role of radiation in MOMP is independent of the activation of both "initiator" and "effector" caspases. Furthermore, both proapoptotic and antiapoptotic Bcl-2 family proteins were involved in radiation-induced apoptotic signaling. Overall, our study indicated that radiation specifically triggered the intrinsic apoptotic signaling pathway through Bcl-2 family protein-dependent mitochondrial permeabilization, which indicates targeting mitochondria is a promising strategy for cancer radiotherapy.

1. Introduction

Radiation treatment has a critical role in cancer therapy. Given its vital role in cell death initiation, exploring the detailed molecular mechanisms of the death signal mediated by radiation is generally considered the key part of radiotherapy research. Apoptosis is thought to be the major mechanism of radiation-induced cell death and the disorder of apoptosis signaling will cause radiotherapy resistance. Apoptosis occurs *via* two pathways: the extrinsic pathway initiated by extracellular receptors and the intrinsic pathway activated by mitochondrial outer membrane permeabilization (MOMP). Although the molecular mechanism of cell death induced by radiation has been widely explored [1,2], it is still unclear which apoptotic signaling pathway is the main form as both the extrinsic pathway [3,4] and the intrinsic pathway [5,6] were reported to be responsible for radiation-induced apoptosis.

Mitochondria regulate caspase activation and cell death through an event termed MOMP; this leads to the release of various mitochondrial intermembrane space proteins that activate caspases, resulting in apoptosis. During apoptosis, the caspases are activated by the intrinsic or extrinsic pathway to break the cell into small membrane-bound structures. The caspase family proteins are categorized into two major groups: the "initiator" caspases and the "effector" caspases [7], and both of these groups were observed to be activated after radiation [3,8]. Serving as a marker for radiation-triggered apoptosis, activation of the effector caspase-3/7 has been detected in various types of cells. For instance, cleaved caspase-3/7 has been detected in neural precursor cells [9], murine breast cancer [10] and human lymphocytes [11] after radiation, and unavoidable apoptotic signaling subsequently occurred. Caspase-8 and caspase-9 are two "initiator" caspases, which are initiating the extrinsic and intrinsic pathways, respectively. Activation of caspase-8 and caspase-9, has also been reported after radiation. Radiation induces apoptosis in radiosensitive THP-1 cells through caspase-8-mediated pathways, and impaired caspase-8 make it resistant to

Abbreviations: MOMP, mitochondrial outer membrane permeabilization; $\Delta\psi_{mito}$, depolarization of mitochondrial membrane potential

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ionizing radiation [4]. Reducing caspase-9 expression or inhibiting caspase-9 activity is able to block depolarization of $\Delta\psi_{mito}$ [12–15]. These studies suggest that both the extrinsic pathway (activated at the plasma membrane) and the intrinsic pathway (activated in the mitochondria) might be initiated during radiation-induced apoptosis.

The Bcl-2 family is a cluster of proteins regulating cell death signaling on the out member of mitochondria. They can be divided into three groups: the prosurvival proteins (such as Bcl-xL and Mcl-1), the proapoptotic effector proteins Bak and Bax and the BH3-only proteins (such as Puma). The activation of Bak and Bax leads to MOMP that results in the release of cytochrome *c* from the mitochondrial intermembrane space into the cytoplasm. Studies have already demonstrated the essential role of Bak and Bax in radiation-induced cell death [16]. Although Bak and Bax are the two key proteins regulating MOMP, MEFs deficient in either Bax or Bak alone display intact apoptosis. This functional overlap has also been reported in various stress-induced forms of apoptosis [17,18]. During MOMP, Bak and Bax are activated by BH3-only proteins and inhibited by prosurvival Bcl-2 family proteins. The BH3-only protein Puma is one of the major mediators of this process, binding directly to Bak and Bax to induce their homo-oligomerization [19]. The antiapoptotic Bcl-2 family proteins Bcl-xL and Mcl-1 reside in the outer mitochondrial membrane and the mitochondrial matrix [20]. Reducing Bcl-xL and Mcl-1 levels dramatically induces cell death at the basal level [21,22], while overexpression of Bcl-xL and Mcl-1 has been shown to increase resistance to various apoptotic stimuli, especially radiation [21,23]. Moreover, a previous study demonstrated that radiation can upregulate Bcl-xL and Mcl-1 expression [24,25]; however, more evidence of radiation-induced decreases in Bcl-xL and Mcl-1 expression has emerged [25,26].

Although radiation-triggered events commonly linked to apoptosis have been reported, the detailed mechanism of radiation-induced apoptotic signaling remain unclear. In particular, how the extrinsic/intrinsic signaling pathway mediating radiation-induced apoptosis is not well understood. Therefore, a comprehensive and systematic investigation is necessary to clarify the mechanism of death signaling due to radiation. In this paper, MEF deficient in one or more caspases and different Bcl-2 family proteins together with HCT116 cells were investigated to elucidate the cell death signaling triggered by radiation. Our data indicate that radiation induced MOMP and activated caspase-3/7 through Bcl-2 family protein regulation and provide direct evidence that radiation specifically initiates the intrinsic apoptotic signaling pathway.

2. Results

2.1. Apoptosis is the major form of cell death induced by radiation

Apoptosis is a mode of cell death that is currently of intense research interest in radiation cell biology. Cell death is an important process in which several irreversible signaling cascade reactions can be activated. Caspase-3 and caspase-7 are two highly related "effector" caspases, and MEFs deficient in the expression of both caspases are markedly resistant to both the intrinsic and extrinsic apoptosis pathways. To explore the cell death signaling induced by radiation, we first investigated whether caspase-3 and caspase-7 were essential to mediate the cytotoxic effects of X-ray treatment. Radiation-induced cell death was examined in MEFs lacking caspase-3 (caspase-3-KO), caspase-7 (caspase-7-KO), or both (caspase-3/7-DKO) as well as their wild-type (WT) counterparts (Fig. 1A). Radiation induced significant cell death in WT, caspase-3-KO and caspase-7-KO MEFs, whereas caspase-3/7-DKO MEFs were significantly resistant to radiation exposure (Fig. 1B). Moreover, cell death was much more in the caspase-7 knockout than in the caspase-3 knockout, indicating that caspase-3 plays a more important role in radiation-induced cell death than caspase-7 (Fig. 1B). The essential roles of caspase-3 and caspase-7 indicate that cell death induced by 5–15 Gy X-ray radiation is largely attributable to apoptosis.

PARP is a known substrate of caspase-3 and caspase-7, which triggers apoptotic processes. To further demonstrate the role of apoptosis in radiation-induced death signaling, the cleavage of PARP was detected. As shown in Fig. 1C–D, PARP cleavage was found in WT, caspase-3-KO and caspase-7-KO MEFs but not in their caspase-3/7-DKO counterparts upon radiation treatment, further validating that apoptosis is the major form of cell death induced by 5–15 Gy X-ray radiation.

2.2. Radiation-induced MOMP is independent of caspase-3 and caspase-7

MOMP has been considered a "one-way ticket" in both the intrinsic and extrinsic apoptotic pathways [27,28]. To further explore radiation-initiated apoptotic signaling, we studied the involvement of caspase-3 and caspase-7 in two key events of MOMP: depolarization of mitochondrial membrane potential ($\Delta\psi_{mito}$) and release of cytochrome *c* from mitochondria into the cytosol. Depolarization of $\Delta\psi_{mito}$ was detected by JC-1. After radiation, mitochondrial potential was decreased in WT, caspase-3-KO, caspase-7-KO, and caspase-3/7-DKO MEFs, as evidenced by increased green fluorescence (Fig. 2A). To further quantify the decrease in mitochondrial potential, the fluorescence intensity of the red and green forms of JC-1 was determined by a biochemical approach [29]. Fig. 2B shows that radiation caused an equivalent decrease in mitochondrial potential among all the MEFs. These results indicate that radiation-induced MOMP occurs upstream of "effector" caspase activation.

To further study the sequential order of MOMP and caspase-3/7, the release of cytochrome *c* in the cytosol and mitochondria of MEFs was determined after radiation. As shown in Fig. 2C–D, cytochrome *c* was released from the mitochondria into the cytosolic fraction in all four MEFs, further confirming that MOMP occurs upstream of "effector" caspase activation after radiation. Overall, these results provide more evidence that radiation triggers MOMP independent of caspase-3 and caspase-7 activation.

2.3. Radiation-induced apoptosis is independent of caspase-8

Caspase-8 initiates the extrinsic pathway in response to extracellular apoptosis-inducing ligands. And caspase-9 activates the intrinsic pathway in response to insults that trigger the release of cytochrome *c* from mitochondria. To systematically investigate which apoptotic pathway is involved, we first examined MEFs deficient in caspase-8 and their wild-type counterparts (Fig. 3A). Upon treatment with X-ray, no significant changes in cell death (Fig. 3B) and caspase-3/7 activation (Fig. 3C) were observed between WT and caspase-8-KO MEFs. Additionally, X-ray treatment triggered the release of cytochrome *c* and the cleavage of caspase-3 and PARP in both WT and caspase-8-KO MEFs (Fig. 3D–E). To further investigate the role of caspase-8 in radiation-initiated apoptotic signaling, we studied the involvement of caspase-8 in cytochrome *c* release and depolarization of $\Delta\psi_{mito}$ induced by radiation. Consistent with its effects on cell viability and caspase-3 activation, deficiency in caspase-8 expression did not affect the ability of radiation to induce cytochrome *c* release and depolarization of $\Delta\psi_{mito}$ (Fig. 3F–I). These data indicate that the caspase-8-mediated extrinsic pathway does not play a pivotal role in radiation-induced apoptosis.

2.4. Radiation exclusively triggered the mitochondria-dependent intrinsic apoptosis pathway

Next, we investigated whether the "initiator" caspase-9 was responsible for radiation-induced apoptosis. For this purpose, caspase-9-KO and wild-type MEFs were used (Fig. 4A). Flow cytometry analysis of cell viability showed that radiation induced more cell death in wild-type than in caspase-9-KO MEFs (Fig. 4B). Furthermore, radiation failed to evoke any caspase-3/7 activation or PARP activation in MEFs lacking caspase-9 expression (Fig. 4C–E). Importantly, Western blot results also showed that no caspase-3 activation was detected in caspase-9-KO

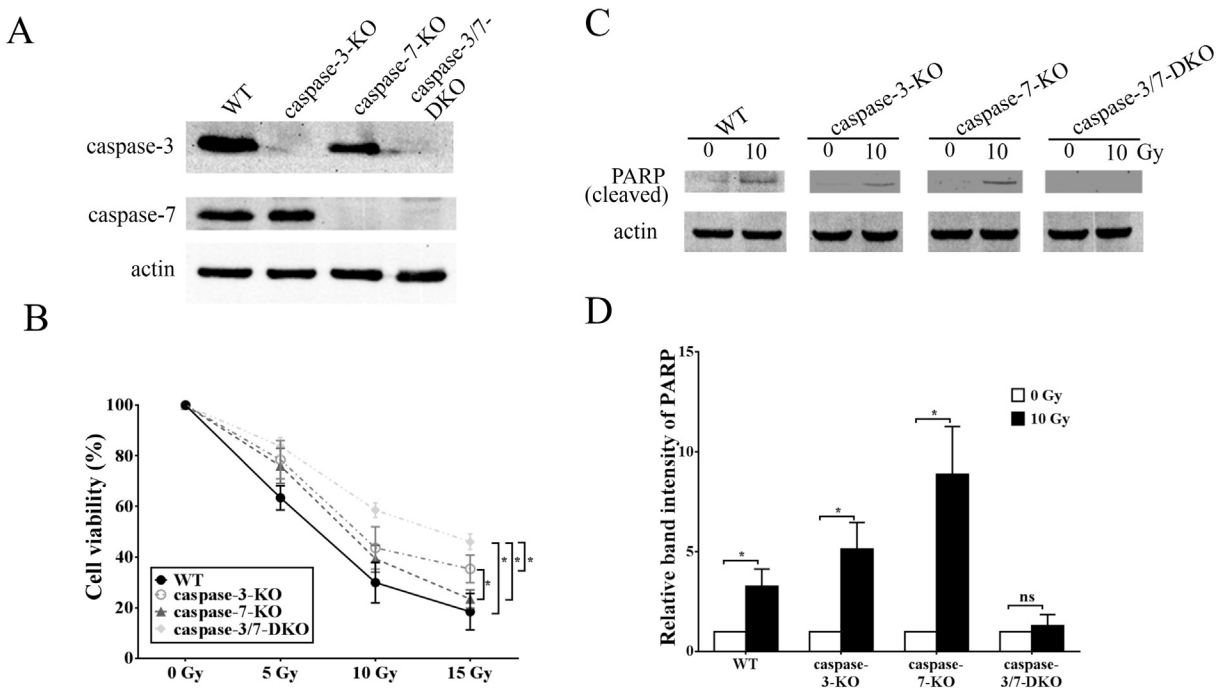

Fig. 1. Caspase-3 and caspase-7 are essential for radiation-induced cell death. (A) Caspase-3 and caspase-7 were stably knocked out in MEFs, and the expression levels of caspase-3 and caspase-7 were determined by Western blot. (B) MEFs were cultured for 24 h after the indicated radiation doses, and cell viability was measured by propidium iodide exclusion test. (C) Cleavage of PARP was determined by Western blot from MEF whole-cell extracts 24 h after radiation. (D) The intensities of PARP in MEFs (C) were quantified after normalization to actin using ImageJ software (NIH). All of the data are mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate $p < 0.05$ (*); ns, not significant; Student's unpaired *t*-test.

MEFs after radiation (Fig. 4D). These collaborative data suggest that X-ray exposure induces apoptotic signaling largely through activating the mitochondria-dependent intrinsic apoptotic pathway.

2.5. Radiation-induced MOMP is independent of caspase-9

To illuminate the roles of caspase-9 in MOMP mediated by radiation, we performed the JC-1 test to measure $\Delta\psi_{mito}$ in WT and caspase-9-KO MEFs. In contrast to its effects on cell viability and caspase-3/7 activation, deficiency in caspase-9 expression did not affect the ability of radiation to induce depolarization of $\Delta\psi_{mito}$, as green fluorescence predominated after radiation (Fig. 4F). Quantitative microplate experiments also confirmed that knocking out caspase-9 did not affect the decrease of mitochondrial potential induced by raditaion. (Fig. 4G), indicating that caspase-9 was not involved in radiation-induced MOMP. To further validate this assumption, we performed cytochrome *c* redistribution in the mitochondrial and cytosolic fractions upon radiation treatment by Western blot. As shown in Fig. 4H–I, X-ray treatment evoked the release of cytochrome *c* from mitochondria to the cytosol regardless of caspase-9 expression, indicating that caspase-9 was downstream of mitochondrial depolarization. Overall, these data have demonstrated that radiation causes acute MOMP independently of any "initiator" caspase. Taken together, our data suggest that radiation evokes the intrinsic apoptotic pathway, in which MOMP is independent of "initiator" and "effector" caspases.

2.6. Proapoptotic Bcl-2 family proteins are involved in radiation-induced apoptosis

The Bcl-2 family proteins exist in dynamic equilibrium, such that increasing the proapoptotic or decreasing the antiapoptotic proteins would induce Bak/Bax-dependent cytochrome *c* release. To further characterize radiation-induced apoptotic signaling, we then investigated the role of Bak and Bax after radiation treatment. Experiments were performed in $Bak^{-/-}Bax^{-/-}$ MEF cells, it's stably reexpressed only Bak or Bax, as well as their wild-type counterparts (Fig. 5A). As shown in Fig. 5B–C, the lowest level of caspase-3/7 activation and the highest level of cell viability were observed in $Bak^{-/-}Bax^{-/-}$ MEFs after X-ray radiation, indicating that Bak and Bax were crucial in radiation-induced apoptosis. Furthermore, Bak and Bax appear to function redundantly, as similar levels of cell viability and caspase-3/7 activation were found between the Bak and Bax re-expressing groups after radiation. Consistent with the cell viability data, the Western results also showed that cytochrome *c* release, cleavage of caspase-3 and PARP were found in WT MEFs and $Bak^{-/-}Bax^{-/-}$ MEFs re-expressing only Bak or Bax, but not in $Bak^{-/-}Bax^{-/-}$ MEFs upon radiation treatment (Fig. 5D–G), further demonstrating that Bak and Bax are involved in apoptotic signaling after radiation.

Next, to further explore radiation-initiated apoptotic signaling, we studied the involvement of Bak and Bax in mitochondrial potential depolarization. Due to the presence of green fluorescent protein in the $Bak^{-/-}Bax^{-/-}$ MEFs reexpressing either Bak or Bax, HCT116 cells deficient in the expression of Bak (Bak-KO), Bax (Bax-KO), or both ($Bak^{-/-}Bax^{-/-}$), as well as their WT counterparts, were used (Fig. 6A). As shown in Fig. 6B–E, dramatic mitochondrial potential depolarization and cytochrome *c* release was observed in WT, Bak-KO and Bax-KO cells, but less mitochondrial potential depolarization and cytochrome *c* release was observed in $Bak^{-/-}Bax^{-/-}$ HCT116 cells. Overall, these results provide strong evidence that radiation-induced apoptosis depends on Bak and Bax to activate MOMP.

2.7. The role of Bcl-2 family proteins in radiation-induced apoptosis

We then examined whether antiapoptotic Bcl-2 family proteins were able to alter radiation-induced cell death. Mcl-1, Bcl-xL knockout MEF cells and Puma knockout HCT116 cells were used (Fig. 7A). Mcl-1 and Bcl-xL are antiapoptotic Bcl-2 family proteins, which are thought to be responsible for inhibiting the activation of apoptotic signaling. As shown in Fig. 7B, losing the expression of Mcl-1 and Bcl-xL remarkably decreased the cell viability induced by radiation. In contrast, because

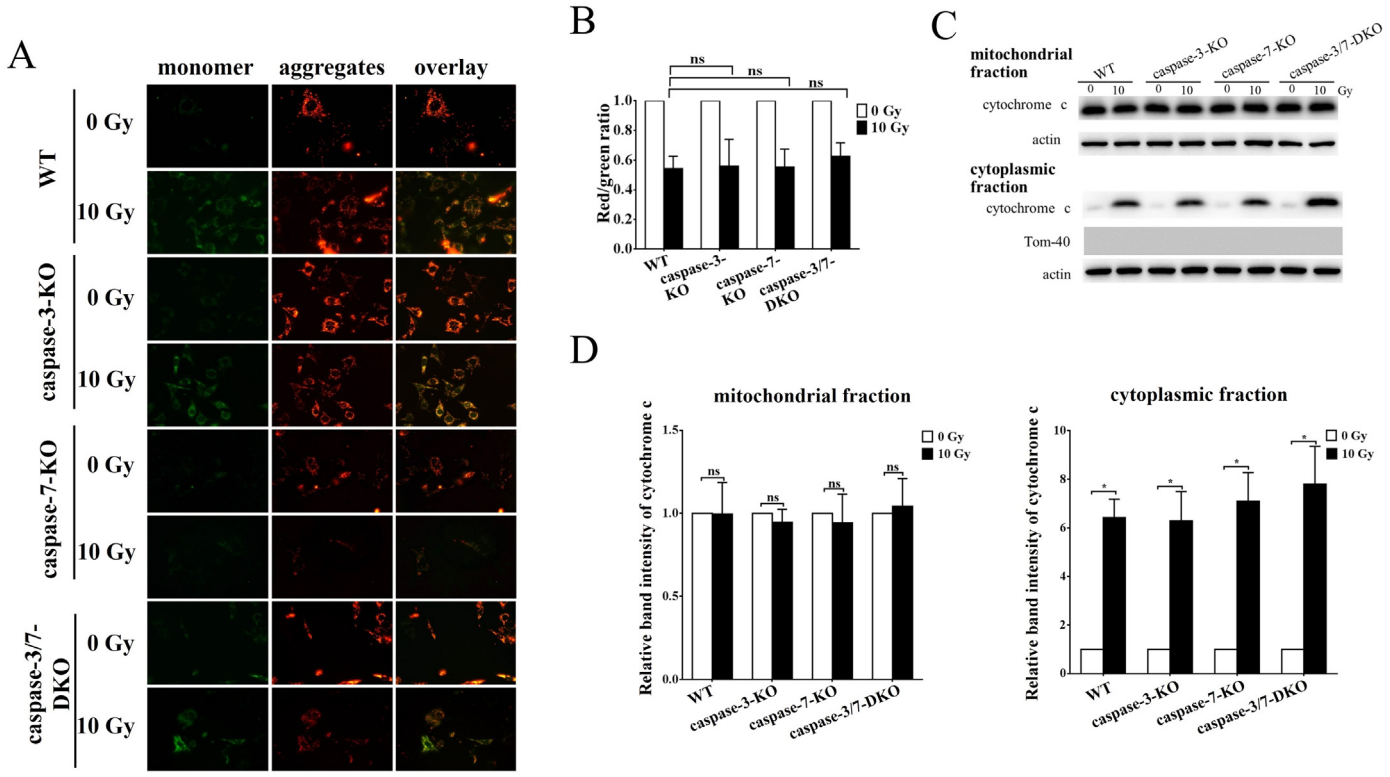

Fig. 2. Caspase-3 and caspase-7 are not required for mitochondrial depolarization induced by radiation. (A) MEFs were treated with X-ray, and 24 h later JC-1 were used to observe fluorescence images of MEFs. (B) MEFs treated with either 0 or 10 Gy for X-ray radiation, and loaded with JC-1 24 h later. The red (aggregates) and green (monomers) fluorescence were determined by microplate reader. (C) The cytosolic fractions and mitochondrial fractions were acquired, and representative Western blot images of the isolated fractions are shown. The absence of Tom-40 in the cytosolic fractions under all conditions indicated that the cytosolic fractions were free of mitochondria. (D) The intensities of cytochrome *c* in mitochondrial and cytoplasmic fraction (C) were quantified after normalization to actin using ImageJ software (NIH). All of the data are mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate $p < 0.05$ (*); ns, not significant; Student's unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the BH3-only Bcl-2 protein Puma possesses a proapoptotic function, knocking out Puma significantly promotes the cell viability after radiation treatment (Fig. 7C). In summary, these results reveal the important role of Bcl-2 family proteins in radiation-induced cell death, suggesting that the intrinsic apoptotic pathway is the main signaling pathway in radiation-induced cell death (Fig. 7D).

3. Discussion

Although the mechanism in radiation-induced cell death have been widely studied [1,2], the detailed signaling pathway has not been fully elucidated. It is still unknown which apoptotic signaling is the main form of radiation-induced apoptosis as both the extrinsic pathway and the intrinsic pathway were reported previously [3–6]. Furthermore, previous studies largely focus on investigating the involvement of one or two important apoptotic regulators, particularly caspases, in radiation-induced apoptotic signaling [30–34]. More often than not, these studies reveal some aspects of apoptosis evoked by radiation, but not all of it. As far as we know, our study is the first one thoroughly examining apoptosis pathways involved in radiation using MEF cells with or without expression of major “initiator” caspases, “effector” caspases or various Bcl-2 family proteins. Furthermore, some of the conclusions drawn from MEF cells were validated by the studies with human colon carcinoma HCT116 cells. Our systematical investigation provides comprehensive information about X-ray-induced apoptosis signaling. Our results elucidated that caspase-3/7 and caspase-9, but not caspase-8, are necessary for radiation-induced apoptosis in MEFs, indicating that the radiation-induced pathway is the mitochondria-dependent intrinsic pathway.

Caspases are a family of endopeptidases that play a key role in the controlled initiation, execution, and regulation of apoptosis [35]. Caspase-8 and 9 are two apical components of cell death pathways controlling the extrinsic and mitochondria-dependent intrinsic pathways, respectively. Emerging evidence suggests that both caspase-8 and caspase-9 are activated and play a major role in apoptotic signaling after radiation [3,8,36]. For example, using caspase-8 siRNA, caspase-8-mediated cell death was partially blocked after radiation [3]. Nevertheless, caspase-9 null embryonic stem cells and embryonic fibroblasts are insensitive to the apoptotic stress induced by UV and γ-irradiation [37]. In this study, using caspase knockout MEFs, the evidence that caspase-9, but not caspase-8, is essential for radiation-induced apoptotic signaling indicates that radiation selectively triggers the mitochondria-dependent intrinsic apoptotic pathway in MEFs (Figs. 3B–E & 4B–E). Therefore, events in which the extrinsic pathways are activated after radiation treatment [3,4] are likely secondary responses to MOMP. In mammalian cells, MOMP activates the downstream protein caspase-9 during most intrinsic apoptosis. However, some evidence suggests that caspase-9 activation also play a role in MOMP during several apoptosis paradigms. For example, inhibiting caspase-9 activity is able to block depolarization of membrane potential [12–15]. Guerrero et al. found caspase-9-induced signaling cascade cleaves anti-apoptotic proteins Bcl-2 and Bcl-xL, leading to amplification of mitochondrial disruption [12]. Hence, to clarify the roles of caspase-9 in MOMP mediated by radiation, membrane potential was measured in WT and caspase-9-KO MEFs. As shown in Fig. 4F–I, mitochondrial potential depolarization and cytochrome *c* release were detected in caspase-9 knockout MEFs after radiation, indicating that MOMP occurs upstream of caspase-9.

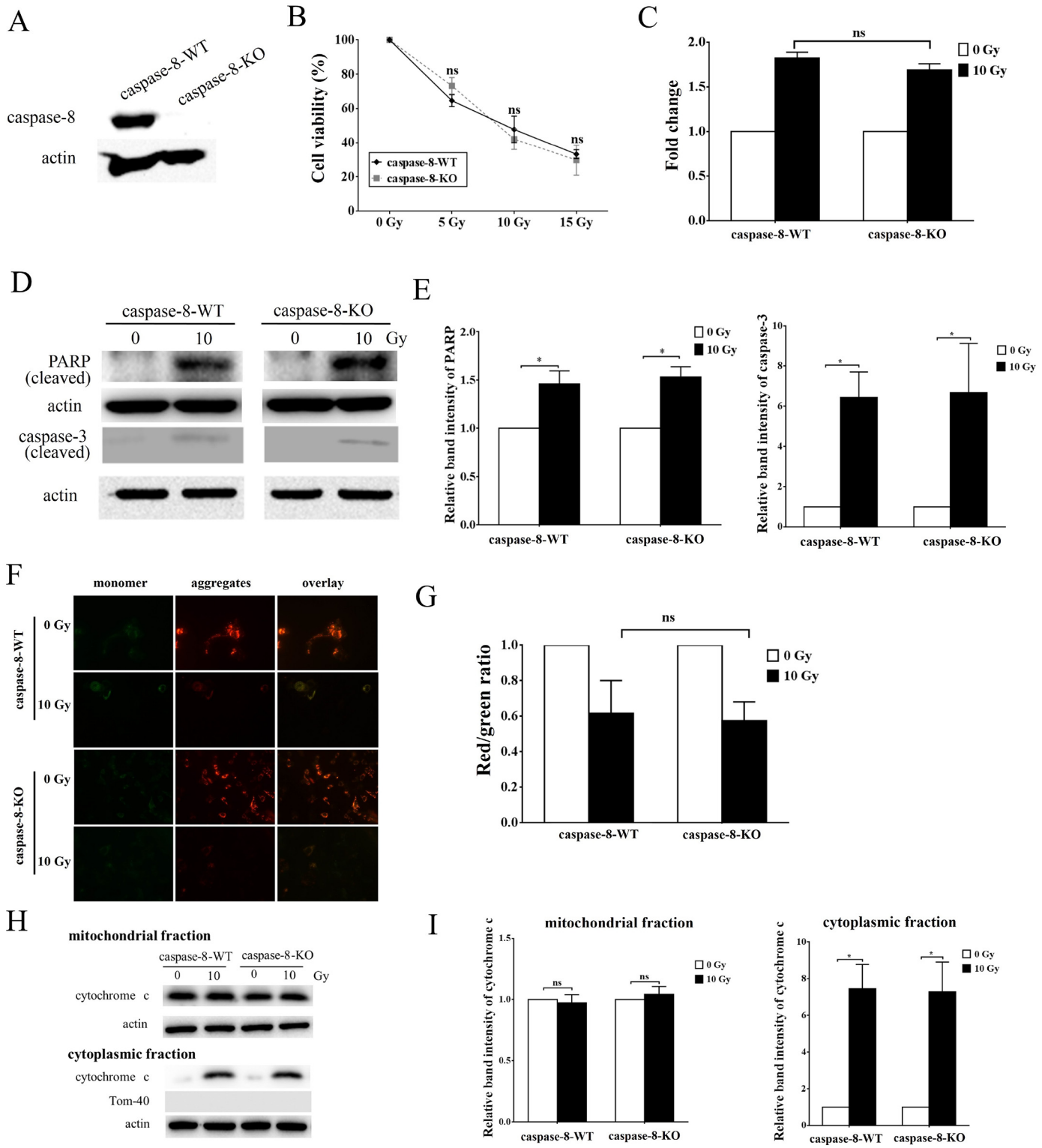

Fig. 3. Caspase-8 is not essential for radiation-induced cell death. (A) The expression levels of caspase-8 were determined by Western blot analysis in WT and caspase-8-KO MEFs. (B) MEFs were cultured for 24 h after the indicated radiation doses, and cell viability was measured by propidium iodide exclusion. (C) MEFs were plated in 96-well plates, caspase-3/7 activity was measured 24 h after radiation, and the levels were normalized to the control sample. (D) Cleavage of caspase-3 and PARP was determined by Western blot from MEF whole-cell extracts 24 h after radiation. (E) The intensities of PARP and caspase-3 in MEFs (D) were quantified after normalization to actin using ImageJ software (NIH). (F) Fluorescence images of MEFs were observed by JC-1 staining after radiation. (G) MEFs treated with either 0 or 10 Gy radiation, and loaded with JC-1 24 h later. The red and green forms were determined by a microplate reader. (H) MEFs were treated with radiation and recultured for 24 h. The cytosolic fractions and mitochondrial fractions were acquired, and representative Western blot images of isolated fractions are shown. (I) The intensities of cytochrome *c* in mitochondrial and cytoplasmic fraction (H) were quantified after normalization to actin using ImageJ software (NIH). All of the data are mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate $p < 0.05$ (*); ns, not significant; Student's unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

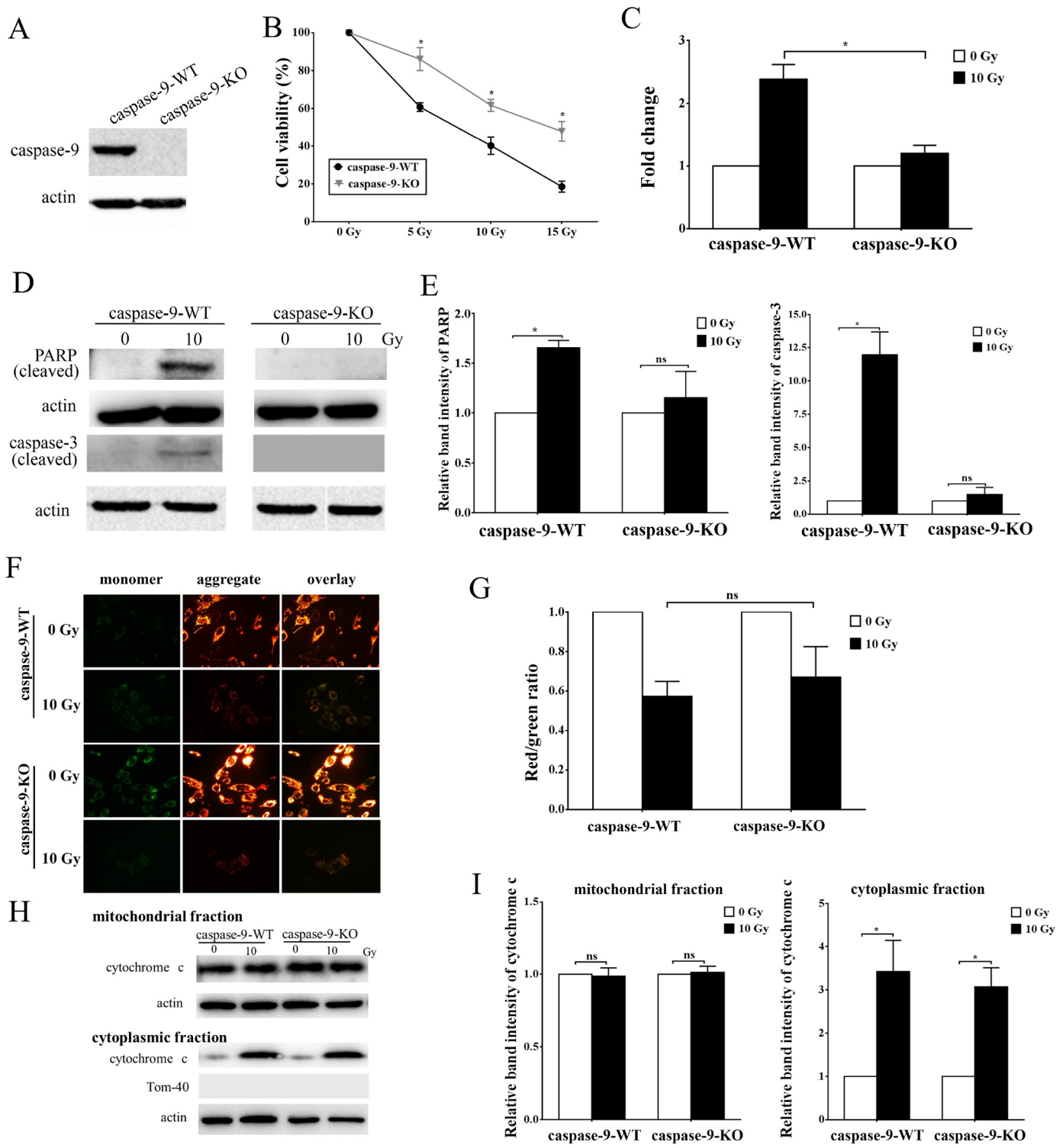

Fig. 4. Radiation induced caspase-9 activation occurs downstream of MOMP. (A) The expression levels of caspase-9 were determined by Western blot analysis in WT and caspase-9-KO MEFs. (B) MEFs were cultured for 24 h after the indicated radiation doses, and cell viability was measured by propidium iodide exclusion. (C) MEFs were plated in a 96-well plate, caspase-3/7 activity was measured 24 h after radiation, and the levels were normalized to the control sample. (D) Cleavage of caspase-3 and PARP was determined by Western blot from MEF whole-cell extracts 24 h after radiation. (E) The intensities of PARP and caspase-3 in MEFs (D) were quantified after normalization to actin using ImageJ software (NIH). (F) Fluorescence images of MEFs were used to observe JC-1 staining after radiation. (G) MEFs treated with either 0 or 10 Gy radiation, and loaded with JC-1 24 h later. The red and green forms were determined by microplate reader. (H) MEFs were treated with radiation and recultured for 24 h. The cytosolic fractions and mitochondrial fractions were acquired, and representative Western blot images of isolated fractions are shown. The isolated cytosolic fractions were not contaminated with mitochondria, as shown by the absence of Tom-40. (I) The intensities of cytochrome *c* in mitochondrial and cytoplasmic fraction (H) were quantified after normalization to actin using ImageJ software (NIH). All of the data are mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate $p < 0.05$ (*); ns, not significant; Student's unpaired *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

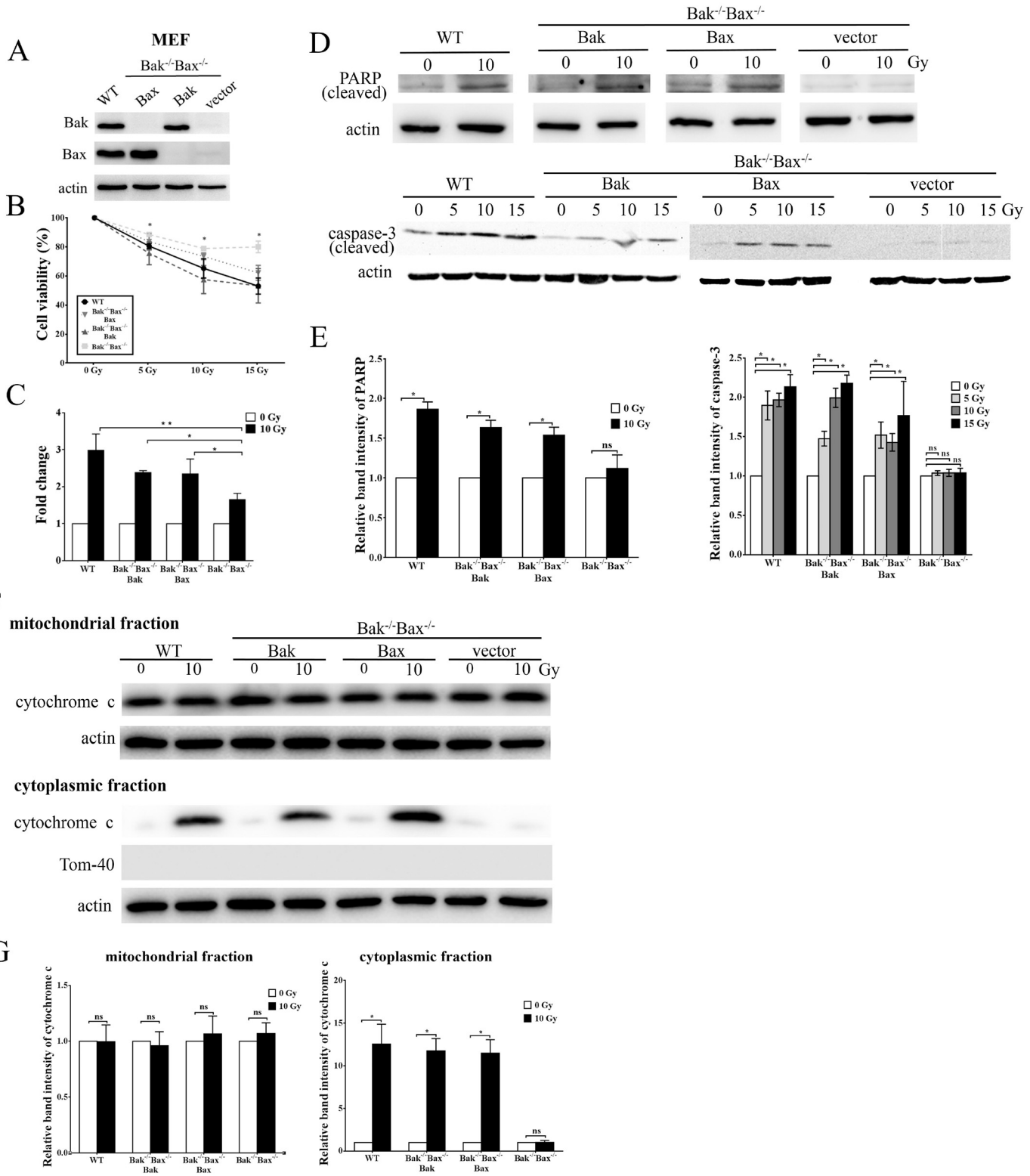

Fig. 5. Bak and Bax are required for radiation-induced cell death in MEFs. (A) Bak, Bax and empty vector were reexpressed in MEFs. The expression levels of Bak and Bax were determined by Western blot analysis. (B-C) MEFs were cultured for 24 h after the indicated radiation treatment, and cell viability and caspase-3/7 activation were measured. (D) Cleavage of PARP and caspase-3 was determined by Western blot from whole-cell extracts. (E) The intensities of PARP and caspase-3 in MEFs (D) were quantified after normalization to actin using ImageJ software (NIH). (F) MEFs were treated with radiation and recultured for 24 h. The cytosolic fractions and mitochondrial fractions were acquired for Western blot. (G) The intensities of cytochrome *c* in mitochondrial and cytoplasmic fraction (F) were quantified after normalization to actin using ImageJ software (NIH). All of the data are mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate $p < 0.05$ (*); $p < 0.01$(**); ns, not significant; Student's unpaired *t*-test.

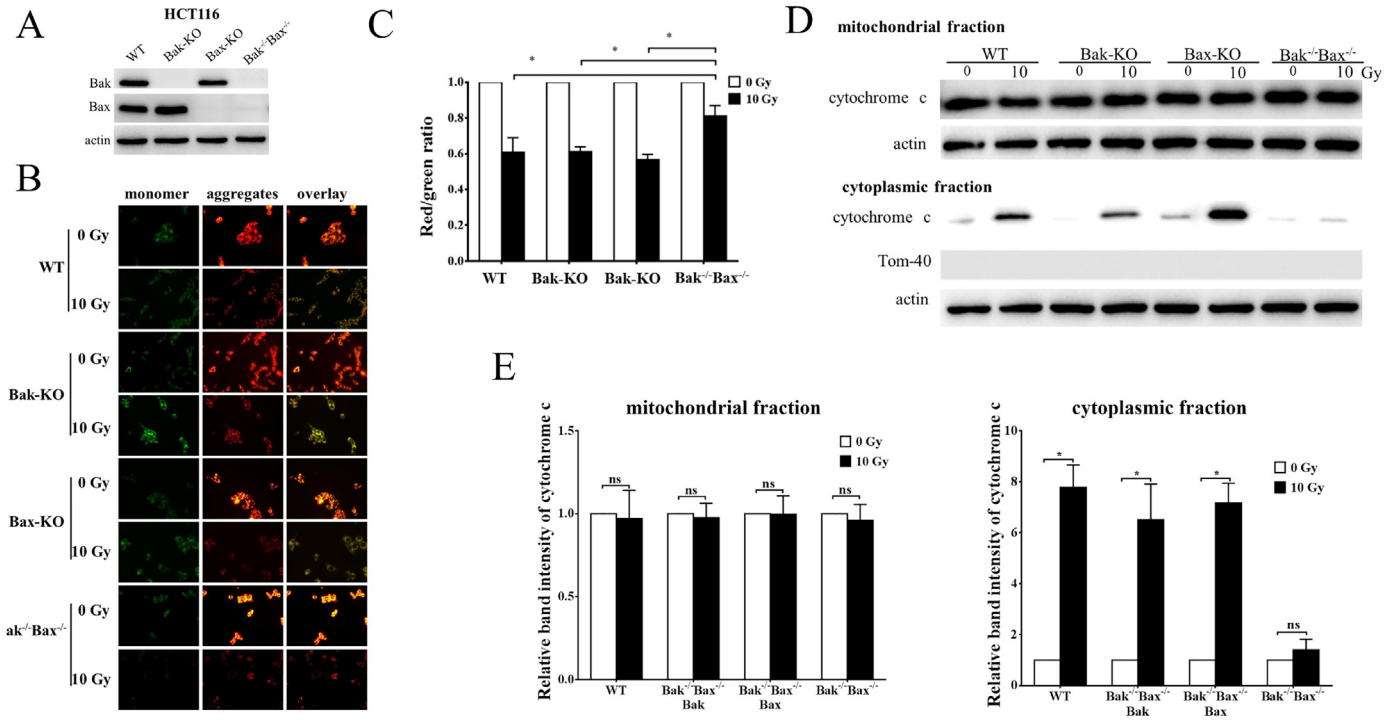

Fig. 6. Bak and Bax are involved in radiation-induced MOMP in HCT116. (A) The expression levels of Bak and Bax were determined by Western blot analysis in WT, Bak-KO, Bax-KO and Bak$^{-/-}$Bax$^{-/-}$ HCT116 cells. (B) Fluorescence images of HCT116 cells stained by JC-1 after radiation. (C) HCT116 cells were treated with either 0 or 10 Gy radiation, and loaded with JC-1 24 h later. The red and green forms were determined by a microplate reader. (D) HCT116 cells were treated with radiation and recultured for 24 h. The cytosolic fractions and mitochondrial fractions were acquired for Western blot. (E) The intensities of cytochrome *c* in mitochondrial and cytoplasmic fraction (D) were quantified after normalization to actin using ImageJ software (NIH). All of the data are mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate $p < 0.05$ (*); $p < 0.01$(**); ns, not significant; Student's unpaired t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Caspase-3 and caspase-7 are the most famous caspases in death signaling; they are activated by caspase-8 or caspase-9 and perform the downstream execution steps of apoptosis by cleaving key cellular substrates. Caspase-7 is highly related to caspase-3, and these two caspases are activated in both the intrinsic and extrinsic pathways [38,39]. Studies show that losing caspase-3 and caspase-7 activity are markedly resistant to both mitochondrial and death receptor-mediated apoptosis [40,41]. In this study, we also found that loss caspase-3/7 could induce resistance to the cell death caused by radiation (Fig. 1B–C). Although no significant differences in cell death were detected in relation to caspase-3 and caspase-7, a tendency for a higher survival rate in caspase-3-KO could still be assumed (Fig. 1B). The reason for this could be that the activation of caspase-3 is upstream of caspase-7. Similar results were also found for N-(3-Oxo-acyl)-homoserine lactone-induced apoptosis in MEF cells [42].

Bcl-2 family proteins have been studied intensively for their importance in the regulation of apoptosis. It is generally believed that MOMP is a "one-way ticket" in both the intrinsic and extrinsic apoptotic pathways [27,43]. Two important events that MOMP leads to the release of cytochrome *c* from the mitochondrial intermembrane space and depolarization of mitochondrial membrane potential. Westphal et al. have reported that both Bak and Bax are crucial to MOMP during apoptotic signaling [44]. However, the role of these two proteins in radiation-induced MOMP still needs to be further confirmed. Here, using Bak or Bax knockout MEF and HCT116 cells, we found that both Bak and Bax participated in a proapoptotic function after radiation treatment, and Bak and Bax functioned redundantly (Figs. 5–6). Consistent with previous studies [45,46], using MEF and HCT116 cells, either Bak or Bax can directly induce cytochrome *c* release and mitochondrial depolarization, and cells deficient in both Bak and Bax expression are markedly resistant to radiation-induced apoptosis. Moreover, other Bcl-2 family proteins, Puma, Bcl-xL and Mcl-1, were also found to play a major role in radiation-induced apoptosis (Fig. 7). Overall, Bcl-2 family proteins can commit a cell to programmed death after radiation by causing outer mitochondrial membrane permeabilization.

Recent studies suggested that radiation-induced apoptotic signaling were also observed in the 3D and *in vivo* culture models. Wang et al. found that p53-target gene Puma mediates radiation-induced apoptosis *via* a cell-intrinsic mechanism in 3D mouse crypt culture [47]. In 3D human tissue models, DNA double-strand breaks and apoptosis in bystander cells were increased after radiation [48]. For *in vivo* studies of radiation, Sarosiek et al. found that mitochondria of many adult somatic tissues, are profoundly refractory to pro-apoptotic signaling, leading to cellular resistance to radiation [49]. Furthermore, treatment of irradiated mice with the anti-apoptotic Bcl-2/xL protein inhibitor ABT-263 after exposure to IR can significant remove senescent cells [50]. Those above studies together with our data indicated that intrinsic apoptosis might be triggered in both *in vitro* and *in vivo* after radiation.

In conclusion, our results indicate that radiation induces a unique mitochondrial-dependent apoptotic signaling pathway in which radiation leads to MOMP, releasing cytochrome *c* to activate caspase-9, caspase-3/7, and the subsequent downstream apoptosis cascade in MEF cells, which indicates targeting mitochondrial-dependent apoptotic pathway is a promising strategy for cancer radiotherapy.

4. Materials and methods

4.1. Reagents

Mitochondrial membrane potential assay kit with JC-1 and DTT were purchased from Beyotime Biotechnology (Jiangsu, China). Propidium iodide (PI) was obtained from Thermo Fisher Scientific.

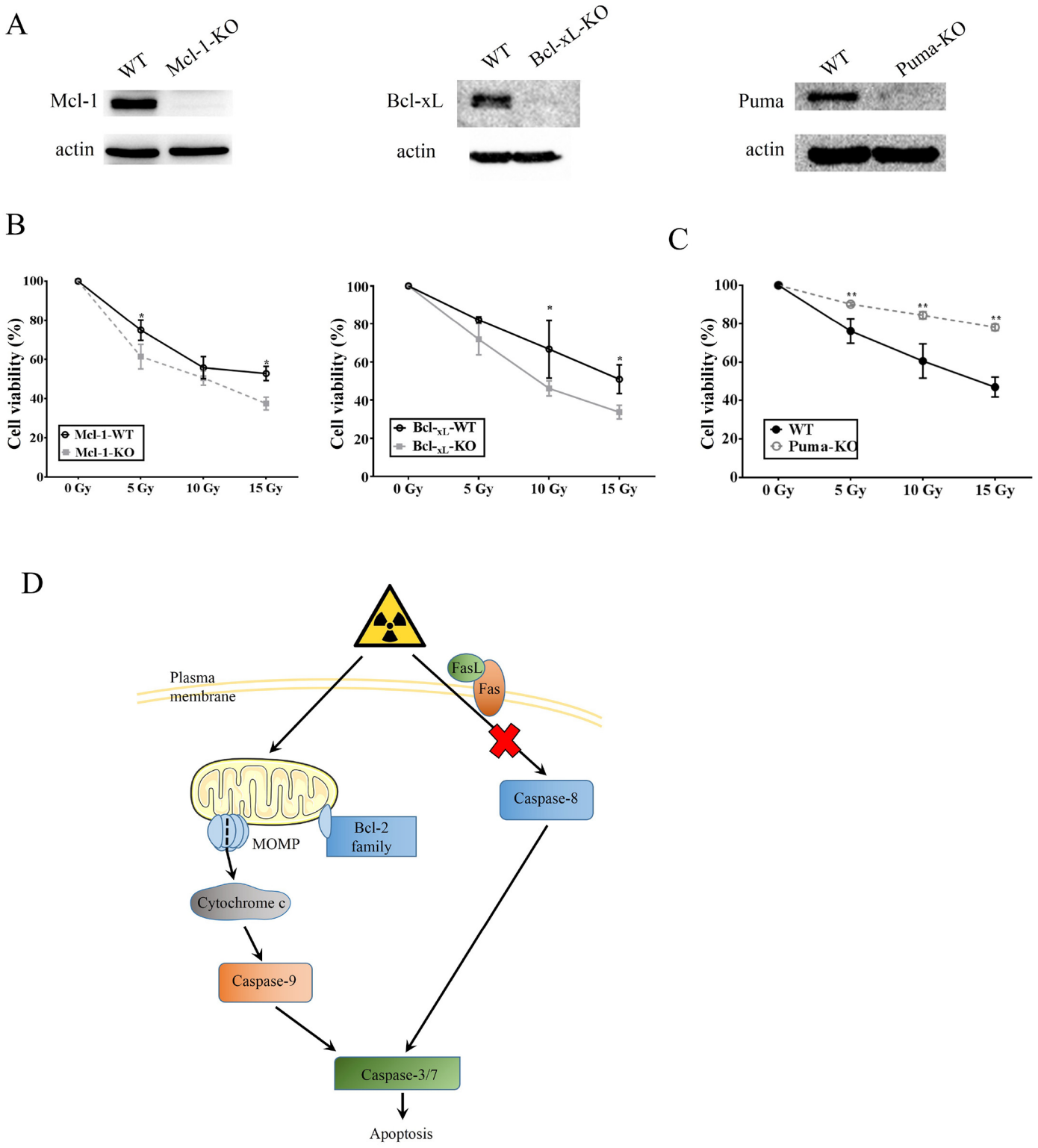

Fig. 7. Radiation-induced cell death is dependent on Bcl-2 family proteins. (A) The expression of Mcl-1 and Bcl-xL in MEF cells and Puma in HCT116 cells was examined by Western blot. (B) Cell viability of WT and Mcl-1-KO or Bcl-xL MEFs were measured 24 h after treatment with different doses of radiation. (C) Cell viability of WT and Puma-KO HCT116 cells were measured 24 h after treatment with different doses of radiation. (D) Radiation induces a unique mitochondrial-dependent apoptotic signaling pathway in which radiation leads to MOMP, releasing cytochrome *c* to activate caspase-9, caspase-3/7, and the subsequent downstream apoptosis cascade. All of the data are mean ± standard deviations of three independent experiments performed in triplicate. Asterisks indicate $p < 0.05$ (*); $p < 0.01$(**); Student's unpaired t-test.

High-glucose DMEM was obtained from HyClone (Logan, UT, USA). Fetal bovine serum was purchased from Biological Industries (#04-001-1ACS) (Beit Haemek, Israel). The Caspase-Glo assay 3/7 kit was purchased from Promega (Shanghai, China). A protease inhibitor cocktail was obtained from Roche Inc., (Switzerland). Anti-β-actin mAb (#TA-09; mouse) was obtained from ZSGB-BIO (Beijing, China). Anti-

caspase-3 pAb (#9662; rabbit), anti-caspase-7 pAb (#9662; rabbit), anti-caspase-8 pAb (#4790; rabbit), anti-caspase-9 pAb (#9508; rabbit), anti-Bak pAb (#3814; rabbit), anti-PARP pAb (#9532; rabbit), anti-Mcl-1 pAb (#5453; rabbit), anti-Mcl-1 pAb (#5453; rabbit) and anti-cytochrome *c* pAb (#4272; rabbit) were purchased from Cell Signaling (Danvers, MA, USA). Anti-cytochrome *c* pAb (#10993-1-AP; rabbit), anti-Tom40 pAb (#18409-1-AP; rabbit), anti-Bax pAb (#50599-2-Ig; rabbit), anti-Bcl-xL pAb (#26967-1-AP; rabbit) and anti-Puma pAb (#55120-1-AP; rabbit) were obtain from Proteintech (Wuhan SANYING, China). Secondary antibodies Peroxidase AffiniPure goat anti-mouse IgG (H + L) (#115-035-003) and Peroxidase AffiniPure goat anti-rabbit IgG (H + L) (#111-035-003) were purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA).

4.2. Cell lines and radiation

MEFs were immortalized by infection with SV40 largeT antigen-expressing lentivirus and caspase-8-KO MEF and their wild-type counterparts were provided by Professor David Vaux (Walter and Eliza Hall Institute of Medical Research, Parkville, VIC Australia) [51]. Caspase-9-KO MEF and their wild-type counterparts were obtained from Professor Jerry Adams (Walter and Eliza Hall Institute of Medical Research) [52]. Caspase-3-KO, caspase-7-KO, caspase-3/7-DKO, or their wild-type counterparts MEFs were obtained from Professor Richard Flavell (Yale University) [53]. Bak/Bax-KO/DKO MEFs and wild-type counterparts were obtained from Craig Thompson (Memorial Sloan Kettering Cancer Center) [54]. Bcl-xL-KO MEF and wild-type counterparts were obtained from Dr. Chi Li (University of Louisville) [55]. Mcl-1-KO MEF and wild-type counterparts were kindly provided by Dr. Joseph Opferman (St. Jude Children's Research Hospital) [56]. Puma-KO HCT116 and wild-type counterparts were acquired from Dr. Bert Vogelstein (John Hopkins University) [57]. HCT116: Bak-KO, Bax-KO, Bak/Bax-DKO, WT were acquired from Dr. Richard Youle (National Institutes of Health) [58]. All of the cell lines were cultured in high-glucose DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were all cultured in a 95% air and 5% CO_2 humidified incubator at 37 °C.

The irradiation was carried out in an X-ray irradiator, X-RAD 320 (Precision X-Ray Inc., USA). Cells are irradiated by high-energy X-rays (U = 320 kV, I = 12.5 mA, SSD = 40 cm, 2 mm aluminum filter) produced by an energized X-ray tube. And the indicated radiation dose was determined by the total radiation time basis on the dose rate 4.987 Gy/min, controlled by the compute automatically. The equipment was maintained and calibrated every year by the manufacturer to ensure the precision of radiation dose. The radiation dose at five sampling points in X-RAD 320 was fall within a 95% confidence limit.

4.3. Cell viability assays

The indicated cells were plated in 35 mm dish tissue culture plates with 2×10^6 cells and cultured for 24 h. Following treatment with the indicated radiation dose, the cells were then cultured for another 24 h. All cells were harvested in the presence of 2 μg/ml PI. Cell viability was measured by PI exclusion using flow cytometry (FACSCalibur, Beckon Dickinson).

4.4. Caspase-3/7 activities

The activities of caspase-3/7 were measured using a microplate reader, according to the manufacturer's protocol, using a Caspase-Glo 3/7 Assay kit (#G8093). Twenty-four hours before the treatment, cells were plated in 96-well plates. At the indicated time points following treatment with various radiation doses, cells were mixed with the CellTiter-Glo reagent, and luminescence was quantified by a spectra Max i3x microplate spectrofluorometer (Molecular Devices). Data were normalized to the respective control group.

4.5. Western blot analysis

Cells (1×10^6) were treated with radiation, collected 24 h later, and then washed with ice-cold PBS twice before addition of lysis buffer (RIPA), which included a protease inhibitor cocktail (Roche Inc., Switzerland). Total protein was boiled for 10 min in a loading buffer and chilled on ice. Twenty micrograms of total protein was separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Roche Inc., Switzerland). Each membrane was blocked with 5% BSA or nonfat dried skimmed milk/TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, adjust pH to 7.4–7.6; *w/v*) at room temperature for 1 h and then incubated with an appropriate primary antibody at 4 °C overnight. After washing three times in TBST, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at 37 °C for 1 h. The protein bands were visualized using ECL reagent (Boster, Wuhan, China) and quantified with ImageJ software. Tom-40 or β-actin was detected as a loading control.

4.6. Measuring $\Delta\psi_{mito}$ using imaging microscopy of JC-1

JC-1 has been widely used to detect the mitochondrial membrane potential. We used two methods to measure the mitochondrial membrane potential: quantitative analysis by a microplate reader and imaging analysis by fluorescence microscope. For the quantitative analysis assay, first, 0.7 × 104 cells/well were seeded in 96-well plates for 24 h, after radiation treatment, cells were incubation with JC-1 solution for 20 min at 37 °C in the dark 24 or 48 h after radiation. The green fluorescence intensity (excitation: 490 nm; emission: 530 nm) and red fluorescence intensity (excitation: 525 nm; emission: 590 nm) was determined by a spectra Max i3x microplate spectrofluorometer (Molecular Devices). For imaging assay, first, cells were plated on a glass coverslips, after radiation treatment, cells were incubating with JC-1 for 20 min at 37 °C in the dark. After washing 3 times, cells were analyzed for fluorescence imaging using a fluorescence microscope (OLYMPUS CKX 41) by capturing the images at 40× magnification. Images were corrected for background using regions without cells. At least three separate experiments were averaged for the graphs.

4.7. Subcellular fractionation

Cells were plated into 15-cm tissue culture dishes for 24 h and cultured for another 24 h in DMEM following the indicated radiation exposure. Subcellular fractionation was carried out as described previously with some modification [59]. Briefly, cells were collected and washed with 1 × PBS and resuspended in 200 μl MIB with protease inhibitors (Roche) and 5 μg/μl BSA. The cells were broken by passing through a 1 ml syringe with a 25 g (for MEF cells) or 27 g (for HCT116) needle, then centrifuged at 400 × *g* (Sigma) for 10 min at 4 °C to eliminate large cellular debris and nuclei. The supernatants were centrifuged at 12,000 × *g* for 10 min at 4 °C. The pellet was mitochondria fraction and the supernatants were the cytosolic fractions. Protein concentrations in the isolated fractions were evaluated by BCA assays (Thermo Fisher Scientific). Equivalent amounts of mitochondria fractions (5 μg) and the cytosolic fractions (20 μg) were electrophoresed. The presence of cytochrome *c*, Tom40, and β-actin was detected by Western blot.

4.8. Statistical analysis

All experiments were performed at least three times. The results are presented as the mean ± SD. Statistical analysis was performed using Student's unpaired *t*-test. A p value < 0.05 was considered significant.

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Declaration of competing interests

The authors declare that they have no conflict of interest.

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