Disruption of Chromosomal Architecture of cox2 Locus Sensitizes Lung Cancer Cells to Radiotherapy

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Despite treatment of lung cancer with radiotherapy and chemotherapy, the survival rate of lung cancer patients remains poor. Previous studies demonstrated the importance of up-regulation of inflammatory factors, such as cyclooxygenase 2 (cox2), in tumor tolerance. In the present study, we investigated the role of cox2 in radiosensitivity of lung cancer. Our results showed that the combination treatment of radiation with aspirin, an anti-inflammatory drug, induced a synergistic reduction of cell survival in A549 and H1299 lung cancer cells. In comparison with normal human lung fibroblasts (NHLFs), the cell viability was significantly decreased and the level of apoptosis was remarkably enhanced in A549 cells. Mechanistic studies revealed that the reduction of cox2 by aspirin in A549 and H1299 was caused by disruption of the chromosomal architecture of the cox2 locus. Moreover, the disruption of chromatin looping was mediated by the inhibition of nuclear translocation of p65 and decreased enrichment of p65 at cox2-regulatory elements. Importantly, disorganization of the chromosomal architecture of cox2 triggered A549 cells sensitive to γ-radiation by the induction of apoptosis. In conclusion, we present evidence of an effective therapeutic treatment targeting the epigenetic regulation of lung cancer and a potential strategy to overcome radiation resistance in cancer cells.

INTRODUCTION

Lung cancer is the leading cause of cancer-related mortality worldwide, and it is classically divided into two major histological subtypes, non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Approximately 85% of all lung cancer cases comprises NSCLC. Although substantial advances have been made for the treatment of NSCLC, such as targeted molecular therapy and immunotherapy, the survival rate of these patients has not significantly improved in the past >30 years. Radiation therapy is the most common method of treatment for lung cancer patients. Radiation induces irreversible damage in targeted cells, including the induction of both single- and double-stranded DNA breaks, and damage to cell membrane, which ultimately lead to the activation of downstream pathways that contribute to cell death. In fact, studies have reported that ionizing radiation activates apoptosis by regulating genes involved in apoptotic pathways, such as p53, bcl-2, B cell lymphoma 2 (bcl-2), bel-2-associated X protein (bax), and caspases. However, aberrantly high expression of cyclooxygenase (cox2) in lung cancer cells limits the efficacy of radiation therapy.

cox2 is the rate-limiting enzyme in the conversion of arachidonic acid to prostanooids, and it is involved in regulating cell growth and proliferation and detected at high levels in many types of lung cancer cells. Previous studies showed that the occurrence of apoptosis, disease, and cancer was related to the disorder of cox2 expression. For instance, inhibition of cox2 expression significantly enhances genotoxic stress-induced apoptosis in several types of normal human cells. Many studies of human cancers have highlighted the frequent overexpression of cox2 in a variety of malignancies. For instance, constitutive expression of cox2 was detected in colorectal, prostate, lung, breast, and other cancers. Additional studies showed that elevated cox2 expression in tumors was associated with increased angiogenesis, tumor invasion, and resistance to radiation-induced apoptosis. However, the mechanisms by which cox2 exerts cytoprotection are not completely understood. Gene expression is regulated by the combined action of multiple trans-acting factors that bind to regulatory elements of cox2. Detailed studies of several model loci, such as the beta-globin locus and HoxD cluster, have shown that associated regulatory elements can spread over very large genomic regions and can be located distal to the target gene. It is increasingly postulated that distal elements regulate genes through the formation of direct physical associations between regulatory elements and their target genes. Chromosome conformation capture (3C) technology has rapidly become an established research tool to
study the formation of long-range looping interactions between genes and regulatory elements.\(^n\) A previous study confirmed that disruption of CTCF- and cohesin-mediated higher-order chromatin structures by DNA methylation downregulated \(\text{cox2}\) expression.\(^{24}\) Our recent study provided evidence that \(\text{cox2}\) expression was regulated by p65- and p300-mediated chromatin looping between \(\text{cox2}\) promoter and enhancer regions.\(^{25}\) Therefore, the regulation of the chromosomal conformation of \(\text{cox2}\) locus may be targeted for cancer treatment.

Studies have shown that some chemotherapeutic agents induce expression of apoptosis-related genes by regulating chromosomal conformation. For example, camptothecin was demonstrated to diminish \(bcl2\) chromatin looping and directly induce apoptosis.\(^{26}\) Owing to its anti-tumor effects, aspirin has recently drawn attention as a novel chemotherapeutic drug.\(^{27}\) The molecular mechanism of aspirin was previously demonstrated to inhibit \(\text{cox2}\) activity, thereby blocking the production of prostaglandins.\(^{28}\) In the present study, we used normal and lung cancer cells to study the combinatorial therapeutic effects of radiation and aspirin and the underlying mechanism. We demonstrated that pre-treatment with aspirin at sublethal doses significantly sensitized NSCLC cells to radiation but showed lower sensitization effects on normal human lung fibroblasts (NHLFs) and human colon cancer cells (HCT116). Using 3C analysis, we showed that aspirin disrupted the chromosomal architecture of the \(\text{cox2}\) locus by inhibiting p65 nuclear translocation, which enhanced the efficacy of radiation treatment and induced cell apoptosis. This study proposed a novel therapeutic approach of combining aspirin with radiation to treat lung cancer and deciphered the mechanism of \(\text{cox2}\) suppression by aspirin.

### RESULTS

#### The Role of \(\text{cox2}\) Expression in Radiosensitivity of Lung Cancer Cells

To overcome radiation resistance in cancer cells, combination therapy with chemotherapeutic agents has been demonstrated to be effective in many different human cancers.\(^{29}\) Aspirin, an anti-inflammatory drug, enhanced cell death in human colon and prostate cancer.\(^{30,31}\) Before we carried out the combination study, aspirin (0, 0.5, 1, 2, and 5 mM) and radiation (0, 1, 2, 5, and 8 Gy) were tested, respectively, for their toxicity (Figures S1 and S2), and 1 mM aspirin with little toxicity and 5 Gy \(\gamma\)-radiation, which normally is used to treat lung cancer cells in the clinical experiment,\(^{32}\) were finally selected for further study.

To examine whether aspirin enhanced the radiosensitivity of lung cancer cells, cell survival was determined by colony formation assay for A549 cells. As shown in Figure 1A, cells treated with a combination of aspirin and radiation exhibited significantly decreased survival following treatment, compared to cells treated with radiation alone. Similarly, pre-treatment with aspirin in other NSCLC cells (H1299 cells) also resulted in significant radiosensitivity (Figure S3). Furthermore, due to the difficulty of colony formation for NHLF cells, we compared the difference of radiosensitivity between cancer lung cells (A549) and NHLF cells, with the endpoints of apoptosis and cell viability, by fluorescence-activated cell sorting (FACS) and 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Our results showed that, compared with NHLF cells, A549 cells pre-treated with aspirin were more sensitive to radiation, exhibiting higher levels of apoptosis (Figures 1B and 1C) and a significant reduction of cell viability at 24 and 48 hr post-irradiation (Figure 1D; Figure S4). To further determine whether there was the radiosensitivity of aspirin for other cancer cells, HCT116 human colon cancer cells were selected and treated with a combination therapy of aspirin and radiation to validate its efficacy in other cancers, but a lower sensitization effect was found (Figure S5). Together, our data demonstrated that combination treatment of aspirin and radiation was more effective in targeting lung cancer cells than either single treatment. Moreover, the combination treatment was not much toxic for normal lung cells and colon cancer cells, suggesting that the combination therapy may be specific to lung cancer.

Several studies reported high expression of \(\text{cox2}\) in lung cancer cells, which may contribute to radiation resistance.\(^{33}\) Therefore, to explore the molecular mechanism underlying the aspirin-mediated radiosensitization of lung cancer cells, we examined \(\text{cox2}\) protein expression by western blot analysis. As shown in Figure 2A, protein levels of \(\text{cox2}\) significantly decreased in aspirin-treated A549 cells compared to untreated cells. In addition, \(\text{cox2}\) expression reduced to the same levels in cells treated with a combination of aspirin and radiation, indicating that aspirin irreversibly inhibited \(\text{cox2}\) expression, which was consistent with previous reports.\(^{28}\) A similar result was proven in H1299 cells (Figure S6). To investigate the role of \(\text{cox2}\) in the radiosensitization of lung cancer cells, we performed transient knockdown of \(\text{cox2}\) using small interfering RNA (siRNA) (Figure S7). Cell viability and colony formation assays were performed to validate our hypothesis. As shown in Figure 2B and Figure S8, \(\text{cox2}\) knockdown sensitized A549 cells at 24 and 48 hr post-irradiation, as cell viability significantly decreased compared to untreated cells. In a long-term colony formation assay, \(\text{cox2}\) knockdown partially decreased cell survival, whereas, in addition to radiation, it almost eliminated cell survival (Figure 2C). Therefore, we demonstrated that a high expression of \(\text{cox2}\) correlated with resistance to radiation in NSCLC cells.

#### The Disruption of Higher-Order Chromatin Structure Was a Cause of Aspirin-Induced \(\text{cox2}\) Suppression

Previous studies have shown that gene regulation is controlled by dynamic chromatin-looping structures.\(^{34}\) Looped structures have been identified at numerous gene loci in a fashion that juxtaposes important genetic elements, such as promoter and enhancer regions.\(^{35}\) To understand the mechanism of aspirin-mediated \(\text{cox2}\) suppression, we analyzed the changes in chromatin interactions at the \(\text{cox2}\) locus by using 3C analysis techniques in A549 cells. Figure 3A illustrates the chromatin-looping structure of \(\text{cox2}\). We observed changes in chromatin interaction between the \(\text{cox2}\) promoter (A1) and downstream enhancer regions at 6.5 kb (A2), in our previous study.\(^{35}\)
Treatment with aspirin drastically disrupted the interaction between A1 and A2 (Figure 3B, left), suggesting aspirin suppressed cox2 expression by affecting the chromatin structure. Moreover, this effect was irreversible. The specificity of PCR products was validated by DNA sequencing (Figure 3C). The disruption of chromosomal interaction at the cox2 locus was also detected in H1299 cells (Figure S9), showing that the A1-A2 interaction was significantly decreased after treatment with aspirin by 3C assay. Our results demonstrated that aspirin suppressed cox2 expression via the disruption of chromatin interactions at the cox2 gene locus.

The Role of p65 in Aspirin-Mediated Disruption of Chromatin Interactions at the cox2 Gene Locus

To elucidate the mechanisms underlying the disruption of chromatin looping at the cox2 locus, we identified chromatin-modifying factors that may contribute to the aspirin-mediated disruption and cox2 suppression. p65 is the most abundant form of nuclear factor kappa light-chain enhancer of activated B cells (nuclear factor kB [NF-kB]), and it plays roles in the expression of various genes involved in immune and inflammatory responses and cell survival. To determine whether p65 modulated aspirin-mediated disruption of chromatin interactions in A549 cells, we first examined the p65 levels in the cytoplasm and nucleus. Figure 4A shows a reduction of nuclear expression of p65 and accumulation of cytosolic p65 in A549 cells upon aspirin treatment. Chromatin immunoprecipitation (ChIP) analysis further depicted reduced binding of p65 on cox2 promoter and enhancer regions in aspirin-treated A549 cells, compared to untreated cells (Figure 4B). To validate the importance of p65 to chromatin interactions at the cox2 locus, we knocked down p65 expression and examined the changes in chromatin looping via 3C assay. A1-A2 looping was significantly decreased in cells transfected with p65 siRNA, and additional aspirin treatment further disrupted the interaction (Figure 4C). These results strongly suggested that aspirin facilitated the disruption of chromatin looping by inhibiting nuclear translocation of p65.

The Regulation of Radiation-Induced Apoptosis by the Disruption of Chromatin Interaction

Apoptosis is a vital component of various processes, including normal cell turnover, development and function of the immune system, and

Figure 1. Cytotoxicity of Combination Treatment of Aspirin and Radiation

Cells were treated with radiation (5 Gy) with or without pre-treatment of 1 mM aspirin. (A) Cell death was measured by colony formation assay (*p < 0.05). (B) FACS analysis of A549 and NHLF cells stained with Annexin V-FITC and PI. The lower right and upper right quadrants represent apoptotic populations. These results were obtained from three independent experiments. (C) Quantification of FACS analysis (*p < 0.05). (D) Cell viability of A549 and NHLF cells was measured by MTT assay with 24 hr post-irradiation (***p < 0.001).
Since our study showed the combination treatment of radiation with aspirin induced a synergistic reduction of cell survival by apoptosis examination in lung cancer cells (Figures 1B and 1C), to investigate the mechanism in regulating radiosensitivity, we examined the expression of proteins involved in apoptosis by western blot analysis. Our results showed that A549 cells pre-treated with aspirin and exposed to radiation exhibited increased levels of pro-apoptotic proteins, such as Bax and Bak, and decreased levels of an anti-apoptotic protein, Bcl-2 (Figure 5A). In comparison, the pro-apoptotic and anti-apoptotic protein expression showed no significant changes in NHLF cells (Figure 5B). To validate whether the suppression of cox2 was involved in radiation-induced apoptosis, expression of these apoptotic proteins was examined in cells transfected with cox2 siRNA. Our results showed that expression levels of pro-apoptotic and anti-apoptotic proteins in A549 cells treated with both cox2 siRNA and radiation increased and decreased, respectively, more significantly than those in cells treated with radiation alone (Figure 5C).

Our previous study proved that cox2 expression was regulated by dynamic chromosomal interactions and p65 was required for stabilizing this chromosomal conformation by binding to the cox2 promoter and enhancer regions. To characterize the molecular mechanism by which changes in chromosomal organization at the cox2 locus regulated cell death, we analyzed the function of associated chromatin-modifying factors in radiation-induced cell apoptosis. Our results showed that knockdown of p65 in A549 cells led to significant cell death after treatment with radiation, compared to control cells treated with radiation (Figure 5D). Therefore, we demonstrated that aspirin mediated the disruption of chromatin looping by reducing p65 expression and further suppressed cox2 expression to increase the radiosensitivity of NSCLC cells.

DISCUSSION
Radiosensitization of tumor cells is critical for the effective radiation therapy of human cancers, which ultimately determines patient prognosis. Thus, one approach to improving the outcome of radiotherapy is to identify molecular targets related to radiation resistance in tumors. Overexpression of epidermal growth factor receptor (EGFR) was reported in the development and growth of malignant tumors, leading to poor clinical outcome in head and neck squamous cell carcinoma. Another study showed that aberrant Wnt signaling mediated radiation resistance in breast cancer. cox2 is a pro-inflammatory enzyme that activates the expression of inflammatory factors and is involved in various cellular processes, including immune response, apoptosis, disease progression, and tumorigenesis. Several previous studies have demonstrated that cox2 overexpression is associated with tumor cell resistance to radiation. Therefore, cox2 may be a potential therapeutic target to enhance radiation-induced apoptosis of lung cancer cells that express high levels of cox2. In the present study, we uncovered the role of cox2 in the regulation
of radiosensitivity in lung cancer cells, and we explored the underlying molecular mechanism.

A previous study demonstrated that aspirin irreversibly inhibited cyclooxygenases.46 In the present study, 1 mM aspirin was selected as a cox2 inhibitor to investigate the radiosensitivity of lung cancer cells. This concentration has been demonstrated as not triggering significant cell death in the HeLa cell line.30 Even to 5 mM, aspirin was not found to inhibit leukocyte attack (LA) and the triggered reactive proliferation of smooth muscle cells (SMCs), which are key events for the development of early atherosclerosis and restenosis in vitro.47 Our results showed that though 1 mM aspirin did not increase cellular toxicity itself, it significantly sensitized lung cancer cells to radiation through cox2 suppression. To validate the relationship between cox2 and radioresistance, RNAi techniques were used to knock down cox2 expression, and our results showed that downregulation of cox2 increased the radiosensitivity of A549 cells. Increasing evidence supports our hypothesis that the activation of cox2 signaling promotes a pro-survival response in irradiated human cervical cancer (HeLa) cells.48 In fact, cox2 suppression by aspirin only sensitized A549 cells to radiation, whereas NHLF and HCT116 cells were not significantly affected, likely because of low cox2 expression in both cell lines (Figure S10). Therefore, selective inhibition of cox2 might be an effective therapeutic approach to treating cancer cells characterized with cox2 overexpression.49

Because of the relationship between cox2 expression and radiosensitivity of cancer cells, cox2 may be a potential molecular target for cancer treatment. The higher-order packing of chromosomes in the cell nucleus is both driven by and leads to physical interactions between genomic regions.50 These dynamic chromosomal interactions have been proven to direct significant functions in the regulation of gene expression.51 For instance, special AT-rich sequence-binding protein 1 (SATB1)-mediated chromatin looping was reported as a requirement for the activation of bcl2 in response to apoptotic signals.26 Furthermore, cohesion-mediated intra-chromosomal looping of the oct4 gene was demonstrated to be a critical epigenetic barrier to the induction of pluripotency.52 Our recent study demonstrated that graphene oxide stimulated cox2 activation by increasing the chromosomal interactions between the cox2 promoter (A1) and downstream enhancer regions (A2).25

In the present study, we investigated the mechanism by which aspirin mediated dynamic higher-order chromosomal conformation to suppress cox2 in A549 cells. Our results showed that aspirin treatment alone significantly decreased A1-A2 interactions. The disruption of chromatin looping was irreversible with the combination treatment of aspirin and radiation (Figure 4B). Furthermore, our data showed
that aspirin disrupted chromatin looping by inhibiting p65 activation and nuclear translocation, reducing interactions between cox2 promoter and enhancer regions. The underlying mechanism probably involved the inhibition of phosphorylation of IκB-α and the reduction of ubiquitin-dependent proteasomal degradation, which retained p65 in the cytoplasm in an inactive form. However, p65 knockdown did not completely abolish A1-A2 chromatin looping (Figure 4C), suggesting the presence of other regulatory factors involved in regulating chromosomal conformation at the cox2 locus. Therefore, we postulated that the epigenetic regulation of cox2 expression might be critical in targeting lung cancer. Because p65 plays a key role in chromatin looping at the cox2 locus, we knocked down p65 expression, and our results showed that p65 knockdown resulted in significantly more cell death following radiation compared to control cells that were irradiated (Figure 5D). Altogether, our results demonstrated that disruption of chromatin interactions at the cox2 locus significantly increased the radiosensitivity of A549 cells.

In conclusion, we uncovered an important mechanism of radiosensitivity in NSCLC cells. The suppression of cox2 played a crucial role in sensitizing cancer cells and enhancing cell death in response to radiation. We proposed a schematic of the mechanism underlying aspirin-mediated sensitization of cancer cells to radiation in Figure 6. Therefore, our study provided evidence to support a novel therapeutic program that targets the regulation of dynamic chromatin structures in cancer. Moreover, this therapeutic strategy represented a potential approach to overcome radiation resistance in cancer cells and reduce the side effects of chemotherapeutic agents.

MATERIALS AND METHODS

Cell Culture

Human NSCLC cell lines A549 and H1299 and the NHLF cell line were cultured in DMEM (Gibco–BRL, Life Technologies, USA) and α-MEM (Gibco-BRL, Life Technologies, USA), respectively, in a humidified 5% CO2 incubator at 37°C. Cell culture media were supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin and streptomycin (Beyotime).

Cytotoxicity Assay

To test the cytotoxicity of aspirin, A549 cells (2 × 10^5) were seeded in 24-well plates. Cells were treated with aspirin at various concentrations for 24 hr. After drug treatment, cells were stained with 0.4% trypan blue solution (Solarbio Science & Technology, Beijing, China). The number of live cells per 1,000 was counted by excluding dead cells positive for trypan blue staining. To test cytotoxicity of aspirin combined with radiation, A549 cells (2 × 10^5) were seeded in 35-mm plates overnight, and then treated with aspirin for 24 hr. Culture medium was changed prior to radiation by ^137 Cs γ-iradiator. Cell viability was evaluated by MTT assay. MTT is reduced by cellular

![Figure 4. The Role of p65 in Modulating Aspirin-Mediated Disruption of Chromatin Architecture](image_url)

(A) Aspirin treatment inhibited nuclear translocation of p65. A549 cells were incubated with 1 mM aspirin for 24 hr. Levels of nuclear and cytosolic p65 were determined by western blot analysis. Values represent mean ± SD (**p < 0.01). (B) Left: aspirin treatment reduced binding of p65 on cox2 promoter and enhancer regions. After aspirin treatment aspirin for 24 hr, the cox2 promoter region (A1) and enhancer regions (A2) in the chromatin precipitate were amplified by PCR. Rabbit IgG was included as a negative control. Right: error bars represent SEM of three independent ChIP assays (*p < 0.05). (C) A549 cells were transfected with control siRNA or p65 siRNA prior to aspirin treatment, and then 3C assay was used to measure the interaction between A1 and A2 (*p < 0.05).
dehydrogenases to generate a soluble, orange formazan product, and the amount of formazan produced is directly proportional to the number of live cells. Therefore, absorbance was measured at 450 nm on a Versamax microplate reader.

**Colony Formation Assay**

A549 cells (2.0 × 10⁵) were seeded in 35-mm plates overnight and then treated with aspirin for 24 hr. Culture medium was changed prior to radiation by ¹³⁷Cs γ-iradiator. Cells were plated at the same density (1,500 cells/well) in 60-mm dishes in triplicate and incubated for 10 days. Finally, cells were stained with Giemsa, and colonies containing more than 50 cells were counted.

**Western Blotting**

After being washed with PBS, pelleted cells were lysed in RIPA buffer (Beyotime, Jiangsu, China), supplemented with protease inhibitor cocktail (Roche, Switzerland), for 45 min. Protein concentrations were determined by bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, USA). Equal amounts of proteins were used to perform SDS-PAGE and western blotting. The following primary antibodies were used: anti-cx2 (1:1,000, Cell Signaling Technology, USA), anti-bax (1:2,000, Proteintech, USA), anti-bak (1:1,000, Proteintech, USA), anti-bcl-2 (1:1,000, Proteintech, USA), and anti-β-actin (1:2,000, ZSGB-Bio, Beijing, China). The following secondary antibodies were used: goat anti-mouse horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (1:3,000, Sigma, St. Louis, MO, USA) and goat anti-rabbit HRP-conjugated IgG (1:3,000, Sigma, St. Louis, MO, USA). Western blotting signals were visualized with chemiluminescence reagent (Boster, Wuhan, China), and intensities of the autoradiograms were determined using ImageJ software.

**3C assay**

3C assay was performed as previously described by Dekker et al.²³,²⁴ Briefly, cells (1.0 × 10⁷) were cross-linked with 2% formaldehyde for 10 min at room temperature and then stopped by adding 0.125 M glycine. Cells were lysed in lysis buffer (10 mM Tris-HCl [pH 8.0], 10 mM NaCl, and 0.2% NP-40), supplemented with fresh protease inhibitors, for 90 min at 4°C, with rotation. Nuclei were pelleted, suspended in 1× restriction enzyme buffer with 0.3% SDS, and then incubated at 37°C for 1 hr. Triton X-100 (final concentration of 1.8%) was added to sequester the SDS. An aliquot of isolated nuclei

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**Figure 5. The Role of Chromosome Conformation in radiosensitivity by regulating apoptosis**

(A and B) Expression levels of apoptotic proteins were examined by western blot analysis in A549 (A) and NHFL (B) cells (*p < 0.05). (C) Expression levels of apoptotic proteins in cells treated with or without cox2 siRNA were examined by western blot analysis (*p < 0.05). (D) FACS analysis of cells transfected with or without p65 siRNA prior to radiation. Cells were stained with PI.

**Figure 6. Schematic Diagram of the Molecular Mechanism of Aspirin-Mediated Radiosensitization of A549 Cells**

Aspirin suppresses cox2 expression by disrupting chromatin architecture at the cox2 locus, sensitizing cells to radiation-induced apoptosis.

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Prior to radiation by ¹³⁷Cs γ-iradiator. Cells were plated at the same density (1,500 cells/well) in 60-mm dishes in triplicate and incubated for 10 days. Finally, cells were stained with Giemsa, and colonies containing more than 50 cells were counted.

**Western Blotting**

After being washed with PBS, pelleted cells were lysed in RIPA buffer (Beyotime, Jiangsu, China), supplemented with protease inhibitor cocktail (Roche, Switzerland), for 45 min. Protein concentrations were determined by bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, USA). Equal amounts of proteins were used to perform SDS-PAGE and western blotting. The following primary antibodies were used: anti-cx2 (1:1,000, Cell Signaling Technology, USA), anti-bax (1:2,000, Proteintech, USA), anti-bak (1:1,000, Proteintech, USA), anti-bcl-2 (1:1,000, Proteintech, USA), and anti-β-actin (1:2,000, ZSGB-Bio, Beijing, China). The following secondary antibodies were used: goat anti-mouse horseradish peroxidase (HRP)-conjugated immunoglobulin G (IgG) (1:3,000, Sigma, St. Louis, MO, USA) and goat anti-rabbit HRP-conjugated IgG (1:3,000, Sigma, St. Louis, MO, USA). Western blotting signals were visualized with chemiluminescence reagent (Boster, Wuhan, China), and intensities of the autoradiograms were determined using ImageJ software.

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(2 \times 10^6) was digested with restriction enzyme NcoI (800 U; New England BioLabs, CA) at 37°C overnight. The digestion reaction was stopped by adding 1.6% SDS, and the aliquot was incubated at 65°C for 20 min. Chromatin DNA was diluted with ligation reaction buffer (30 mM Tris-HCl [pH 8.0], 10 mM MgCl₂, 10 mM DTT, and 1 mM ATP), and DNA fragments (2 μg) were ligated with T4 DNA ligase (4,000 U; TaKaRa, Japan) at 16°C for 4 hr (final DNA concentration of 2.5 μg/mL). Samples were treated overnight with proteinase K (20 mg/mL) at 65°C, to reverse cross-linking, and RNase A (1 μg/mL) for 30 min at 37°C. Then, 3C samples were purified by phenol-chloroform extraction and amplified by PCR, using specific primers. PCR-cycling parameters were 30 s at 95°C, 30 s at 63°C, and 30 s at 72°C, for 35 cycles. Quantitative levels of the 3C assay were analyzed by nest PCR and normalized by input. The primers of the first PCR were as follows: A1, 5'-ATTAGCCCAATAAGCCAGGCAAC-3' and A2, 5'-TGACATGATGCTGTGGAGCTG-3'. The primers of the second PCR were as follows: A1, 5'-ATTAGGGGATTACAGGCGTG-3' and A2, 5'-TGACATCGATGCTGTGGAGCTG-3'.

ChiP
ChiP assays were performed using the ChiP assay kit as described by the manufacturer (Beyotime, Jiangsu, China). Briefly, A549 cells were fixed with 1% formaldehyde for 10 min at 37°C and washed twice with ice-cold PBS containing a protease inhibitor. Then, the fixed cells were re-suspended in lysis buffer and subjected to sonication to trim the DNA into fragments within the range of 200–1,000 bp. After centrifugation (12,000 \times g for 5 min), an aliquot of chromatin was saved as an input control, and the rest was diluted in ChIP dilution buffer. The diluted chromatin was incubated with 2 μg anti-p65 antibody or normal IgG. Thereafter, the immunoprecipitated DNA was purified and amplified by PCR. The PCR products were separated by agarose gel electrophoresis (2%) and visualized ethidium bromide staining. The primer sequences were as follows: 5'-GGGAAAGAAAAGACATCTGGC-3', 5'-AGGAAAGCTGCCCATTGGG-3', 5'-GTGCTGGGATACAGGCGTG-3', and 5'-CCTTTTCTCCTTGTAAGGGCG-3'.

Cell Death Analysis via FACS
A549 and NHFL cells were pre-treated with aspirin for 24 hr and then washed with PBS prior to radiation treatment (5 Gy) for 24 hr. In comparison, cells were also treated with aspirin or radiation alone. Following treatment, cells were pelleted by centrifugation at 500 \times g and washed with PBS three times. Cells (1.0 \times 10^6) were stained with Annexin-V-fluorescein isothiocyanate (FITC) (5 μL) and propidium iodide (PI; 5 μL) for 15 min at room temperature in the dark, according to the manufacturer’s instructions (BD Biosciences). Cells (1.0 \times 10^6) were subjected to FACS analysis using FlowJo software.

Statistical Analysis
All cell culture experiments were performed at least three independent times. The significance of mean difference for two or more treated groups relative to an untreated group was analyzed by a one-way ANOVA test or t test. Data are presented as mean ± SD. Statistical significance was determined as p < 0.05 (*p < 0.05, **p < 0.01, and ***p < 0.001).

SUPPLEMENTAL INFORMATION
Supplemental Information includes ten figures and can be found with this article online at https://doi.org/10.1016/j.ymthe.2018.08.002.

AUTHOR CONTRIBUTIONS
Y.S. and H.D. conceived and designed the experiments. Y.S. executed western blot, ChIP, and 3C experiments and wrote the manuscript. X.C. performed the FACS analysis. Y.Z. performed the colony formation assay. S.C. and L.W. commented on the project. All authors discussed the results and commented on the manuscript.

CONFLICTS OF INTEREST
The authors declare that they have no competing interests.

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