

# circFOXO3 protects cardiomyocytes against radiation-induced cardiotoxicity

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**Abstract.** Radiation therapy, one of the major treatment options for cancer, can cause delayed heart damage. The circular RNA (circRNA) circFOXO3 (hsa\_circ\_0006404) is associated with cancer progression. However, the functions of circFOXO3 in radiation-induced cardiotoxicity remains unknown. The present study aimed to identify the functions of circFOXO3 in radiation-induced cardiotoxicity. The present study established circFOXO3-knockdown (KD) or -over-expressing (OE) cardiomyocytes. Functional assay results showed that KD of circFOXO3 in cardiomyocytes significantly increased DNA damage and apoptosis after radiation. By contrast, OE of circFOXO3 reduced DNA damage and apoptosis rates in response to radiation. Mechanistically, KD of circFOXO3 elevated the levels of Bax, caspase 3 and caspase 7, and decreased Bcl-2 expression, whereas OE of circFOXO3 decreased Bax, caspase 3 and caspase 7 expression, and increased Bcl-2 expression. Thus, the present study indicated that circFOXO3 protected cardiomyocytes from radiation-induced cardiotoxicity by reducing DNA damage and apoptosis. circFOXO3 may be a potential therapeutic target against radiation-induced cardiotoxicity.

## Introduction

Radiation therapy, which is used for the treatment of some cancer types, can cause delayed heart damage (1-3). In the past two decades, it was found that radiation therapy increases the risk of radiation related to cardiac damage in cancer survivors (4,5). A significant increase in death rates in the follow-up after 10 years was found in patients post-radiation therapy in the US (6). A previous study also revealed that radiation therapy increased cardiovascular mortalities in females treated for the left breast compared with those who were

treated only for the right breast from earlier studies during the 1970s and 1980s (7). However, the underlying causes and biomarkers of radiation-induced cardiotoxicity are currently unknown, prompting the need for studies investigating the differences in sensitivity and resistance to the development of radiation-induced cardiotoxicity (8-10).

Circular RNAs (circRNAs) are a recently recognized type of functional non-coding RNA that consist of a circular configuration through a typical 3' to 5' phosphodiester bond. Since circRNAs do not contain a free 5' or 3' terminus, they are much more stable compared with linear RNAs in cells (11). The functional roles of circRNAs in cardiovascular diseases, such as myocardial infarction, ischemia-reperfusion injury, atherosclerosis, cardiomyopathy and cardiac fibrosis have been increasingly reported (12-16). circRNA\_101237 has been demonstrated to mediate anoxia/reoxygenation injury by targeting let-7a-5p/insulin-like growth factor 2-binding protein 3 in cardiomyocytes (12). A previous study reported that circFndc3b modulates cardiac repair after myocardial infarction via the fused in sarcoma/VEGF-A axis (15). circRNA-Ttn105-110 was found to play a protective role in doxorubicin-induced cardiotoxicity (16). Moreover, increasing evidence has revealed that circRNAs may serve as endogenous competing RNAs and affect gene expression by binding to microRNAs (miRs/miRNAs) as sponges (17). circRNAs can also interact with proteins as sponges, decoys or scaffolds (18). Additionally, circRNAs that are retained in the nucleus can interfere with transcription and promote alternative splicing (19).

circFOXO3 was first identified by a research group in Toronto. Several findings have been reported regarding circFOXO3 function. Lower levels of circFOXO3 have been detected in breast cancer, however the expression was revealed to increase when cancer cells underwent apoptosis (20). Subsequent studies found that circFOXO3 also played a role in the progression of acute myeloid leukemia (21), glioblastoma (22), breast cancer (23) and cardiac senescence (24). However, the role of circFOXO3 in radiation-induced cardiotoxicity remains to be elucidated.

The present study aimed to identify the functions of circFOXO3 in radiation-induced cardiotoxicity. The current study established circFOXO3-knockdown (KD) or -overexpression (OE) models in cardiomyocytes and the effects of circFOXO3 on DNA damage and cell apoptosis were observed. In addition, the effect of circFOXO3 on the apoptotic pathway was investigated. The data indicated that circFOXO3 protected

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cardiomyocytes from radiation-induced cardiotoxicity by reducing DNA damage and apoptosis. Thus, circFOXO3 may be a potential therapeutic target against radiation-induced cardiotoxicity.

## Materials and methods

**Cell culture.** Human cardiomyocytes (AC16; American Type Culture Collection) were cultured at 80% confluency in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). Cells were incubated at 37°C with 5% CO<sub>2</sub>.

**RNA Fluorescence in Situ Hybridization (FISH).** circFOXO3 probes were designed and synthesized by Guangzhou RiboBio Co., Ltd. The probe signals were detected with a FISH kit (Guangzhou RiboBio Co., Ltd.) according to the manufacturer's instructions. Briefly, 5x10<sup>4</sup> AC16 cells were fixed in 4% paraformaldehyde for 1 h at room temperature. Pre-hybridization were conducted with 200  $\mu$ l pre-hybridization buffer at 37°C for 30 min. Then, 0.5  $\mu$ M circFOXO3 FISH Probe Mix or controls (U6 and 18S) were incubated overnight at 37°C. Fluorescent microscopy (magnification, x400) were used to detect the signal.

**Radiation treatment.** Cells in culture were irradiated with an irradiator (Gammacell 3000 Elan; Atomic Energy of Canada Ltd.) using a 137Cs source at a dose rate of 6 Gy.

**Construction of circFOXO3-KD or -OE cell models.** OE and KD of circFOXO3 were achieved by the infection of circFOXO3-OE or circFOXO3-targeting short hairpin RNA (shRNA) lentiviruses. circFOXO3 expression in AC16 cells was assessed in circFOXO3-KD, circFOXO3-OE and negative control (NC) groups. circFOXO3-KD or -OE cells were constructed as previously described (22).

**Reverse transcription-quantitative PCR (RT-qPCR).** For RT-qPCR, RNA was extracted using TRIzol<sup>®</sup> (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed using the Prime Script RT Master Mix at 37°C (15 min) (Takara Bio, Inc.). PCR was performed using a PCR Master Mix (2X; Thermo Fisher Scientific, Inc.). To quantify the levels of circRNA and mRNA, qPCR was performed using a SYBR Premix Ex Taq kit (Takara Bio, Inc.) with GAPDH as the internal control. The following primers were used: circFOXO3 forward, 5'-attgtccatggagacagcccgcg-3' and reverse, 5'-gtggggaactcactgtgctaag-3'; and GAPDH forward, 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse, 5'-ACC ACCCTGTTGCTGTAGCCAA-3'. The qPCR cycling conditions were as follows: Initial denaturation for 3 min at 95°C, followed by 45 cycles at 95°C (10 sec) and 58°C (45 sec); data were acquired at the end of the annealing/extension phase. Each sample was replicated three times and the data were analyzed by comparing Cq values (25).

**Comet assay.** Cells grown in 100-mm<sup>3</sup> dishes were radiated at dose rates 0 or 6 Gy. At the specified times, the cells were suspended at a concentration of 1x10<sup>5</sup> cells/ml and mixed

with melted, low-melting point agarose (Trevigen, Inc.) at a 1:10 ratio and transferred onto a Comet slide (Trevigen, Inc.). The slides were placed at 4°C for 30 min before being immersed in lysis solution (Trevigen, Inc.) for 1 h at 4°C. Subsequently, the slides were run on a horizontal electrophoresis apparatus for 10 min 20 V. Propidium iodide (Becton-Dickinson and Company) was added to the slides and stained at room temperature for 15 min. The slides were visualized on a Leica microscope (Leica Microsystems, Inc.). The olive tail moment (OTM) was recorded for each cell using ImageJ 1.52 (National Institutes of Health).

**Flow cytometry (FCM) analysis.** Cell apoptosis was measured using Annexin V-FITC staining. Briefly, cells were radiated at dose rates of 0 or 6 Gy and cultured for 24 h. The cells were then harvested, washed twice with PBS, stained with Annexin V-FITC and PI in binding buffer and detected by FCM (Beckman gallios, Beckman Coulter, Inc.; Flowjo 10.07, BD Biosciences) after 15 min incubation at room temperature in the dark. Early apoptotic cells (Annexin V<sup>+</sup>/PI<sup>-</sup>) and late apoptotic cells (Annexin V<sup>+</sup>/PI<sup>+</sup>) were quantified.

**Western blotting.** Cell lysates were prepared using RIPA buffer (Beyotime Institute of Biotechnology) containing protease inhibitors. Protein concentration was determined using a bicinchoninic acid protein assay kit. A total of 30  $\mu$ g protein was separated via 10% SDS-PAGE, followed by transfer to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc.) and blocking with 5% non-fat milk in TBS-Tween buffer 7 (0.12 M Tris-base, 1.5 M NaCl, 0.1% Tween-20) for 1 h at room temperature. Immunoreactive bands were detected using an ECL kit (cat. no. PI32209; Pierce; Thermo Fisher Scientific, Inc.). Primary antibodies targeting the following proteins were used at 1:1,000: Bax (cat. no. 2772; Cell Signaling Technology, Inc.), Bcl-2 (cat. no. 4223; Cell Signaling Technology, Inc.), caspase 3 (cat. no. 9662; Cell Signaling Technology, Inc.), cleaved-caspase 3 (cat. no. 9602; Cell Signaling Technology, Inc.), cleaved-caspase 7 (cat. no. 8438; Cell Signaling Technology, Inc.) and actin (cat. no. AB0035; Abways Technology). HRP-conjugated goat anti-mouse (cat. no. SA00001-1) and goat anti-rabbit (cat. no. SA00001-2) antibodies were used as secondary antibodies (1:10,000; ProteinTech Group, Inc.). Images are representative of four independent experiments. Image-Pro Plus 6.0 software (Media Cybernetics, Inc.) was used to semi-quantify the relative band intensities from western blotting images.

**Statistical analysis.** Data are presented as the mean  $\pm$  SD from at least three replicates. Student's two-tailed unpaired t-test was used to determine differences between two groups. One-way ANOVA followed by Tukey's post hoc test was applied to determine differences among at least three groups. Statistical analyses were performed using SPSS v17 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

## Results

**circFOXO3 is significantly upregulated in cardiomyocytes after radiation.** AC16 cells with or without radiation were collected. qPCR results showed that circFOXO3 expression

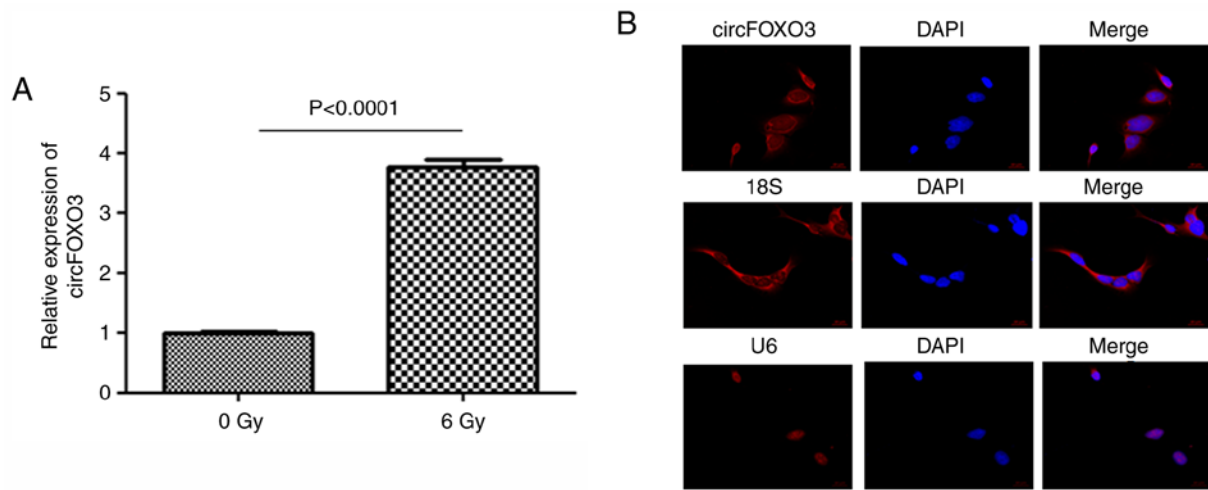


Figure 1. Expression and intracellular localization of circFOXO3 following radiation in cardiomyocytes. (A) Expression of circFOXO3 in AC16 cells after radiation. (B) Intracellular localization of circFOXO3 after radiation in AC16 cells. All experiments were repeated at least three times.  $P<0.0001$  vs. control. circ, circular RNA.

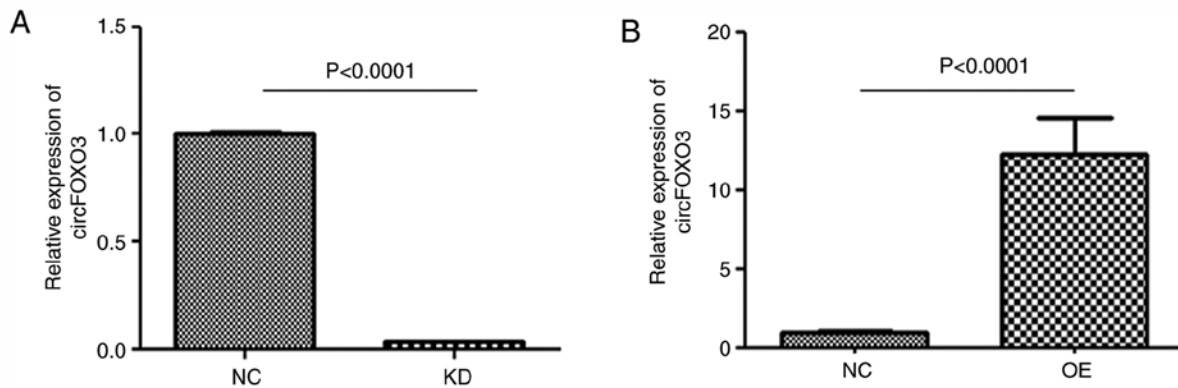


Figure 2. Relative expression of circFOXO3 after KD or OE in cardiomyocytes. (A) RT-qPCR analysis of circFOXO3 in AC16 cells with or without circFOXO3 KD. (B) RT-qPCR analysis of circFOXO3 in AC16 cells with or without circFOXO3 OE. All experiments were repeated at least three times.  $P<0.0001$  vs. NC. RT-qPCR, reverse transcription-quantitative PCR; circ, circular RNA; OE, overexpression; KD, knockdown; NC, negative control.

was significantly upregulated after radiation (Fig. 1A). The subcellular localization of circFOXO3 in AC16 cells after radiation was then examined. As shown in Fig. 1B, circFOXO3 was mainly localized in the cytosol, consistent with 18S (cytosol localization) (Fig. 1B). U6 served as a nuclear localization control (Fig. 1B). These results indicated that circFOXO3 might be involved in radiation-induced cardiotoxicity.

**Successful establishment of circFOXO3-KD or -OE cardiomyocytes.** circFOXO3 expression was significantly downregulated in the KD group ( $P<0.001$  vs. NC) and upregulated in the OE group ( $P<0.001$  vs. NC) (Fig. 2), indicating that circFOXO3-KD or -OE cardiomyocytes were successfully established.

**circFOXO3 elevates the DNA repair processes after radiotherapy in cardiomyocytes.** A comet assay was performed to determine the effects of circFOXO3 on radiation-induced DNA damage. The results showed that circFOXO3 KD significantly increased DNA damage (Fig. 3A and B). However, circFOXO3 OE significantly reduced DNA damage (Fig. 3C and D).

**circFOXO3 decreases the percentage of apoptotic cells after radiotherapy in cardiomyocytes.** FCM was performed to test the effects of circFOXO3 on cell viability. Quantitative analysis indicated that apoptosis was increased in circFOXO3-KD cells after radiation ( $P=0.0026$  vs. NC; Fig. 4A and B). Conversely, the apoptosis rate was suppressed in circFOXO3-OE cells ( $P<0.001$  vs. NC; Fig. 4C and D).

**circFOXO3 suppresses apoptotic protein expression after radiotherapy in cardiomyocytes.** The apoptotic signaling pathway serves a vital role in radiation-induced cardiotoxicity. The expression levels of Bax, Bcl-2, caspase 3 and caspase 7 were investigated using western blotting. The results showed that circFOXO3 KD suppressed the expression of Bcl-2 ( $P<0.001$  vs. NC), whereas it increased the expression of Bax, caspase 3 and caspase 7 ( $P<0.001$  vs. NC) (Fig. 5A and B). By contrast, circFOXO3 OE elevated the expression of Bcl-2 ( $P<0.001$  vs. NC), whereas it decreased the expression of Bax, caspase 3 and caspase 7 ( $P<0.001$  vs. NC) (Fig. 5C and D).

To further clarify the anti-apoptotic function of circFOXO3 on AC16 cells in the absence of radiation, the levels of Bax, caspase 3 and Bcl-2 in circFOXO3-KD,

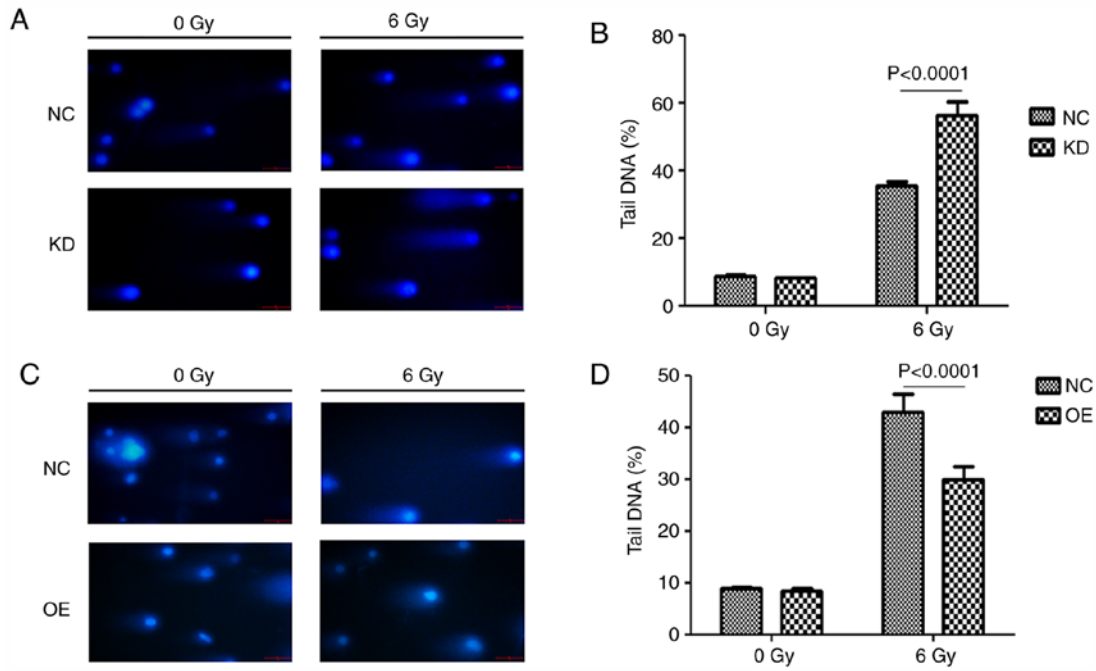


Figure 3. circFOXO3 elevates the DNA repair processes following radiation in cardiomyocytes. (A and B) DNA damage was analyzed using a comet assay following radiation in AC16 cells with or without circFOXO3 KD. (C and D) DNA damage was analyzed using a comet assay following radiation in AC16 cells with or without circFOXO3 OE.  $P < 0.0001$  vs. NC. circ, circular RNA; OE, overexpression; KD, knockdown; NC, negative control.

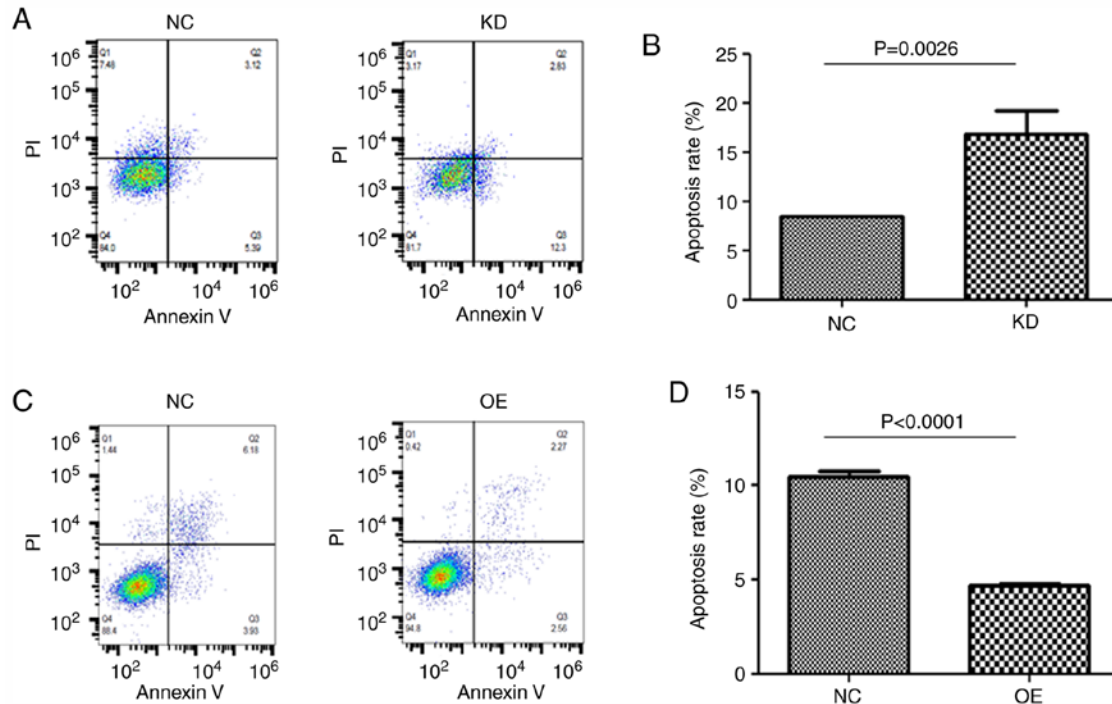


Figure 4. circFOXO3 decreases cell apoptosis after radiation in cardiomyocytes. (A and B) Cell apoptosis was analyzed using FCM following radiation in AC16 cells with or without circFOXO3 KD. (C and D) Cell apoptosis was analyzed using FCM following radiation in AC16 cells with or without circFOXO3 OE.  $P < 0.0001$  vs. NC. circ, circular RNA; OE, overexpression; KD, knockdown; NC, negative control; FCM, flow cytometry.

-OE and NC AC16 cells in the absence of radiation were measured. As shown in Fig. S1, the protein levels of Bax, caspase 3 and Bcl-2 were not notably altered in the absence of radiation among these groups of cells, suggesting that the anti-apoptotic effects of circFOXO3 is dependent on radiation treatment.

## Discussion

The present study constructed circFOXO3-KD or -OE cardiomyocytes in order to assess the function of circFOXO3 on radiation-induced cardiotoxicity. The results revealed that downregulation of circFOXO3 significantly increased

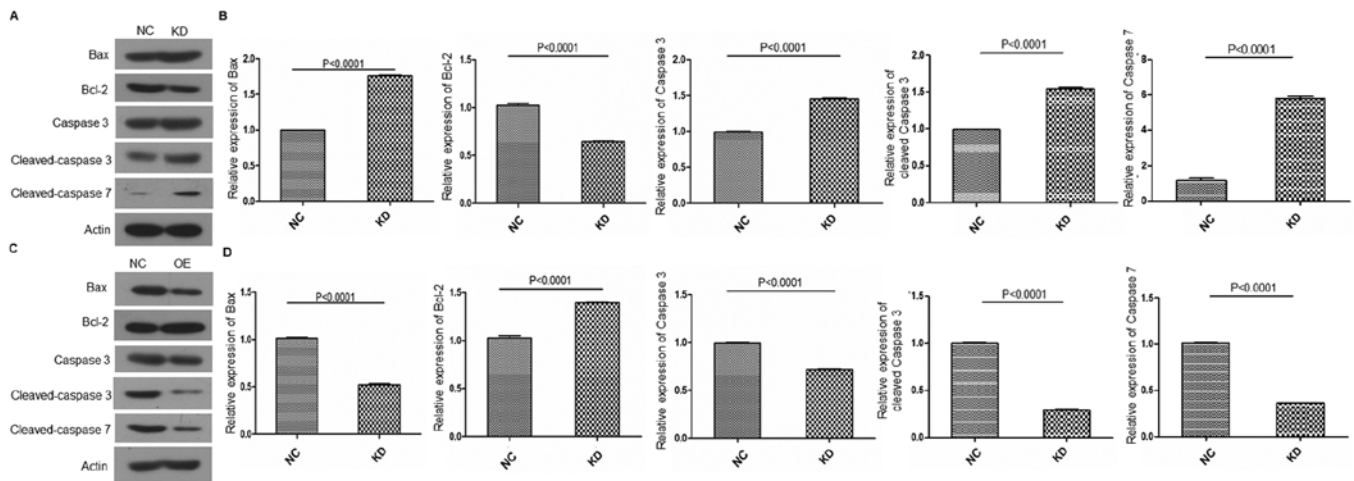


Figure 5. circFOXO3 inhibits the activation of the apoptotic pathway following radiation in cardiomyocytes. (A) Protein expression levels of Bax, Bcl-2, pro- and cleaved-caspase 3 and cleaved-caspase 7 were evaluated by western blotting in AC16 cells with or without circFOXO3 KD after radiation. (B) Relative expression of Bax, Bcl-2, pro- and cleaved-caspase 3 and cleaved-caspase 7 was semi-quantified using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.). (C) Protein expression levels of Bax, Bcl-2, pro- and cleaved-caspase 3 and cleaved-caspase 7 were evaluated by western blotting in AC16 cells with or without circFOXO3 OE following radiation. (D) Relative expression of Bax, Bcl-2 and caspase 3 was semi-quantified using Image-Pro Plus 6.0 software. All experiments were repeated at least three times. P<0.0001 vs. NC. circ, circular RNA; OE, overexpression; KD, knockdown; NC, negative control.

DNA damage and apoptosis after radiation, whereas the upregulation of circFOXO3 showed the opposite results. Mechanistically, inhibition of circFOXO3 increased the expression of pro-apoptotic proteins Bax and caspase 3, while decreasing the expression of Bcl-2 in radiated cardiomyocytes.

Several studies have focused on the function of circRNAs in cancer biology. For instance, it was found that circ-ABCA promoted proliferation and reduced apoptosis in ovarian cancer by negatively regulating miR-1271, miR-1252 and miR-203 (26). Another study observed that circ-ITCH suppressed cell proliferation and promoted apoptosis via sponging miR-10a (27). Circulating miRNAs have been proposed as biomarkers of radiation-induced cardiac toxicity in non-small cell lung cancer (28). However, to the best of our knowledge, the function of circFOXO3 in radiation-induced cardiotoxicity has not yet been reported. The present study observed that downregulation of circFOXO3 significantly suppressed cell survival after radiation, whereas upregulation of circFOXO3 exerted the opposite results. These data suggested that circFOXO3 is involved in radiation-induced cardiotoxicity. However, Du *et al* (20) reported that circFOXO3 expression increased in breast cancer in cells undergoing apoptosis by enhancing FOXO3 activity. A possible explanation for this discrepancy might be related to the novel functions of FOXO3 and circFOXO3 in tumor progression. Recently, it was reported that FOXO3A promoted glioblastoma multiforme (GBM) cell proliferation and invasion (29). Moreover, circFOXO3 enhanced GBM progression (22).

The apoptotic pathway is closely associated with the progression of radiation-induced cardiotoxicity. It was previously reported that irradiation can induce the upregulation of Bax and downregulation of Bcl-2 in cardiomyocytes, leading to apoptosis and subsequent development of fibrosis (30,31). In the present study, while circFOXO3 KD promoted apoptosis, OE of circFOXO3 decreased the expression of pro-apoptotic proteins Bax and caspase 3 while increasing the expression

of Bcl-2 in radiated cardiomyocytes. Thus, the present results showed that circFOXO3 could effectively inactivate the apoptotic pathway.

In conclusion, the present study demonstrated that circFOXO3 served a role in regulating cell apoptosis in cardiomyocytes and may be a potential therapeutic target for radiation-induced cardiotoxicity.

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#### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

#### Authors' contributions

YQ was involved in the conceptualization, performed the experiments, formal analysis, writing the original draft and reviewing and editing the manuscript. XX and LL were involved in the investigation, formal analysis and data validation. All authors read and approved the final manuscript.

#### Ethics approval and consent to participate

Not applicable.

#### Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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