

# MicroRNA-206 relieves irradiation-induced neuroinflammation by regulating connexin 43

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**Abstract.** Radiation therapy has been widely used for the treatment of various types of cancer; however, it may cause neuroinflammation during the pathological process of the disease. Astrocytes, the most abundant cell type in the central nervous system, have been confirmed to play vital roles in various diseases. Connexin (Cx)43, the main Cx type in astrocytes, which has been identified as a direct target gene of microRNA (miR)-206, was found to be involved in diseases pathologies in regions with astrocytes. The aim of the present study was to investigate the mechanism through which  $\gamma$ -radiation may cause astrocyte neuroinflammation and determine the specific mechanism underlying the effects of miR-206 in irradiation-induced HA-1800 cells. A dual-luciferase reporter system was used to predict and verify the target binding site between Cx43 and miR-206. HA-1800 cell viability and apoptosis were determined using a MTT assay and flow cytometry, respectively. In addition, the HA-1800 cells were induced by  $\gamma$ -radiation, then the protein and mRNA expression levels of Cx43, miR-206 and cleaved-caspase-3 were determined using western blot and reverse transcription-quantitative PCR analyses, respectively. ELISA was also performed to evaluate the concentrations of different inflammatory cytokines (TNF- $\alpha$ , IL- $\beta$ , IL-6 and IFN- $\gamma$ ). The dual-luciferase reporter system indicated that Cx43 was a direct target of miR-206. miR-206 mimics increased the expression level of miR-206 in the astrocytes. Irradiation suppressed cell proliferation, increased apoptotic cells and enhanced cleaved-caspase-3 expression and inflammatory cytokines secretion in astrocytes. Furthermore, miR-206 was found to be downregulated and its expression was inversely associated with that of Cx43 in  $\gamma$ -radiation-induced astrocytes.

Overexpression of miR-206 enhanced miR-206 and suppressed Cx43 expression, while Cx43 was upregulated in HA-1800 cells transfected with miR-206 mimic + Cx43-plasmid. However, the expression level of miR-206 was not significantly different in the Cx43-plasmid transfected group. In addition, it was found that miR-206 mimics relieved irradiation-induced neuroinflammation, which was confirmed by increased cell viability, and reduced cell apoptosis and cleaved caspase-3 protein expression, as well as decreased inflammatory cytokine secretion. Furthermore, all the effects of miR-206 mimics on  $\gamma$ -radiation-induced astrocytes were reversed by Cx43-plasmid. In summary, the results of the present study indicated that miR-206 may relieve irradiation-induced neural damage by regulating Cx43, which may provide a novel research direction and a potential therapeutic target for the clinical treatment of inflammation-associated neuronal injury following irradiation.

## Introduction

Radiotherapy has been widely used for the treatment of several types of cancer, including prostate cancer (1), gynecological cancer (2), lung cancer (3) and pituitary adenomas (4). It is estimated that 80% of cancers may regress following radiation treatment (5). However, radiotherapy may result in a series of long-term systemic and local side effects, such as secondary intracranial tumors (6), pituitary dysfunction (7) and stroke (8). When radiotherapy is used to treat pituitary adenomas, it may increase the risk of ischemic stroke, and neuroinflammation is the main pathological process. Therefore, it is crucial to prevent and/or reduce the impact of such complications on patients.

Astrocytes, the most widely distributed cells in the central nervous system (CNS), play key roles in providing structural support for nerve cells, and participate in the formation of the blood-brain barrier (9). Doron *et al* (10) confirmed that inflammatory activation of astrocytes promoted melanoma brain tropism via the C-X-C motif chemokine ligand 10/C-X-C motif chemokine receptor 3 signaling axis. However, Tsunemi *et al* (11) reported that astrocytes protected human dopaminergic neurons from  $\alpha$ -synuclein accumulation and propagation (11). Therefore, astrocytes may act as a double-edged sword in disease, and it is crucial to identify effective therapies for reducing the harmful effects and enhancing the neuroprotective function of astrocytes.

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Connexin (Cx) is widely distributed in the body, such as in astrocytes and hepatocytes; however, the distribution of Cx varies among different cell types (12,13). Cx43 is the main gap junction protein in cardiomyocytes and is also the main Cx protein found between ventricular myocytes (14). Abnormal expression levels of Cx43 have been associated with tumor occurrence and development. Previous reports have confirmed that the decreased level or lack of Cx43 expression may result in abnormal intercellular communication, weakened ability of the body to monitor and regulate cells, and excessive clonal cell growth (15). In addition, Cx43 plays an important role in neuroinflammation, including promoting the assembly of gap junctions and increasing the intercellular signal exchange (16-18). Thus, elucidating the association between the immune and nervous systems may be important for preventing CNS disease.

MicroRNA(miR)-206 has been shown to serve an important role in a variety of tumor types, including glioma (19-21). Duan *et al* (22) reported that miR-206 modulated lipopolysaccharide-mediated inflammatory cytokine production in human astrocytes. Furthermore, miR-206 has been found to alleviate the sevoflurane-induced inhibition of hippocampal astrocyte activation in aged rats, by targeting IGT-1 via suppression of the PI3K/AKT/CREB signaling pathway (23). However, the role of miR-206 in irradiation-induced neuroinflammation remains unknown. Notably, Cx43 was found to be a direct target gene of miR-206 (24). Therefore, we hypothesized that miR-206 may play an important role in irradiation-induced neuroinflammation by regulating Cx43 expression.

Therefore, the present study was designed to investigate the roles of microRNA (miR)-206/Cx43 in an irradiation-induced neuroinflammation cell model.

## Materials and methods

**Cell culture and inflammation model establishment.** The HA-1800 normal astrocyte cell line was purchased from Shang Hai Ze Ye Biotechnology Co., Ltd. (cat. no. AC340443; <http://www.shzysw.cn/>) and cultured in DMEM (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) and 1% penicillin-streptomycin, and maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The HA-1800 cells were treated with 20 Gy  $\gamma$ -radiation for 12, 24 and 48 h to establish the *in vitro* inflammation model (25).

**Cell transfection.** A total of 100 nM mimics control (sense, 5'-UUCUCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; GenePharma Co., Ltd.), 100 nM miR-206 mimics (sense, 5'-UGGAAGUAAGGAAGUGUG UGG-3' and antisense, 5'-ACACACUCCUUAACAUAUCCAUU-3'; GenePharma Co., Ltd.), 1  $\mu$ g Cx43-plasmid (cat. no. sc-400241-ACT; Santa Cruz Biotechnology, Inc.) and 1  $\mu$ g Control CRISPR Activation Plasmid (control-plasmid; cat. no. sc-437275; Santa Cruz Biotechnology, Inc.) were transfected into the HA-1800 cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) at 37°C for 24 h, following the manufacturer's protocol. Reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis were used to detect the expression levels of the related genes and proteins, respectively. Subsequent experiments were performed 24 h after transfection.

**Dual-luciferase reporter assay.** TargetScan bioinformatics software ([http://www.targetscan.org/vert\\_72/](http://www.targetscan.org/vert_72/)) was used to predict the binding sites between miR-206 and Cx43. The 3'-untranslated region of Cx43, which contains the miR-206 binding site or mutated target site, were synthesized using genomic PCR and cloned into pMIR vectors (Ambion; Thermo Fisher Scientific, Inc.) to construct the reporter vector Cx43 wild-type (Cx43-WT) or Cx43 mutated-type (Cx43-MUT). The 293 cells (American Type Culture Collection) were transfected with Cx43 WT or MUT combined with miR-206 mimics or mimics control using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) and incubated for 48 h, according to the manufacturer's protocol. A Dual-Luciferase Reporter Assay system (Promega Corporation) was used to assess the luciferase activity, and the data were normalized to Renilla luciferase activity.

**MTT assay.** HA-1800 cells were treated with 20 Gy  $\gamma$ -radiation for 12, 24 and 48 h, or transfected with mimic control, miR-206 mimic, miR-206 mimic+control-plasmid, or miR-206 mimic+Cx43-plasmid for 24 h and then treated with 20 Gy  $\gamma$ -radiation for 24 h. Then, the HA-1800 cells (10<sup>4</sup> cells per well) were cultured in 96-well plates (BD Bioscience) and incubated for 24 h at 37°C. Then, 10  $\mu$ l MTT (5 mg/ml) solution was added to the cells and continuously incubated for a further 4 h. Subsequently, the solution was removed and 100  $\mu$ l DMSO was added to dissolve the formazan product. Finally, the optical density at 570 nm was assessed using a multifunctional plate reader (BioTek Instruments, Inc.), after 15 min of vibration mixing according to the manufacturer's protocol.

**Flow cytometry analysis.** HA-1800 cells were treated as aforementioned, then harvested by trypsinization and centrifugation (1,000 x g, 5 min, 4°C). HA-1800 cell apoptosis was determined using an Annexin V-FITC/PI apoptosis detection kit (BD Biosciences), according to the manufacturer's protocol. Finally, the apoptotic cells were quantified using a BD FACSCalibur flow cytometer (Becton-Dickinson and Company) and analyzed using CellQuest software (version 5.1; BD Biosciences).

**Western blot analysis.** Total protein was extracted from the HA-1800 cells with RIPA lysis buffer (Beyotime Institute of Biotechnology) and the concentration was determined using a BCA Protein Assay kit (Invitrogen; Thermo Fisher Scientific, Inc.). Then, equal amounts of protein (40  $\mu$ g/lane) were separated using 10% SDS-PAGE and transferred to a PVDF membrane. After blocking with 5% skimmed milk in PBS-0.1% Tween-20 at room temperature for 1.5 h, the membranes were incubated with primary antibodies against  $\beta$ -actin (cat. no. ab8227; dilution, 1:1,000; Abcam), cleaved caspase-3 (cat. no. ab32042; dilution, 1:1,000; Abcam), caspase-3 (cat. no. ab32351; dilution, 1:1,000; Abcam) and Cx43 (cat. no. ab235585; dilution, 1:1,000; Abcam) overnight at 4°C. Subsequently, the membranes were washed and incubated with an anti-rabbit IgG horseradish peroxidase-linked antibody (cat. no. 7074; dilution, 1:2,000; Cell Signaling Technology, Inc.) at room temperature for 1.5 h. Finally, the proteins were assessed using an ECL detection system (MilliporeSigma) in accordance with the manufacturer's instructions.

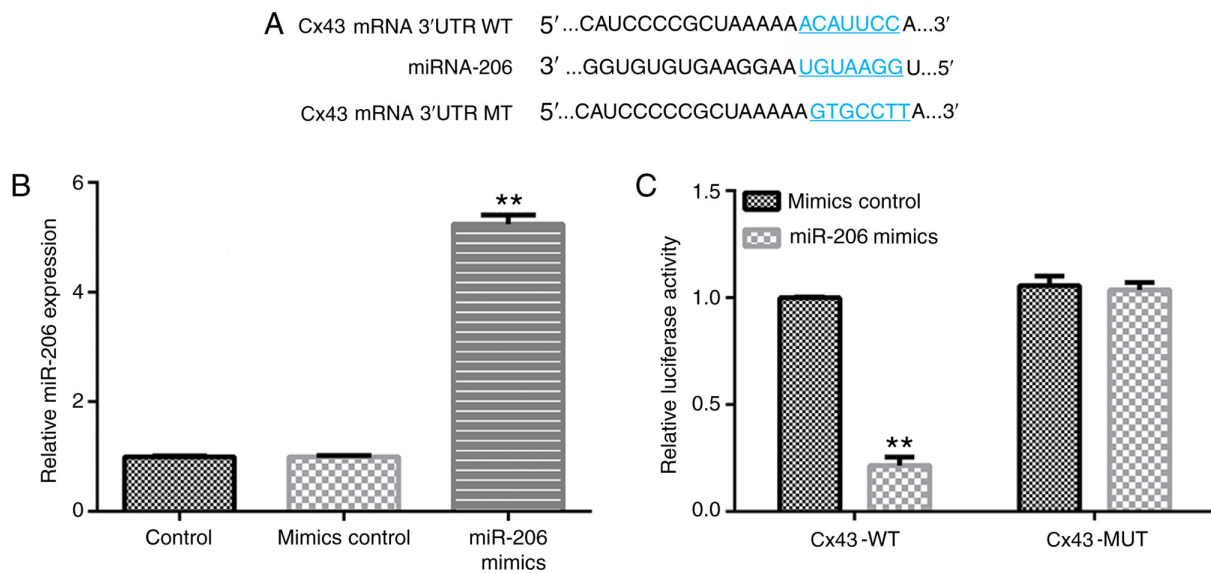


Figure 1. Cx43 is a direct target of miR-206. (A) Schematic showing miR-206 targeted binding with Cx43. (B) Reverse transcription-quantitative PCR analysis of miR-206 expression levels in 293 cells. (C) Dual-luciferase reporter assay confirmed the interaction between miR-206 and Cx43. \*\* $P < 0.01$  vs. mimics control. Cx43, connexin 43; miR, microRNA; WT, wild-type; MUT, mutated.

**ELISA.** Inflammatory factors (TNF- $\alpha$ , cat. no. 430201; IL- $\beta$ , cat. no. 437007; IL-6, cat. no. 430507; and IFN- $\gamma$ , cat. no. 430107) were evaluated using ELISA kits (BioLegend, Inc.) according to the manufacturer's instructions. After the HA-1800 cells were sonicated in 2% sulfuric acid, followed by three freeze/thaw cycles, they then centrifuged for 10 min at 4°C at 10,000  $\times$  g. Subsequently, the concentrations of TNF- $\alpha$ , IL- $\beta$ , IL-6 and IFN- $\gamma$  in the supernatant were detected using ELISA. The OD of each well at 450 nm was measured using a microplate reader (Bio-Tek Instruments, Inc.), in accordance with the manufacturer's instructions.

**RT-qPCR analysis.** Following treatment, total RNA was extracted from the HA-1800 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's instructions. miScript Reverse Transcription kit (Qiagen, Inc.) was used to transcribe total RNA into cDNA. The reverse transcription reaction conditions were as following: 25°C for 5 min, 42°C for 60 min and 80°C for 2 min. An ABI 7000 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the SYBR-Green PCR Master Mix kit (Takara Biotechnology Co., Ltd.) was used to examine the mRNA expression levels of miR-206, Cx43 and GAPDH. The amplification conditions were as follows: Pre-denaturation at 95°C for 10 min; followed by 37 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec and extension at 72°C for 34 sec. Primers were purchased from Sangon Biotech Co., Ltd. The primer sequences were as follows: GAPDH, forward, 5'-CTTTGGTATCGTGAAGGACTC-3' and reverse, 5'-GTA GAGCAGGGATGATGTTCT-3'; Cx43, forward, 5'-TGCTTG GGATAGCTGGGCGGA-3' and reverse, 5'-TGGGGGCAGA GAGAGAAAGCCC-3'; U6, forward, 5'-GCTTCGGCAGCACATACTAAAT-3' and reverse, 5'-CGCTTACGAAATTTG CGTGTCAT-3'; and miR-206, forward, 5'-GGCGGTGGAAT GTAAGGAAG-3' and reverse, 5'-GGCTGTCGTGGACT GCG-3'. Target gene expression level was determined using the  $2^{-\Delta\Delta C_q}$  method (26).

**Statistical analysis.** Statistical analysis was performed using SPSS v20.0 (IBM Corp). All the results are presented as the mean  $\pm$  SD from three independent experiments. The mean differences among groups were calculated using either one-way ANOVA followed by Tukey's post hoc tests or unpaired Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Cx43 directly interacts with miR-206.** First, the association between Cx43 and miR-206 was investigated. As presented in Fig. 1A, Cx43 was a potential target of miR-206. RT-qPCR analysis confirmed that the miR-206 mRNA expression was significantly higher in 293 cells transfected with miR-206 mimics compared with that in the mimics control group (Fig. 1B). Furthermore, the dual-luciferase reporter system confirmed that miR-206 mimics notably suppressed Cx43-WT luciferase activity, while there were no significant changes in Cx43-MUT luciferase activity (Fig. 1C), suggesting that the effects of miR-206 on neuroinflammation may be mediated by targeting Cx43.

**Irradiation suppresses proliferation and induces apoptosis in the HA-1800 cells.** Next, the HA-1800 cells were treated with 20 Gy  $\gamma$ -radiation for 12, 24 and 48 h to establish an *in vitro* inflammatory model. Analysis using MTT assay and flow cytometry suggested that irradiation significantly inhibited HA-1800 cell proliferation (Fig. 2A) and increased the number of apoptotic cells (Fig. 2B and C). In addition, the expression levels of apoptosis-related proteins were investigated using western blot analysis. As displayed in Fig. 2D and E, irradiation markedly enhanced cleaved caspase-3 protein expression (Fig. 2D) and increased the cleaved caspase-3/caspase-3 ratio (Fig. 2E). The aforementioned data demonstrated that irradiation could inhibit the proliferation of HA-1800 cells.

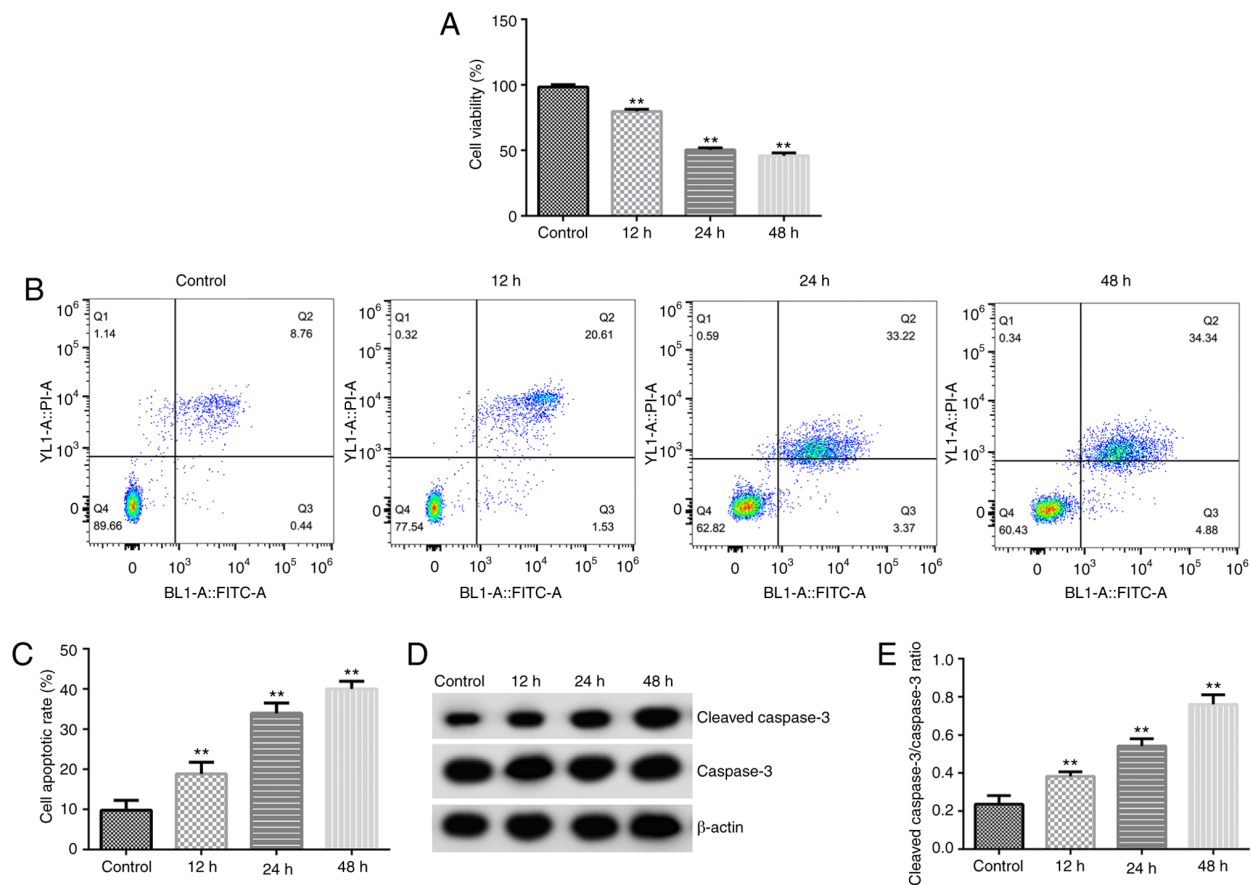


Figure 2. Effects of irradiation on cell proliferation and apoptosis in HA-1800 cells. (A) Cell viability was analyzed using MTT assay. (B) HA-1800 cell apoptosis was analyzed using flow cytometry. (C) Quantitative analysis of the number of apoptotic cells. (D) Western blot analysis of cleaved caspase-3 expression level. (E) Analysis of cleaved-caspase-3/caspase-3 ratio. \*\* $P < 0.01$  vs. control.

*Irradiation promotes inflammatory factor secretion in HA-1800 cells.* Subsequently, the concentration of inflammatory factors (TNF- $\alpha$ , IL- $\beta$ , IL-6 and IFN- $\gamma$ ) in the supernatant of the HA-1800 cells was investigated. Analysis using ELISA revealed that irradiation markedly elevated the secretion of TNF- $\alpha$ , IL- $\beta$ , IL-6 and IFN- $\gamma$  (Fig. 3A-D) compared with the control group. These findings demonstrated that the inflammatory model in the HA-1800 cells induced by  $\gamma$ -radiation was successfully established, and that  $\gamma$ -radiation may promote the detrimental effects of astrocytes.

*miR-206 expression is notably downregulated and Cx43 expression is upregulated in irradiation-induced HA-1800 cells.* Subsequently, the mRNA and protein expression levels of miR-206 and Cx43 were investigated in irradiation-induced HA-1800 cells using RT-qPCR and western blot analyses, respectively. It was found that the expression level of miR-206 was significantly downregulated (Fig. 4A) and the expression level of Cx43 was notably upregulated in irradiation-induced HA-1800 cells compared with the control group (Fig. 4B and C). These findings revealed that the miR-206/Cx43 axis may play a protective role in irradiation-induced neuroinflammation.

*Cx43-plasmid reverses the effect of miR-206 mimics on Cx43 expression level in HA-1800 cells.* To further understand the regulatory association between miR-206 and Cx43 in HA-1800 cells, mimics control, miR-206 mimics, Cx43-plasmid or

control-plasmid were transfected into HA-1800 cells for 24 h and the transfection efficiency was determined using RT-qPCR and western blot analyses. As shown in Fig. 5A, the expression of miR-206 was upregulated in HA-1800 cells transfected miR-206 mimics. In addition, compared with that in the control-plasmid group, the Cx43-plasmid markedly enhanced Cx43 expression in HA-1800 cells (Fig. 5B and C). However, the expression level of Cx43 was lower in HA-1800 cells transfected with miR-206 mimics compared with that in the mimics control group, and this inhibition was increased in miR-206 mimics + Cx43-plasmid co-transfected cells (Fig. 5D and E), indicating that miR-206 could regulate Cx43 expression level in HA-1800 cells.

*miR-206 negatively regulates the expression level of Cx43 in  $\gamma$ -radiation-induced HA-1800 cells.* Previous reports have confirmed that astrocytes play different roles in the CNS (9,27,28); therefore, the roles of miR-206 and Cx43 in neuroinflammation were investigated in the present study. HA-1800 cells were transfected with mimics control, miR-206 mimics, control-plasmid or Cx43-plasmid for 24 h, and induced with 20 Gy  $\gamma$ -radiation for another 24 h. Subsequently, the expression levels of miR-206 and Cx43 mRNA and protein levels were assessed using RT-qPCR and western blot analyses, respectively. The results shown in Fig. 6A-C revealed that miR-206 expression was downregulated and Cx43 was upregulated in  $\gamma$ -radiation-induced HA-1800 cells compared with



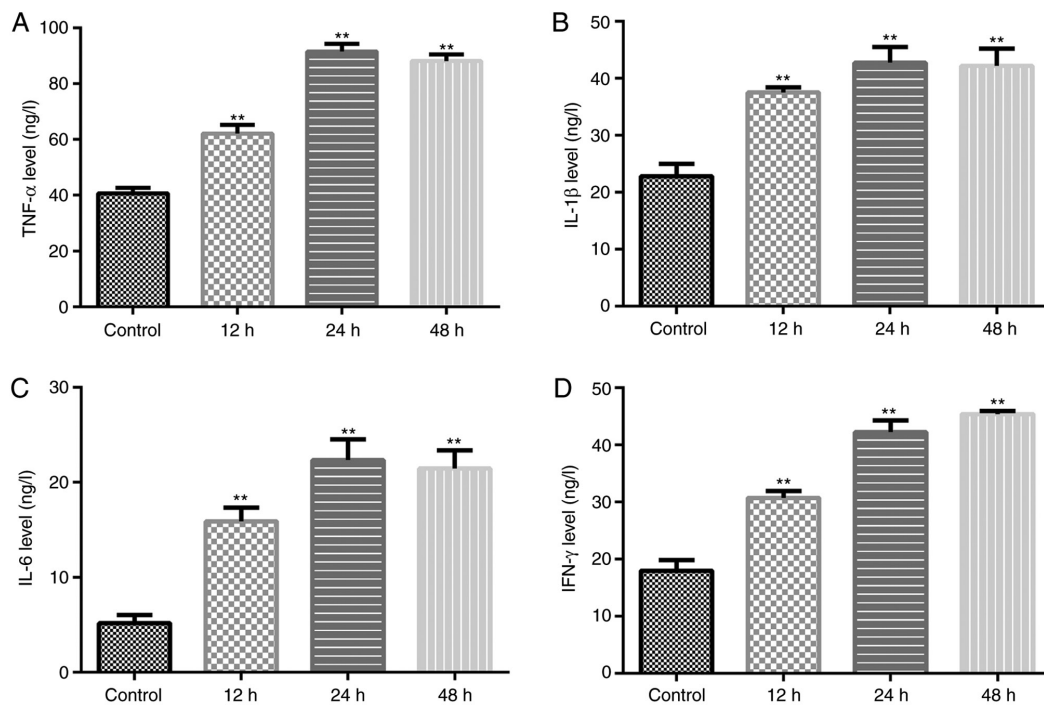


Figure 3. Effects of irradiation on inflammatory factor release in HA-1800 cells. ELISA was conducted to evaluate the concentrations of (A) TNF- $\alpha$ , (B) IL- $\beta$ , (C) IL-6 and (D) IFN- $\gamma$  in the supernatant of the HA-1800 cells. \*\*P<0.01 vs. control.

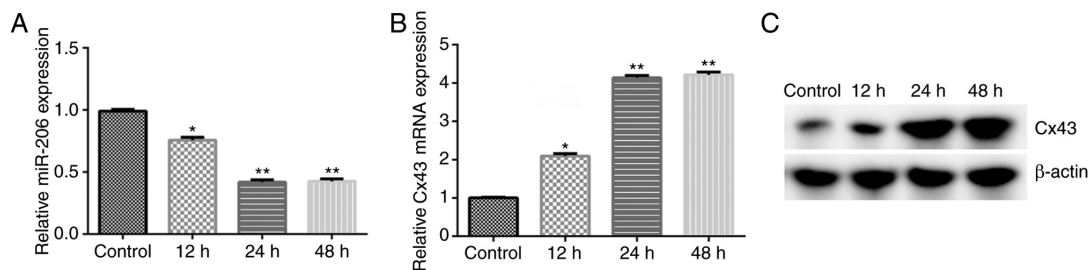


Figure 4. Effects of irradiation on miR-206 and Cx43 expression level in HA-1800 cells. (A) miR-206 and (B) Cx43 mRNA expression in irradiation-induced HA-1800 cells was determined using reverse transcription-quantitative PCR analysis. (C) Detection of Cx43 protein expression level in irradiation-induced HA-1800 cells using western blotting. \*P<0.05 and \*\*P<0.01 vs. control. Cx43, connexin 43; miR, microRNA.

the control group, while miR-206 mimics notably increased miR-206 expression and decreased Cx43 expression. In addition, there was no change in the miR-206 expression in the irradiation + miR-206 mimics + Cx43-plasmid group compared with that in the irradiation + miR-206 mimics + control-plasmid group, while the expression level of Cx43 was higher in the irradiation + miR-206 mimics + Cx43-plasmid group compared with that in the irradiation + miR-206 mimics + control-plasmid group. These data revealed that miR-206 negatively regulated Cx43 expression in  $\gamma$ -radiation-induced HA-1800 cells.

**miR-206 alleviates  $\gamma$ -radiation-induced HA-1800 cell apoptosis by downregulating CX43 expression.** To further elucidate the role of miR-206 in  $\gamma$ -radiation-induced neuroinflammation, HA-1800 cells were transfected with mimics control, miR-206 mimics, control-plasmid or Cx43-plasmid for 24 h, then induced with 20 Gy  $\gamma$ -radiation for another 24 h. Subsequently, the effects of miR-206 mimics on cell viability and apoptosis were investigated. The results of the MTT assay and flow cytometry suggested that  $\gamma$ -radiation markedly

inhibited HA-1800 cell viability (Fig. 7A) and increased the number of apoptotic cells (Fig. 7B and C).

Cell apoptosis is typically regulated via apoptosis-specific genes; therefore, the expression level of apoptosis-associated proteins was determined in the present study. As shown in Fig. 7D and E,  $\gamma$ -radiation increased cleaved caspase-3 expression (Fig. 7D) and the cleaved caspase-3/caspase-3 ratio (Fig. 7E) compared with the control group. In addition, the opposite effects were observed in the HA-1800 cells transfected with miR-206 mimics and induced by irradiation, whereas these effects were reversed by transfection with Cx43-plasmid, indicating that miR-206 protected the cells against  $\gamma$ -radiation-stimulated neuroinflammation by targeting Cx43.

**miR-206 attenuates the inflammatory response in  $\gamma$ -radiation-induced HA-1800 cells by downregulating Cx43 expression.** To investigate the molecular mechanism underlying the role of miR-206 in  $\gamma$ -radiation-induced HA-1800 cells, the release of the inflammatory cytokines TNF- $\alpha$ , IL- $\beta$ , IL-6 and IFN- $\gamma$  was investigated using ELISA. As shown

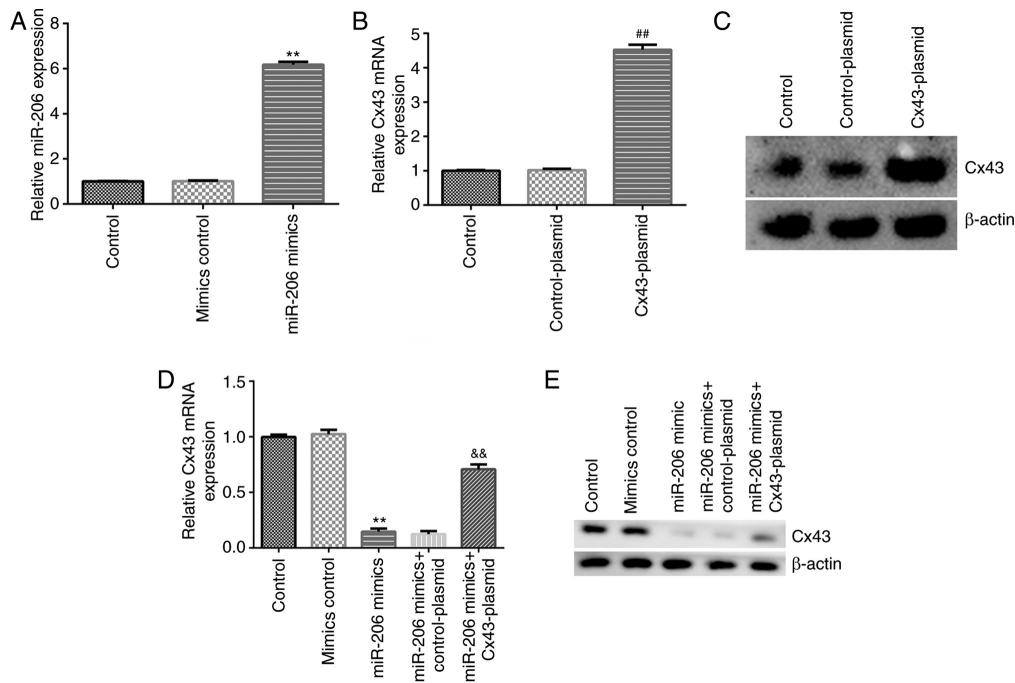


Figure 5. Cx43-plasmid abolishes the effect of miR-206 mimics on Cx43 expression in HA-1800 cells. Mimics control, miR-206 mimics, Cx43-plasmid or control-plasmid were transfected into HA-1800 cells for 24 h. (A) RT-qPCR analysis of miR-206 expression level in HA-1800 cells transfected with mimics control or miR-206 mimics. Cx43 (B) mRNA and (C) protein expression level in HA-1800 cells transfected with Cx43-plasmid or control-plasmid was analyzed using RT-qPCR and western blot analyses, respectively. (D and E) Cx43 (D) mRNA and (E) protein expression level in HA-1800 cells transfected with miR-206 mimics + control-plasmid or miR-206 mimics + Cx43-plasmid was analyzed using RT-qPCR and western blot analyses, respectively. \*\* $P < 0.01$  vs. mimics control; ## $P < 0.01$  vs. control-plasmid; && $P < 0.01$  vs. miR-206 mimics + control-plasmid. RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; Cx43, connexin 43.

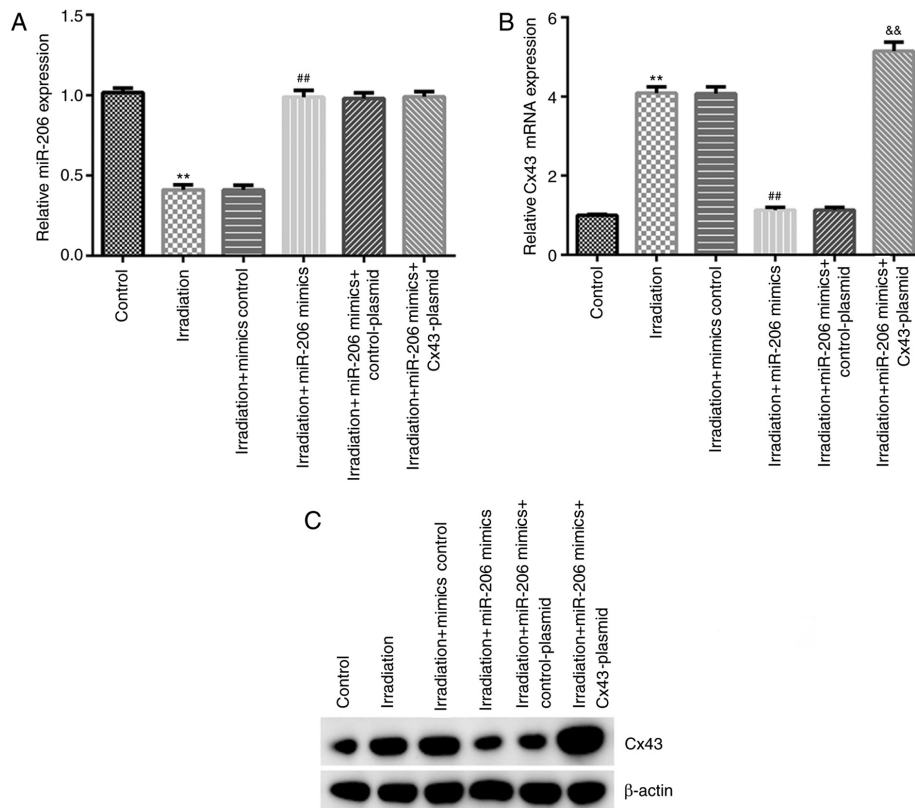


Figure 6. Cx43-plasmid reverses the effects of miR-206 mimics on Cx43 expression in  $\gamma$ -radiation-induced HA-1800 cells. HA-1800 cells were transfected with mimics control, miR-206 mimics, control-plasmid or Cx43-plasmid for 24 h, and induced with 20 Gy  $\gamma$ -radiation for another 24 h. The cells were divided into six groups as follows: Control, irradiation, irradiation + mimics control, irradiation + miR-206 mimics, irradiation + miR-206 mimics + control-plasmid and irradiation + miR-206 mimics + Cx43-plasmid groups. Reverse transcription-quantitative PCR analysis of (A) miR-206 and (B) Cx43 mRNA expression levels in the aforementioned six groups. (C) Detection of Cx43 protein expression levels in the aforementioned six groups. \*\* $P < 0.01$  vs. control; ## $P < 0.01$  vs. irradiation + mimics control; && $P < 0.01$  vs. irradiation + miR-206 mimics + control-plasmid. miR, microRNA; Cx43, connexin 43.

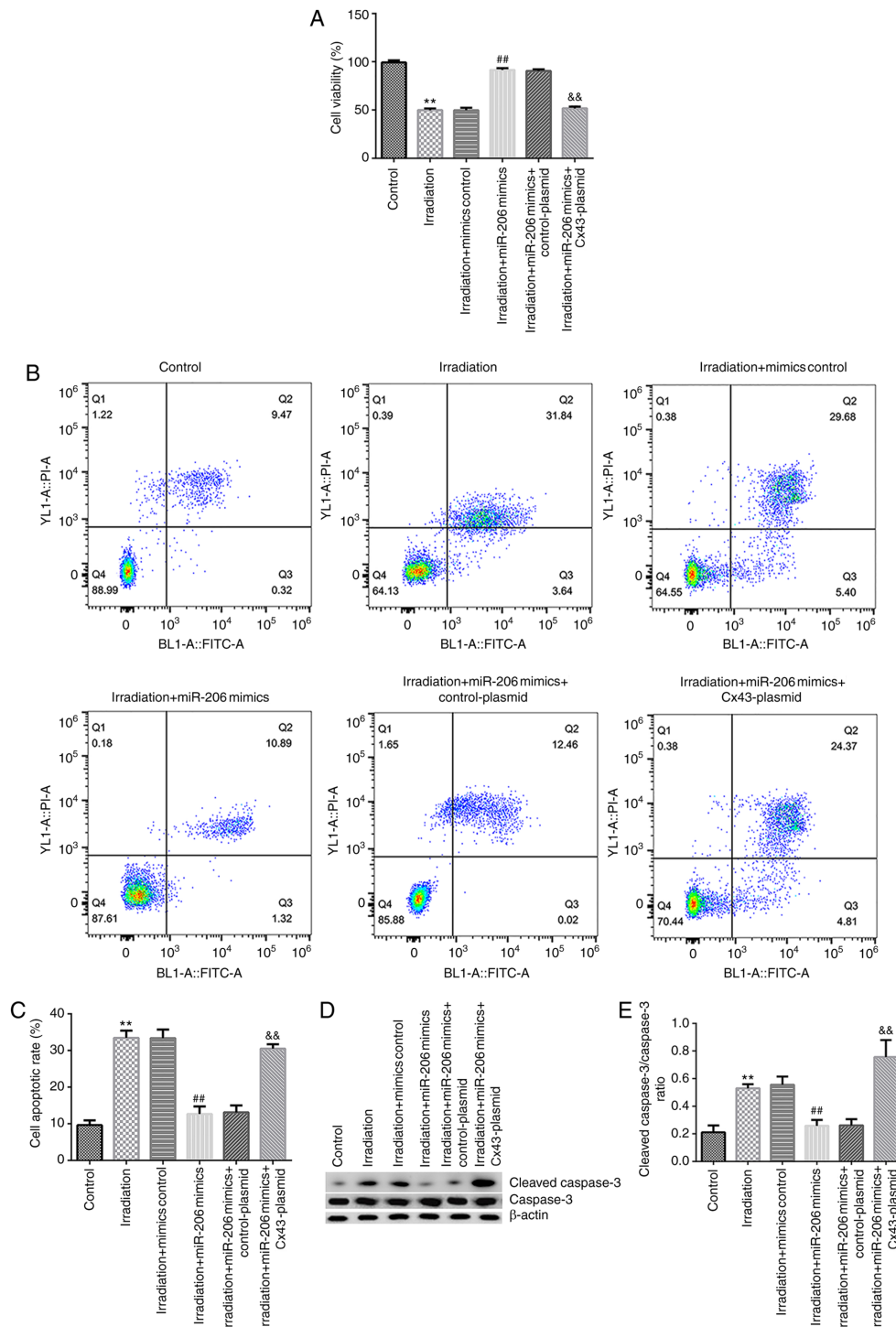


Figure 7. Cx43-plasmid reverses the effects of miR-206 mimics on the proliferation and apoptosis of irradiation-induced HA-1800 cells. Following transfection and radiation stimulation, HA-1800 cells were divided into six groups as follows: Control, irradiation, irradiation + mimics control, irradiation + miR-206 mimics, irradiation + miR-206 mimics + control-plasmid and irradiation + miR-206 mimics + Cx43-plasmid groups. (A) Cell viability was analyzed using an MTT assay. (B) Cell apoptosis was analyzed using flow cytometry. (C) Quantification of apoptotic cells. (D) Western blot analysis of cleaved caspase-3 expression. (E) Determination of cleaved caspase-3/caspase-3 ratio. \*\*P<0.01 vs. control; ##P<0.01 vs. irradiation + mimics control; &&P<0.01 vs. irradiation + miR-206 mimics + control-plasmid. miR, microRNA; Cx43, connexin 43.

in Fig. 8A-D,  $\gamma$ -radiation notably promoted the release of these inflammatory factors, while  $\gamma$ -radiation-induced HA-1800 cells transfected with miR-206 mimics secreted lower levels of inflammatory cytokines compared with those in the irradiation alone group. However, these findings were abolished following transfection with Cx43-plasmid. Taken together, the aforementioned results indicated that miR-206 may reduce the

inflammatory response by negatively regulating Cx43 expression in HA-1800 cells.

## Discussion

Radiation therapy has been widely used to treat several types of cancer, including anal cancer and pituitary adenomas (29,30).

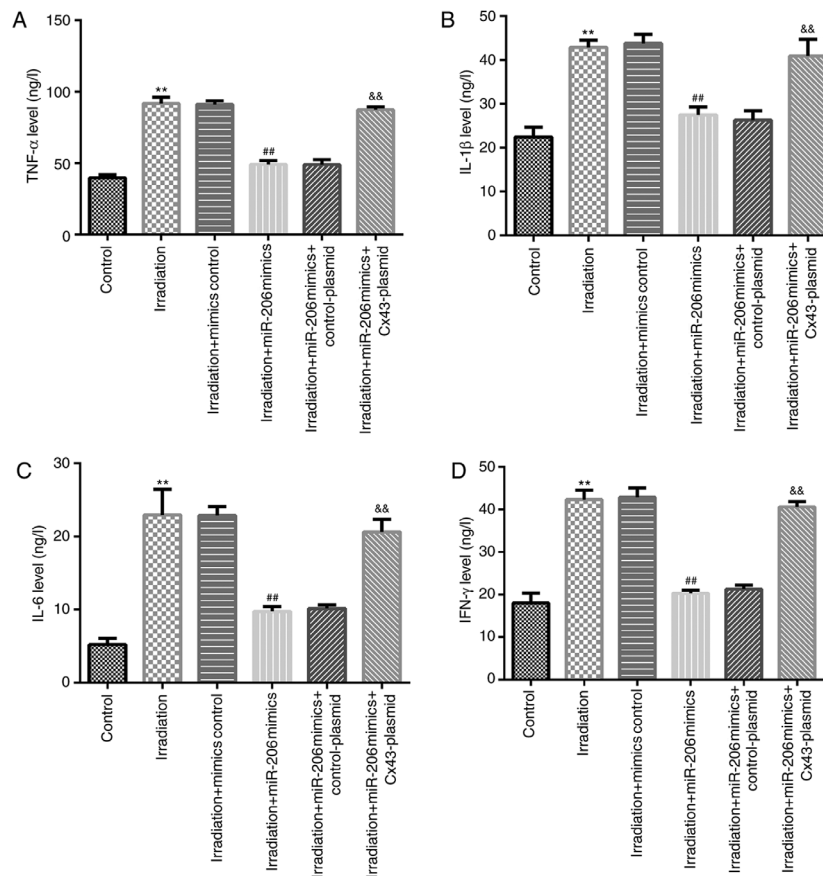


Figure 8. Cx43-plasmid reverses the effects of miR-206 mimics on inflammatory factor release by irradiation-induced HA-1800 cells. Following transfection and radiation stimulation, HA-1800 cells were divided into six groups as follows: Control, irradiation, irradiation + mimics control, irradiation + miR-206 mimics, irradiation + miR-206 mimics + control-plasmid and irradiation + miR-206 mimics + Cx43-plasmid groups. The concentrations of (A) TNF- $\alpha$ , (B) IL- $\beta$ , (C) IL-6 and (D) IFN- $\gamma$  in the supernatant of HA-1800 cells was analyzed using ELISA. \*\*P<0.01 vs. control; ##P<0.01 vs. irradiation + mimics control; &&P<0.01 vs. irradiation + miR-206 mimics + control-plasmid.

Numerous reports have shown that radiation therapy could effectively inhibit tumor growth, with neuroinflammation being the main pathological process. Chang (31) revealed the effect of far-infrared radiation therapy on inflammation regulation in lipopolysaccharide-induced peritonitis in mice. Astrocytes, the most abundant cell type in the CNS, are functionally and structurally associated with numerous pathological responses to disease (32). It has also been reported that astrocytes aggravated ischemic stroke injury; however, they also exert neuroprotective effects by releasing neurotrophic proteins (33,34). At present, certain astrocyte-based neuroprotective treatment strategies include treatment for astrocyte protection (35), astrocyte anti-excitotoxicity and antioxidation (36), as well as astrocyte-derived growth factors (37).

Cxs are transmembrane proteins, which are responsible for intercellular communication (38). Cx43, a member of the Cx family, has been identified as the main component of the gap junction channels in astrocytes. Furthermore, regulating the opening of the Cx43 hemichannel may protect from tissue injury due to excessive activation of the inflammatory response (39). Investigations on the association among neurological diseases, the inflammatory response and Cx channel disorders are currently at the primary stage. Therefore, the present study was designed to establish effective therapy strategies to promote the neuroprotective effects and abolish the detrimental effects of astrocytes.

It has been reported that the upregulation of Cx43 is crucial for radiation-induced neuroinflammation (25). In addition, Cx43 was found to be a direct target gene of miR-206 (24). Thus, in the present study, it was hypothesized that miR-206 may play a protective role in radiation-induced neuroinflammation by regulating Cx43 expression. First, the TargetScan database was used and it was found that Cx43 was a potential target of miR-206. To further investigate the roles of miR-206 in astrocytes, mimics control or miR-206 mimics were transfected into HA-1800 cells. RT-qPCR analysis revealed that the miR-206 expression was significantly higher in HA-1800 cells transfected with miR-206 mimics compared with that in the mimics control group. Dual-luciferase reporter analysis further confirmed that Cx43 directly interacted with miR-206, indicating that the effects of miR-206 on inflammation in astrocytes may be mediated by targeting Cx43.

Astrocytes may exert both harmful and beneficial effects (40). In the present study, the type of astrocyte function induced by  $\gamma$ -radiation was investigated. The HA-1800 cell line was used as a model and cells were induced with 20 Gy  $\gamma$ -radiation for 12, 24 and 48 h to generate an *in vitro* inflammatory model. Then, the effects of  $\gamma$ -radiation on HA-1800 cell viability, apoptosis and inflammatory factor release were analyzed. The results indicated that  $\gamma$ -radiation notably suppressed HA-1800 cell viability, increased the number of apoptotic cells and upregulated release of TNF- $\alpha$ ,



IL- $\beta$ , IL-6 and IFN- $\gamma$ . In addition, the expression of the apoptosis-related proteins, cleaved caspase-3 and caspase-3, was also examined in irradiation-induced HA-1800 cells and the results demonstrated that cleaved caspase-3 protein expression and the cleaved caspase-3/caspase-3 ratio were increased following irradiation. All these findings demonstrated that  $\gamma$ -radiation may promote the detrimental effects of astrocytes. Furthermore, the expression levels of miR-206 and Cx43 in irradiation-treated HA-1800 cells were analyzed. miR-206 was notably downregulated in irradiation-induced HA-1800 cells and the expression level of Cx43 was markedly higher in response to irradiation, compared with that in the control group.

Yang *et al* (41) reported that inhibition of Cx43 and phosphorylated-nuclear receptor subfamily 2 group B member 1 in spinal astrocytes impaired bone cancer pain in mice. Subsequently, it was investigated whether controlling the expression level of Cx43 in astrocytes could eliminate the detrimental effects of irradiation. miR-206 and Cx43 were overexpressed in HA-1800 cells using miR-206 mimics and Cx43-plasmid, respectively, and transfection efficacy was confirmed using RT-qPCR and western blot analyses. Both Cx43 mRNA and protein expression levels were found to be increased in the Cx43-plasmid transfected cells, while miR-206 mimics increased miR-206 expression and reduced Cx43 expression, indicating that miR-206 regulated Cx43 expression in the HA-1800 cells. Research has confirmed that astrocytes play vital roles in the CNS (42); therefore, the roles of miR-206 and Cx43 in  $\gamma$ -radiation-induced HA-1800 cells was investigated. HA-1800 cells were transfected with mimics control, miR-206 mimics, control-plasmid or Cx43-plasmid for 24 h, then induced with 20 Gy  $\gamma$ -radiation for another 24 h. The expression levels of Cx43 were decreased in cells transfected with miR-206 mimics, which indicated that miR-206 could abolish the upregulated Cx43 expression stimulated by  $\gamma$ -radiation. Furthermore, the expression level of Cx43 was higher in the irradiation + miR-206 mimics + Cx43-plasmid group compared with that in the irradiation + miR-206 mimics + control-plasmid group, whereas the miR-206 expression level in the irradiation + miR-206 mimics + Cx43-plasmid group did not change, suggesting that miR-206 negatively regulated Cx43 expression in  $\gamma$ -radiation-induced HA-1800 cells.

To further elucidate the regulatory functions of miR-206 and Cx43 in  $\gamma$ -radiation-induced HA-1800 cells, the viability and apoptosis of astrocytes following irradiation treatment was investigated. It was found that miR-206 mimics relieved irradiation-induced neuroinflammation, which was confirmed by increased cell proliferation, inhibition of apoptosis, and reduced cleaved caspase-3 expression and inflammatory cytokine secretion. These effects were also found to be reversed by Cx43-plasmid, demonstrating that miR-206 protected against  $\gamma$ -radiation-stimulated neuroinflammation by regulating Cx43 expression. Radiotherapy may cause several long-term complications, such as secondary intracranial tumors, hypopituitarism and stroke (6-8). Although radiation therapy is one of the causes of ischemic stroke among patients with pituitary adenoma, the underlying mechanism remains unclear. The findings of the present study indicated that the miR-206/Cx43 axis may be involved in the occurrence and development

of ischemic stroke induced by radiotherapy in patients with pituitary adenoma, providing a theoretical basis for the clinical treatment of such patients. Moreover, miR-206 may serve as a novel potential diagnostic biomarker for ischemic stroke among patients with pituitary adenoma, as this can be measured in the blood or cerebrospinal fluid, which will be further investigated in our future research.

However, this study was only a preliminary *in vitro* study on the role of miR-206 in irradiation-induced neuroinflammation. In order to further verify the role of miR-206 in irradiation-induced neuroinflammation, certain issues must be further explored in depth. For example, it is unclear whether only  $\gamma$ -ray-treated astrocytes can accurately mimic the conditions in the CNS. Furthermore, the interaction between astrocytes and the surrounding microenvironment under the effect of  $\gamma$ -rays must be further examined. In addition, the role of miR-206 in irradiation-induced neuroinflammation should be investigated using animal models. We plan to address these issues in future studies.

In summary, the results of the present study revealed that miR-206 relieved irradiation-induced neural injury by regulating Cx43 expression in astrocytes, suggesting that the miR-206/Cx43 axis may serve as a novel potential diagnostic biomarker for the clinical treatment of inflammation-associated neural injury in radiation-induced diseases.

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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

WZ contributed to the conception and design of the study, data acquisition, analysis and interpretation, and also drafted and critically revised the manuscript. LF contributed to data collection and statistical analysis. HX contributed to data collection, statistical analysis and manuscript preparation. WZ and HX confirmed the authenticity of all the raw data. All authors have 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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