HMGB1 mediates low-dose ionizing radiation-induced Wnt/β-catenin activation in SRA01/04 cells: Mechanistic clues to early cataractogenesis

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Abstract. Emerging evidence from our prior investigations has elucidated the dose-dependent regulatory effects of low-dose ionizing radiation on cellular behaviors including proliferation, migration and differentiation in HLE-B3 lens epithelial cells, with concomitant activation of the canonical Wnt/β-catenin signaling cascade. To extend these findings to alternative cellular models, the present study systematically evaluated the biological responses of the well-characterized human lens epithelial cell line SRA01/04 to low-dose ionizing radiation exposure (0.05-0.2 Gy) versus high-dose radiation (0.5-2 Gy), with particular emphasis on temporal dynamics during acute (0-72 h) and chronic (7 days) phases. Mechanistically, lentivirus-mediated RNA interference was employed to establish stable High mobility group box protein 1 (HMGB1)-knockdown cell models, enabling rigorous interrogation of β-catenin subcellular localization and functional readouts under 0, 0.1 and 0.2 Gy γ-ray exposures. Key findings revealed the following: i) low-dose ionizing radiation within the 0.05-0.2 Gy range significantly potentiated SRA01/04 cell proliferation and migration capacity ($P<0.05$), concomitant with nuclear accumulation of β-catenin; ii) genetic ablation of HMGB1 abolished radiation-induced β-catenin nuclear translocation, resulting in 77% reduction in proliferation rate and 82% suppression of migratory activity compared with wild-type counterparts under equivalent radiation. The experimental evidence identifies HMGB1-mediated signaling as the critical molecular nexus connecting low-dose ionizing radiation exposure to dysregulated Wnt/β-catenin activity in lens epithelium, offering a new therapeutic target for preventing radiation-related cataracts.

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Introduction

With the widespread use of nuclear energy technology across various fields, the likelihood of humans being exposed to low-dose ionizing radiation has significantly increased, and the resulting health issues have garnered attention. Different tissues in the human body exhibit varying levels of tolerance to radiation, with the eye lens being particularly sensitive. A study by Neriishi *et al* (1) on postoperative cataract cases among atomic bomb survivors revealed a significant dose-response relationship between radiation exposure and the incidence of postoperative cataracts. For each 1 Gy increase in radiation dose, the ratio of postoperative cataract incidence (OR) rose by 1.39, with an estimated dose threshold of 0.1 Gy (1). In a nearly 20-year prospective cohort study of radiation technicians in the United States, the risk of cataract was also elevated in the group with the highest occupational exposure to ionizing radiation (average dose 60 mGy) compared with the lowest group (average dose 5 mGy) (2). Radiation-induced cataract is no longer regarded as a typical tissue response with a clearly defined threshold for relatively high doses.

Epidemiological studies have found that chronic occupational radiation exposure significantly increases the risk of posterior subcapsular, cortical and nuclear cataracts, especially posterior subcapsular cataracts, with a higher risk observed in women than in men (3). Following exposure to low-dose ionizing radiation, the lens fails to develop normally, leading to slow DNA damage and repair within lens epithelial cells (LECs) (4), as well as abnormal proliferation and differentiation (5), abnormal degradation of cell organelles (6), and proteomic and liposomal changes (7), ultimately resulting in lens opacity and cataract formation. A previous study demonstrated that low-dose ionizing radiation can enhance the

proliferation and migration of HLE-B3 cells, and upregulates the protein levels of β-catenin, cyclin D1 and c-Myc, which is linked to the activation of the Wnt/β-catenin signaling pathway (8). Vigneux *et al* (9) irradiated HLE-B3 cells with X-rays, which also revealed a clear radiation damage response. The proliferation and migration of HLE-B3 cells significantly decreased after 0.2 Gy irradiation, with proliferation peaking at 7 days post-irradiation (9). By contrast, the specific reaction of SRA01/04 cells to radiation damage has not been reported in detail. SRA01/04 cells and HLE B3 cells are both Human lens epithelial cell lines. The SRA01/04 cell line is widely used in studying pathogenesis of cataracts, such as the oxidative stress model, autophagy regulation and lens fibrosis. According to the literature, whole-genome expression analysis of HLE-B3 and SRA01/04 cells were performed by Illumina Human HT12 Expression Bead Chip microarray. The results showed that both cell lines significantly expressed several genes related to lens biology or cataract, including PAX6, ZEB2, PVRL3, SPARC, COL4A1 and SLC16A12 (10). As another human lens epithelial cell line, SRA01/04 cells may exhibit different biological characteristics under the same experimental conditions. Importantly, based on prior studies, the detailed molecular mechanism by which the Wnt/β-catenin signaling pathway induces lens opacity in response to low-dose ionizing radiation urgently requires further investigation.

A preliminary gene chip analysis results showed that the expression of the HMGB1 gene underwent significant changes in SRA01/04 cells after 0.2 Gy of γ-radiation (11). The expression of HMGB1 is closely related to cell survival, proliferation and migration (12). Shu *et al* (13) found that increased expression of HMGB1 in chondrocytes induces phosphorylation of GSK-3β and upregulates β-catenin expression, thereby promoting chondrocyte apoptosis and cartilage matrix degradation. Wang *et al* (14) found that elevated expression of HMGB1 promotes the proliferation and migration of lung cancer cells, and this oncogenic behavior is mediated through the activation of the Wnt/β-catenin signaling pathway. Chen *et al* (15) also reported that elevated expression of HMGB1 in human bronchial epithelial cells leads to the phosphorylation and inactivation of GSK3β through the PI3K/AKT signaling pathway, which results in the accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus for expression, thereby inducing the occurrence of epithelial-mesenchymal transition (EMT). Therefore, it was hypothesized that the increase of HMGB1 expression in LECs induced by low-dose ionizing radiation can activate the Wnt/β-catenin signaling pathway, thus promoting the proliferation and migration of LECs and inducing lens opacification.

To address the knowledge gaps in understanding the damaging effects of SRA01/04 cells under low-dose ionizing radiation and the molecular mechanisms regulated by the Wnt/β-catenin signaling pathway, SRA01/04 cells were infected with lentivirus to inhibit HMGB1 expression, and the regulatory relationship between HMGB1 expression and the Wnt/β-catenin signaling pathway in these cells following low-dose ionizing radiation exposure. The present study was specifically designed to address two fundamental objectives: First, to delineate the mechanistic relationship between low-dose ionizing radiation-induced alterations in proliferative and migratory capacities of SRA01/04 cells and concurrent Wnt/β-catenin signaling activation. Second, to systematically characterize the molecular circuitry governing radiation-triggered Wnt/β-catenin pathway activation in human LECs, with particular emphasis on identifying key regulatory nodes within this pathological cascade.

Materials and methods

SRA01/04 cell line. SRA01/04 cells are adherent cells obtained from the American Type Culture Collection and are derived from normal LECs of infants with retinopathy of prematurity. The cell line was established by transfecting these cells with a plasmid vector carrying the Simian Virus 40 large T antigen (TagSV40) (16). Cells were authenticated by STR profiling and verified to be mycoplasma-free.

Cell irradiation. In the present study, low doses were set at 0.05, 0.075, 0.1 and 0.2 Gy, while high doses were at 0.5 and 2 Gy, serving as controls. The irradiation methods were as follows: The SRA01/04 cells were irradiated with 0.05, 0.075, 0.1, 0.2 and 0.5 Gy gamma rays using a ^{137}Cs radiation source at a dose rate of 9.73 mGy/min, conducted at the National Secondary Standard Dosimetry Laboratory of the Radiation Safety Institute of the Chinese Center for Disease Control and Prevention. A ferrous sulfate dosimeter was utilized for radiation source calibration to ensure accurate dosing. SRA01/04 cells were also irradiated with 2 Gy γ-rays from a ^{60}Co radioactive source at a dose rate of 1 Gy/min at the Beijing Irradiation Center. The distance from the sample to the cobalt source was 68 cm, and the dose was calibrated by physical measurement using an ionization chamber, with a dose calibration uncertainty of 0.1%.

Cell proliferation assay. For this assay, SRA01/04 cells (6,000 cells/well) were seeded in 100 μl of medium in 96-well plates, with six wells seeded for each group. On the second day after adherent growth, the cells were randomly assigned to the 0-2 Gy irradiation groups. A total of 10 μl of Cell Counting Kit-8 (CCK-8) reagent (Dojindo Laboratories, Inc.) were added to each well at various times post-irradiation, and the plates were incubated for 1 h at 37˚C. Absorbance values were measured at 450 nm using a microplate reader once color development was adequate. This experiment was independently repeated three times. Cell viability was determined using the following formula: Cell viability=(irradiated group optical density (OD) value-blank group OD value)/(0 Gy group OD value-blank group OD value).

Gap closure assay. SRA01/04 cells in favorable growth condition were harvested, and 50 μl of the cell suspension at a density of 2x10^5 cells/ml was seeded into each well of an Ibidi culture insert, placed within a culture dish. A total of 1 ml of medium with 10% FBS was added to the dish surrounding each insert, with three replicate wells for each group (17). The following day, after the cells had adhered, they were irradiated with γ-rays at doses of 0, 0.05, 0.075, 0.1, 0.2, 0.5 and 2 Gy. Immediately after irradiation, the inserts were gently removed using sterile tweezers to create uniform cell-free wounds (scratches). The dishes were then returned to the incubator.

Cell migration into the gap area was monitored at 8, 24, 48 and 72 h using an inverted microscope, and images were captured. Closure of the gap at each time point was measured using ImageJ 1.54 software (National Institutes of Health). The gap closure rate was calculated as follows: The Gap Closure Rate (%)=[(Initial Gap Width-Gap Width at Time t)/Initial Gap Width] x100%. In total, 3 fields of view were analyzed for each dosage and time point, and an average gap closure rate was calculated.

Transwell migration assay. The healthy SRA01/04 cells were inoculated into T-25 culture flasks and subjected to γ-ray irradiation at various doses: 0, 0.05, 0.075, 0.1, 0.2, 0.5 and 2 Gy. At 8 h, 48 h and 7 days post-irradiation, the cells were resuspended at a density of $4x10^4$ cells/ml. A total of 200 μl of the suspension was added to the upper chamber of Transwell inserts (5-μm pore size). The lower chamber received 600 μl of medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) as a chemoattractant. After the cells adhered to the chamber surface, the medium with 10% FBS in the chambers was replaced with serum-free medium. The cells were allowed to migrate in a 37˚C incubator for 8 h. The medium was discarded, and the Transwell inserts were washed twice with phosphate-buffered saline (PBS). Non-migratory cells on the upper surface were wiped off, while migratory cells on the underside of the filter were fixed with 4% paraformaldehyde at room temperature for 10-30 min, immersed in anhydrous methanol for 20 min, and stained with Giemsa. All migratory cells on the entire membrane surface were counted under a light microscope. This experiment was independently repeated three times.

Clone formation experiment. Following the Transwell migration assay, SRA01/04 cells that had migrated to the lower chamber after γ-ray irradiation at various doses were harvested. These cells were trypsinized, resuspended, and seeded into 6-well plates at a density of 1,000 cells per well (three replicate wells per group). Cells were cultured for 7-10 days, with medium changes every 3 days, to allow clonogenic colony formation. Upon observation of clearly defined colonies (cell clusters larger than 50 μm in diameter), cultures were terminated. Cells were gently washed once with 1X PBS, fixed with anhydrous methanol for 30 min, and stained with Giemsa for 30 min. After air-drying at room temperature, cell clones were manually counted and images were captured.

Mouse irradiation and Giemsa staining of posterior lens capsule. Six-week-old Specific pathogen free (SPF) C57BL/6J mice (15 males weighing 20±2 g and 15 females weighing 18±2 g) were ordered from Beijing Vital River Laboratory Animal Technology Co., Ltd. [License no.: (SCXK (Beijing) 2021-0006], raised in the animal room of the Institute of Occupational Health and Poisoning Control, Chinese Center for Disease Control and Prevention. Each cage contained 6 mice housed under 12/12-h light-dark cycle. The temperature was kept at ~22˚C and humidity at 40-60%. Sterile distilled water and SPF-grade feed were provided *ad libitum*. The mice were checked daily for weight, health and behavior.

C57BL/6J mice (three males and three females per group) were subjected to whole-body γ-ray irradiation at doses of 0, 0.05, 0.1, 0.2 and 2 Gy. For the 0.05, 0.1 and 0.2 Gy irradiations, a ^{137}Cs radiation source was used with a dose rate of 9.73 mGy/min. For the 2 Gy irradiation, a ^{60}Co radiation source was employed with a dose rate of 1 Gy/min.

Six months after irradiation at different doses, mice were euthanized by an intraperitoneal injection of an overdose of 1% pentobarbital sodium (150 mg/kg), death was confirmed by the absence of a heartbeat and respiratory arrest, and their eyeballs were enucleated. The lenses were dissected out, with the lens capsules separated. The posterior lens capsules of the mice were spread flat on glass slides with the inner side facing up, and Giemsa staining solution was directly applied, allowing it to stain for 20-30 min. The stained posterior capsules were then examined under an inverted microscope for the presence of stained cells.

All animal experimental procedures were approved by the Experimental Animal Welfare and Ethics Committee of the National Institute for Radiological Protection, Chinese Center for Disease Control and Prevention [approval no. (2022)005; Beijing China].

Western blot assay. To analyze protein expression in SRA01/04 cells, cell lysates were first prepared and mixed with 5X loading buffer, followed by boiling at 100˚C for 10 min to denature the proteins. The BCA method was used for protein quantification. The denatured protein samples (30 μg per lane) were then subjected to electrophoresis on 10% sodium dodecyl sulfate-polyacrylamide gels to separate the proteins based on their molecular weights. After electrophoresis, the separated proteins were transferred onto nitrocellulose membranes. The membranes were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature to prevent non-specific binding of antibodies. A variety of primary antibodies, including rabbit anti-β-catenin (1:1,000; cat. no. 8480; Cell Signaling Technology, Inc.), rabbit anti-cyclin D1 (1:200; cat. no. ab16663; Abcam), rabbit anti-c-Myc (1:1,000; cat. no. 18583; Cell Signaling Technology, Inc.), rabbit anti-HMGB1 (1:10,000; cat. no. ab79823; Abcam), rabbit anti-Phospho-β-catenin (1:1,000; cat. no. 9561; Cell Signaling Technology, Inc.), and mouse anti-GAPDH (1:1,000; cat. no. TA-08; ZSGB-BIO), were added and incubated overnight at 4˚C to allow specific binding to their target proteins. After incubation, the membranes were washed three times with TBST and then incubated with anti-mouse secondary antibodies (1:4,000; cat. no. ZB-5305; ZSGB-BIO) or anti-rabbit secondary antibodies (1:3,000; cat. no. ZB-5301; ZSGB-BIO) for 1 h. Immunoreactive bands were detected using the SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher Scientific, Inc.). The membranes were developed and imaged on the ChemiDoc XRC+ chemiluminescence imaging system (Bio-Rad Laboratories, Inc.). The gray value of the bands was measured using Image Lab (Bio-Rad Laboratories, Inc.). All protein expression experiments were independently repeated three times.

Immunofluorescence microscopy analyses. A cell suspension of $2x10^5$ cells/ml was inoculated onto sterile coverslips in a 24-well plate. The following day, after the cells had adhered and proliferated, they were exposed to 0, 0.05, 0.075, 0.1, 0.2, 0.5 and 2 Gy of γ-radiation. After irradiation, the cells were fixed with 4% paraformaldehyde at room temperature

for 10 min. The coverslips were then carefully oriented with the cell-containing side up, and PBS containing 0.2% Triton X-100 was added to permeabilize the cells for 10 min at room temperature. Next, to block non-specific antibody binding, the coverslips were treated with PBS containing 3% BSA (Beijing Solarbio Science & Technology Co., Ltd.) and incubated on a slow shaker at room temperature for 1 h. The primary antibody rabbit anti-β-catenin (1:80; cat. no. 8480; Cell Signaling Technology, Inc.) was added to the samples. After incubation, the coverslips were washed three times with PBS and then incubated with the secondary antibodies: Anti-rabbit IgG (H+L), F(ab')$_2$ Fragment (Alexa Fluor® 594 Conjugate) (1:600; cat. no. 8890; Cell Signaling Technology, Inc.) for 1 h. VECTASHIELD® Antifade Mounting Medium with DAPI (Vector Laboratories, Inc.) was added to stain the nuclei for 10 min in the dark at room temperature. After washing with 1X PBS, the coverslips were mounted. The cellular localization of β-catenin was observed and photographed using a laser confocal microscope (LSM700; Zeiss GmbH).

shRNA lentivirus vector construction. The construction of the shRNA lentiviral vector was completed by Shanghai GeneChem Co., Ltd. The lentiviral vector skeleton was GV493 (hU6-MCS-CBh-gcGFP-IRES-puromycin). A total of three candidate interference sequences were designed for the knockdown of the HMGB1 gene in cells, as detailed in Table I. Negative control (NC) cell lines were constructed from lentiviral vectors with a control sequence (TTCTCCGAACGTGTCACGT) inserted at the same site.

The shRNA expression vector was co-transfected with two packaging plasmids, pHelper 1.0 and pHelper 2.0, into 293T cells using the transfection reagent. Lentiviral particles were harvested from the culture supernatant of 293T packaging cells at 48 h post-transfection. The collected supernatant was pooled and clarified by centrifugation at 4,000 x g for 10 min at 4˚C, followed by filtration through a 0.45-μm membrane. The viral particles were concentrated via ultracentrifugation at 25,000 rpm for 2 h at 4˚C. The resulting viral pellet was resuspended in a sterile PBS solution, aliquoted, and stored at -80˚C.

Well-grown SRA01/04 cells were seeded into 6-well plates. The following day, after cell attachment, they were transduced with lentivirus at a multiplicity of infection (MOI) of 10. The viral supernatant was replaced with fresh complete medium after 16-24 h of incubation, and the cells were cultured for an additional 48 h. Transduction efficiency was confirmed to exceed 80% by assessing the intensity of green fluorescent protein (GFP) expression under a fluorescence microscope. At 72 h post-transduction, the medium was replaced with a selection medium containing 2 μg/ml puromycin to select for stably transduced cells. After 1 week of selection, stable knock-down cell lines were established. Subsequently, the cells were expanded in medium with a reduced puromycin concentration of 0.67 μg/ml. All experiments were conducted following one week of this expansion phase.

Statistical analyses. SPSS 27.0 software (IBM Corp.) was used for statistical analysis of data. GraphPad Prism 9 software (Dotmatics) was used for plotting. Experimental data were expressed as the mean ± standard deviation (SD).

Table I. Interference sequence.

Sequence name	Interference sequence (5'-3')
sh1-HMGB1	GATGCAGCTTATACGAAATAA
sh2-HMGB1	TCGGGAGGAGCATAAGAAGAA
sh3-HMGB1	TTCCTCTTCTGCTCTGAGTAT

Sh-, short hairpin.

Independent sample t-tests were used for comparisons between two groups, while one-way ANOVA followed by Fisher's LSD post hoc test or the Kruskal-Wallis H test followed by Dunn's post hoc test was applied for comparisons among multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of ionizing radiation on cell proliferation and migration. Cell viability was determined by CCK-8 assay. At 8 h after irradiation with 0.05, 0.1 and 0.2 Gy, cell viability was significantly enhanced compared with the non-irradiated group ($P<0.05$). By 24 h post-irradiation, significant enhancement in viability was observed in cells treated with 0.1, 0.2, and 2 Gy, with statistical significance at $P<0.05$ or $P<0.01$. At 48 h post-irradiation, cell viability in the low-dose groups and the 0.5 Gy group was enhanced compared with the non-irradiated group, with the most significant increase observed after 0.2 Gy irradiation ($P<0.001$). However, cell viability began to decline in the 2 Gy group, showing no significant difference when compared with the non-irradiated group ($P>0.05$). At 72 h post-irradiation, cell viability in the low-dose groups and the 0.5 Gy group remained higher than that of the non-irradiated group, while cell viability in the 2 Gy group was significantly reduced compared with the non-irradiated group ($P<0.01$) (Fig. 1A). Additionally, in Fig. 1B it is also illustrated that the OD values of all groups gradually increased from 0 to 48 h post-irradiation. At 72 h, although the OD values of the low-dose and 0.5 Gy groups showed a slight decrease, they remained higher than the non-irradiated group. Overall, from 0 to 72 h post-irradiation, the OD values of the low-dose and 0.5 Gy groups were higher than those of the non-irradiated group, indicating increased cell proliferation. By contrast, the OD value of the 2 Gy group was lower than the non-irradiated group at 72 h post-irradiation, indicating reduced proliferative capacity. These changes suggest that low-dose ionizing radiation (0.05-0.2 Gy) promotes the proliferation of SRA01/04 cells, particularly after irradiation with 0.1 or 0.2 Gy, where significant enhancements in proliferation capacity were observed between 8 and 48 h ($P<0.05$). Conversely, 2 Gy irradiation weakened cell proliferation capacity.

To exclude the potential stimulation effect of low-dose ionizing radiation on early-stage cell proliferation, the CCK-8 method was utilized to evaluate cell viability 7 days post-irradiation with various doses. As depicted in Fig. 1, the cell viability of the 0.2 Gy group was significantly increased

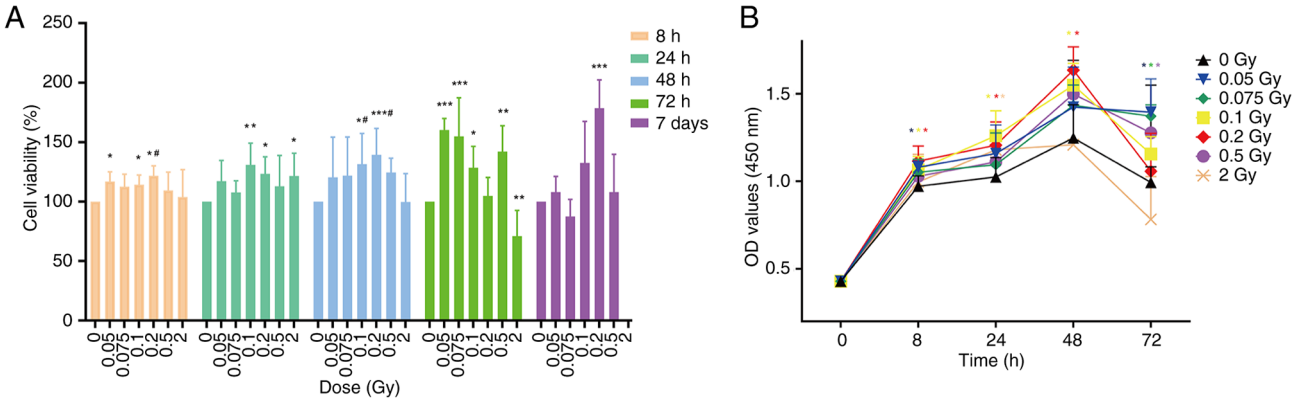

Figure 1. Effects of different doses of γ-rays on the proliferation of SRA01/04 cells. (A) Changes in cell viability at 8-72 h and 7 days after irradiation (n=6). (B) Changes in cell proliferation ability 0-72 h after irradiation (n=6). Data represent the mean±SD of 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.001, compared with the non-irradiated group; $^{\#}$P<0.05 compared with 2 Gy group, One-way ANOVA. OD, optical density.

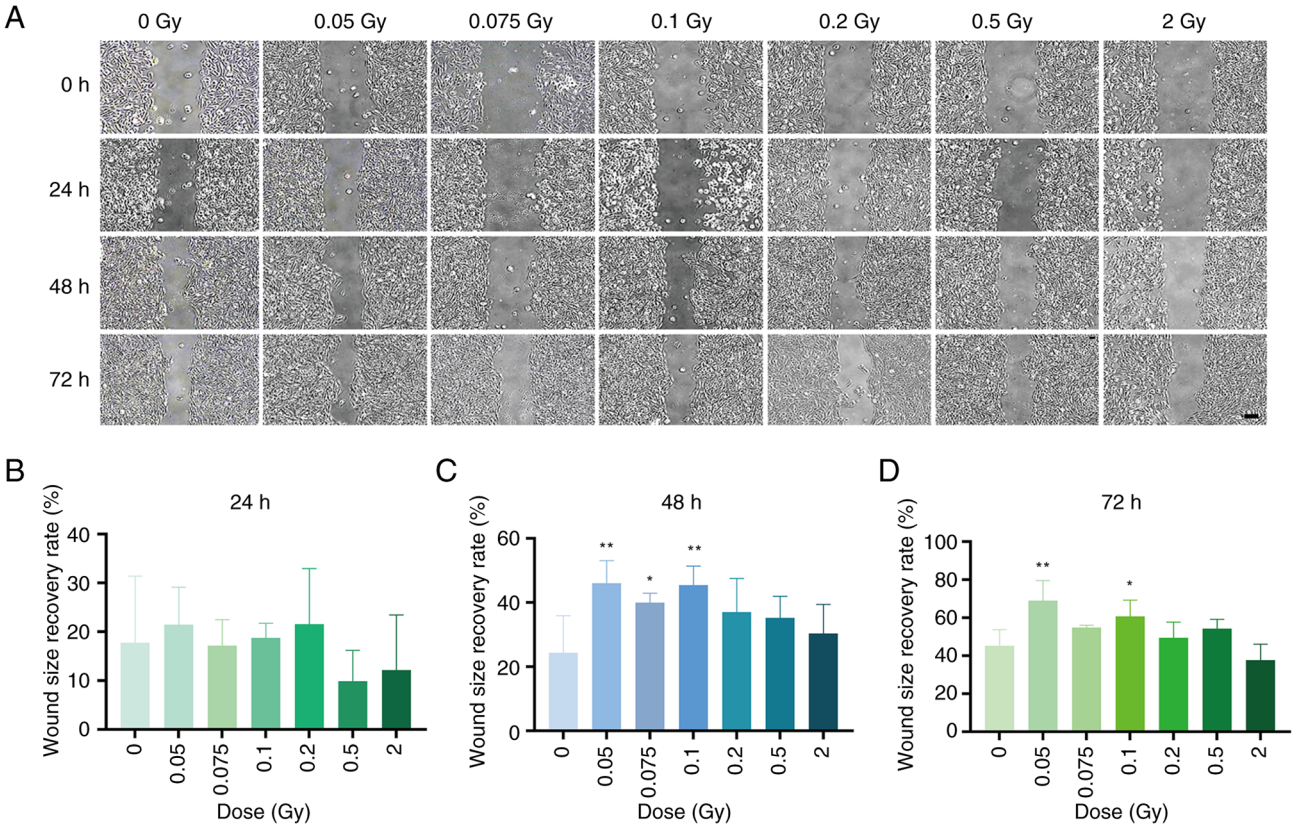

Figure 2. Gap closure assay was used to investigate migration behavior of SRA01/04 cells after exposure to different doses of γ-rays. (A) Representative images of gap closure assay at 0-72 h after scratching. (B-D) Cell gap closure rate at 24 h (B), 48 h (C) and 72 h (D) after irradiation. Magnification, x200. Data represent the mean ± SD of 3 independent experiments. *P<0.05 and **P<0.01, compared with the non-irradiated group, One-way ANOVA.

compared with the non-irradiated group (P<0.001), while the 0.1 Gy group showed a non-significant increase (P>0.05).

The effects of various doses of γ-ray irradiation on SRA01/04 cell migration were evaluated using the gap closure and Transwell migration assay. The gap closure assay indicated that at 48 h post-irradiation with different doses of γ-rays, the gap closure ability of the 0.05, 0.075 and 0.1 Gy groups was significantly enhanced compared with the non-irradiated group (P<0.05 or 0.01). At 72 h post-irradiation, the gap closure ability of the 0.05 and 0.1 Gy groups continued to show significantly increased(P<0.05 or P<0.01), whereas the 2 Gy group displayed a non-significant decrease in gap closure ability (P>0.05) (Fig. 2A and B). The Transwell migration assay revealed that at 8 h after irradiation, all irradiated groups demonstrated a significantly higher number of migratory cells than the unirradiated group (P<0.05 or P<0.01) (Fig. 3A). At 48 h post-irradiation, low-dose irradiated cells exhibited significantly increased migration compared with the non-irradiated group (P<0.05), while no significant difference was observed in the 0.5 and 2 Gy groups (P>0.05) (Fig. 3B).

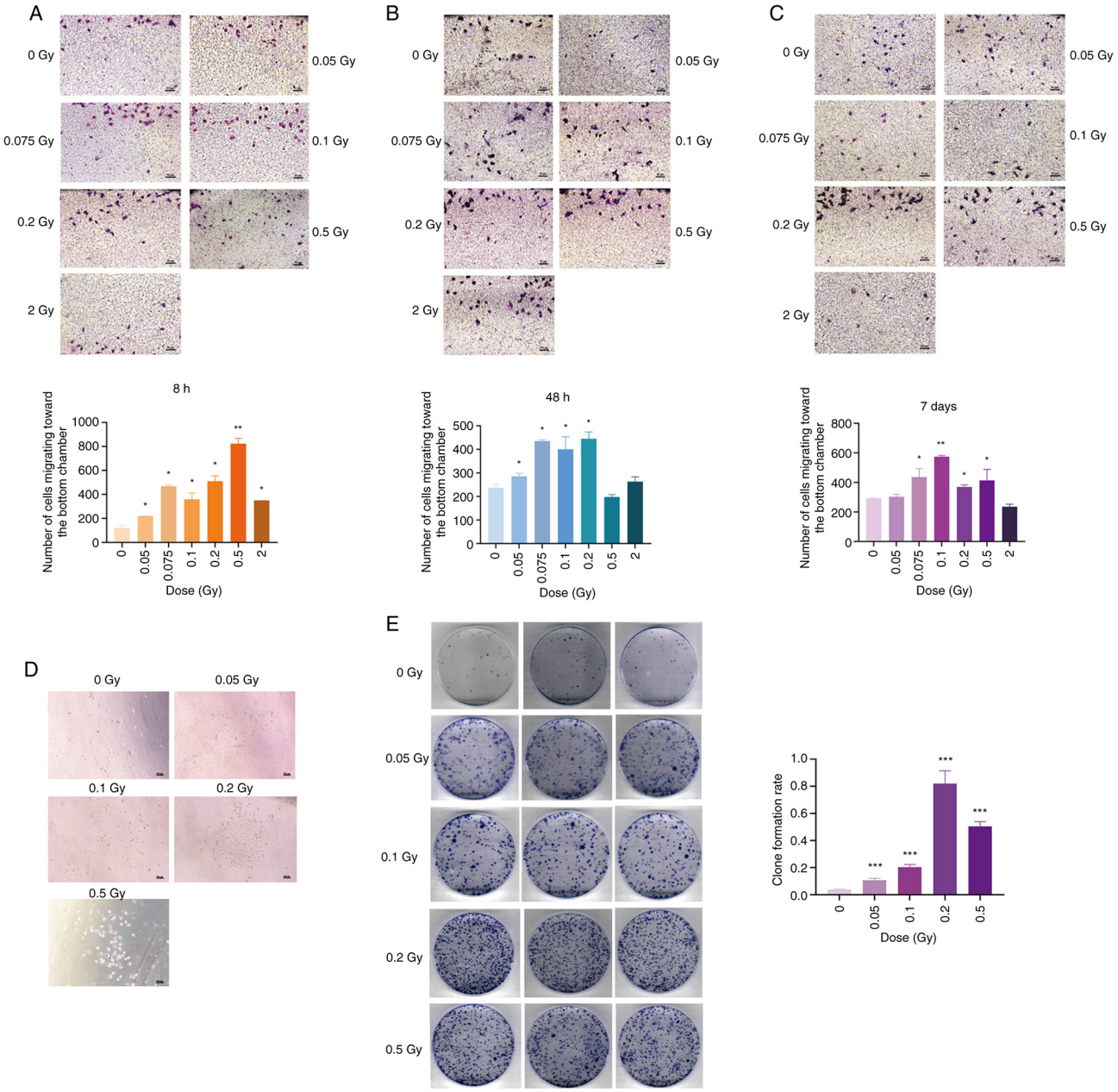

Figure 3. Transwell assay was used to detect the effect of different doses of γ-rays on the migration of SRA01/04 cells. (A-C) Representative images and statistics of Giemsa staining of migratory cells at 8 h (A), 48 h (B) and 7 days (C) after irradiation (n=3). Clone formation experiment was used to detect the proliferation of migratory cells. (D) SRA01/04 cells migrated to culture dishes 7 days after γ-ray irradiation. (E) Representative images and quantitative clonogenic analysis of plate colony formation in SRA01/04 cells (n=3). Scale bar, 50 μm (x200); Scale bar, 100 μm (x100). Data represent the mean ± SD of 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.001, compared with the non-irradiated group, One-way ANOVA.

After 7 days of irradiation with 0.075, 0.1, 0.2 and 0.5 Gy, the number of migratory cells remained significantly higher than in the non-irradiated group (P<0.05 or P<0.01), whereas 2 Gy irradiation resulted in a non-significant decrease in migration ability (P>0.05) (Fig. 3C). Collectively, these findings suggest that low-dose ionizing radiation (0.05-0.2 Gy) promotes SRA01/04 cell migration.

Furthermore, in the Transwell migration assay conducted 7 days after irradiation, it was observed that some SRA01/04 cells not only migrated to the bottom of the chamber but also took up residence within the lower pores, continuing to proliferate along the walls (Fig. 3D). It has been reported that cultured SRA01/04 cells are not sensitive to serum and can survive in a medium containing 1% serum. When serum concentration is restored, these cells can revert to normal proliferation patterns (18). The adherent cells at each dose point were collected, and the proliferation ability of the cells that had migrated into the wells 7 days after irradiation was assessed using a colony formation assay. Results presented in Fig. 3E indicated that 7 days after SRA01/04 cells were irradiated with 0.05, 0.1, 0.2 and 0.5 Gy, the number of clone colonies formed by the migratory cells was significantly higher than in the non-irradiated group (P<0.001). This suggests that low-dose ionizing radiation can promote the proliferation of migratory SRA01/04 cells.

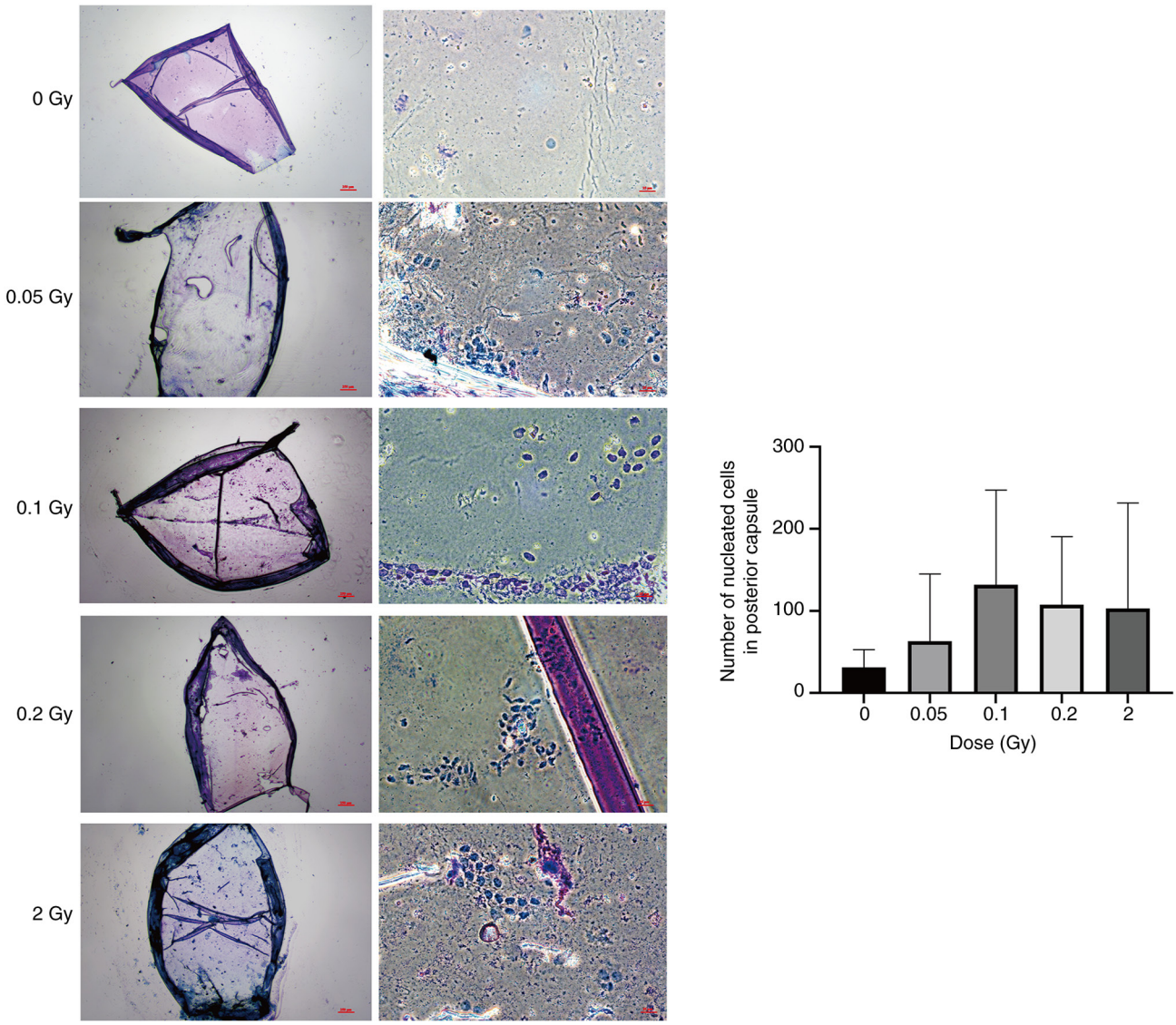

Figure 4. Giemsa staining and statistical images of nucleated cells in the posterior lens capsule of mice at 6 months after irradiation with different doses of γ-rays (n=8). Scale bar, 250 μm (x40); Scale bar, 25 μm (x400). Data represent the mean±SD of 8 mice.

Effect of ionizing radiation on the migration of mouse LECs. In a previous study by the authors, it was reported that in the third month following exposure to low dose ionizing radiation, scattered and randomly distributed migrating cells with regular nuclei were observed within the posterior lens capsule of mice (8). In the current study, both the radiation dose and observation time were escalated. At 6 months after irradiation with low doses (0.05-0.2 Gy) and 2 Gy, scattered nucleated cells with regular nuclear morphology were still observed in the posterior capsule of all irradiated groups (Fig. 4). After 0.1 Gy irradiation, the highest number of nucleated cells in the posterior capsule was observed, consistent with our previous findings. However, at six months post-irradiation, the lens aspect ratio distortion described by Markiewicz *et al* (19) was not observed in either the low-dose or 2 Gy groups. Additionally, no lens opacity was detected.

Effect of low-dose ionizing radiation on β-catenin and its related protein expression in SRA01/04 cells. As shown in Fig. 5, the expression levels of β-catenin and c-Myc in the irradiated group were significantly higher than those in the non-irradiated group at 8 h after exposure to different doses of γ-rays (P<0.05, P<0.01 or P<0.001). Meanwhile, cyclin D1 expression was also significantly elevated in the 0.05 and 0.075 Gy groups relative to the non-irradiated group (P<0.05 or P<0.01) (Fig. 5A). At 24 h post-irradiation, β-catenin and c-Myc expression remained significantly higher in the 0.1 and 0.2 Gy groups (P<0.05, P<0.01 or P<0.001), while c-Myc expression in the 2 Gy group significantly decreased (P<0.001) compared with the non-irradiated group. Moreover, the cyclin D1 expression level was significantly higher in the 0.1 and 2 Gy groups (P<0.001) compared with the non-irradiated group (Fig. 5B). At 48 h, low-dose (0.05-0.2 Gy) and 0.5 Gy irradiation continued to induce significant upregulation of β-catenin and c-Myc compared with the non-irradiated group (P<0.05, P<0.01 or P<0.001). Cyclin D1 expression increased significantly compared with the non-irradiated group 48 h after 0.5 Gy irradiation (P<0.001) but was significantly lower after 2 Gy irradiation compared with the non-irradiated group (P<0.001) (Fig. 5C). These results indicated that

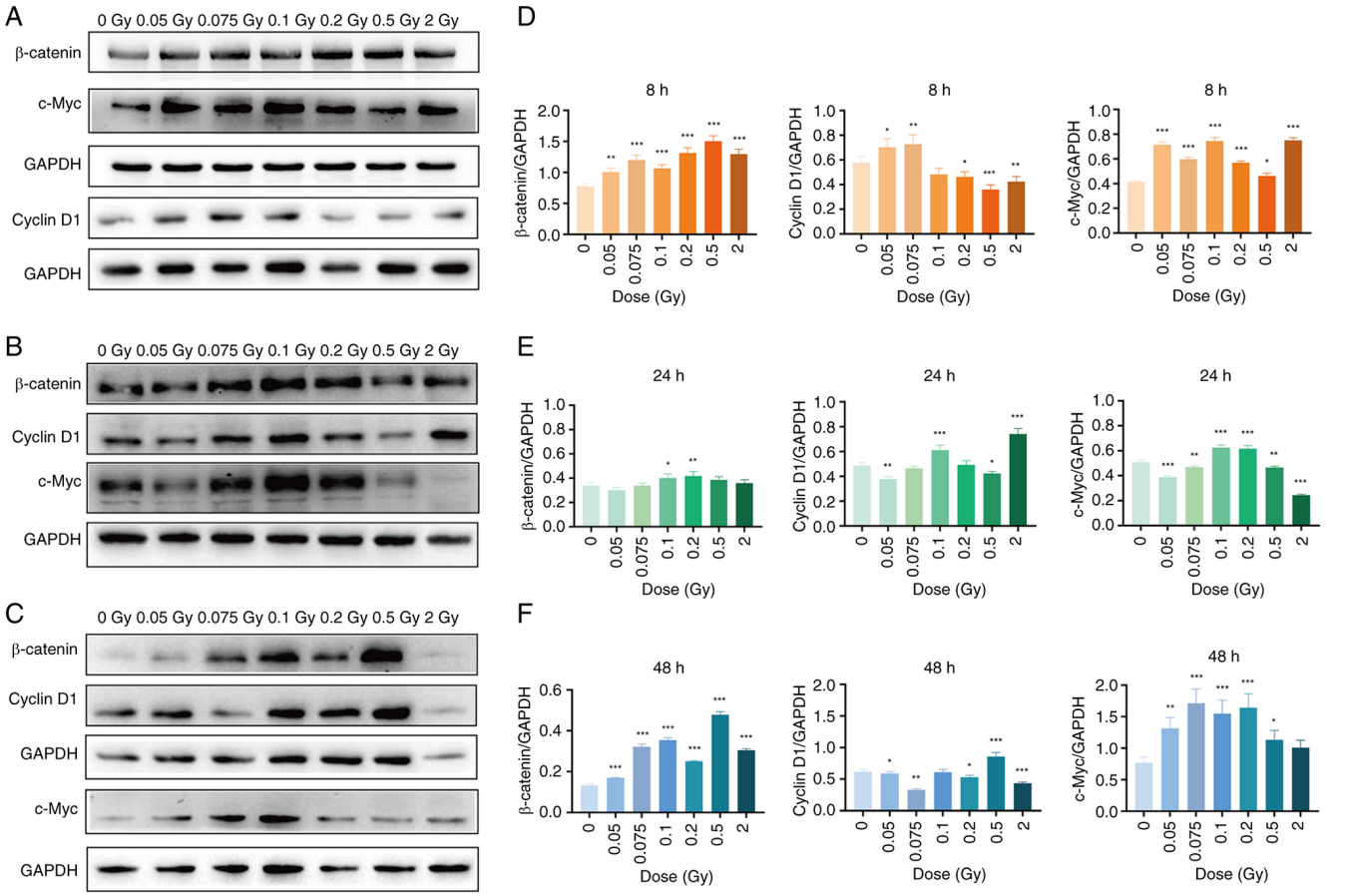

Figure 5. Effect of different doses of γ-rays on the protein expression levels of β-catenin, cyclin D1 and c-Myc in SRA01/04 cells. (A-C) The protein levels of β-catenin, cyclin D1 and c-Myc were analyzed by western blot at 8 (A), 24 (B) and 48 h (C) after irradiation. (D-F) The relative protein expression levels of β-catenin, cyclin D1 and c-Myc were quantitatively determined by the density method at 8 (D), 24 (E) and 48 (F) after irradiation. GAPDH was used as a loading control. Data represent the mean ± SD of 3 independent experiments. *P<0.05, **P<0.01 and ***P<0.001, compared with the non-irradiated group, one-way ANOVA.

low-dose ionizing radiation can differentially upregulate the protein expressions of β-catenin, cyclin D1 and c-Myc in SRA01/04 cells.

To elucidate the impact of γ-ray irradiation on the expression and localization of β-catenin in SRA01/04 cells, immunofluorescence assays were conducted with cells exposed to a spectrum of γ-ray doses. As shown in Fig. 6A and B, β-catenin was mainly localized in the cell membrane and cytoplasm or uniformly expressed on the cell membrane in the non-irradiated group. At 24 and 48 h after low-dose (0.05-0.2 Gy) and 0.5 Gy irradiation, a distinct translocation of β-catenin into the nucleus was observed with notable accumulation within the nucleus. After 2 Gy irradiation, β-catenin was also significantly expressed in the nucleus at 24 h, while the localization of β-catenin did not significantly change compared with the non-irradiated group at 48 h. These results suggested that low-dose ionizing radiation may activate the Wnt/β-catenin signaling pathway in SRA01/04 cells, which is associated with abnormal cell proliferation and migration.

Effect of HMGB1 on the expression and localization of β-catenin after low-dose γ-irradiation. In SRA01/04 cells, HMGB1 expression significantly increased at 24 h after 0.075, 0.1, 0.2 and 0.5 Gy irradiation and at 48 h after 0.1, 0.2, 0.5 and 2 Gy irradiation compared with the non-irradiated group (P<0.001) (Fig. 7A and B). Subsequently, the cells were infected with lentivirus, and the knockout effects of three different interfering sequences targeting HMGB1 were evaluated by western blot analysis. Both sh1 and sh2 interfering sequences markedly reduced HMGB1 protein expression, with the sh1 sequence emerging as the most effective in HMGB1 suppression. The sh1 sequence was selected for subsequent experiments (Fig. 7C). Leveraging this foundation, the intricate relationship between HMGB1 expression in LECs after exposure to low-dose ionizing radiation and the activation of the Wnt/β-catenin signaling pathway were explored.

Previous studies have established that nuclear translocation of β-catenin is a key step in Wnt signaling, while its phosphorylation is the primary mechanism controlling the pathway's 'on' and 'off' states, phosphorylation of β-catenin at Ser33, Ser37 and Thr41 can prevent its nuclear translocation and the activation of target gene transcription (20,21). Western blot analysis was used to assess the expression of total and phosphorylated β-catenin (p-β-catenin) in HMGB1-knockout cells. The results showed that in the non-irradiated groups, there were no significant differences in total β-catenin or p-β-catenin among the different cell groups (P>0.05). However, at 48 h following exposure to low-dose radiation (particularly at 0.2 Gy), the knockdown group exhibited a significantly lower level of total β-catenin compared with

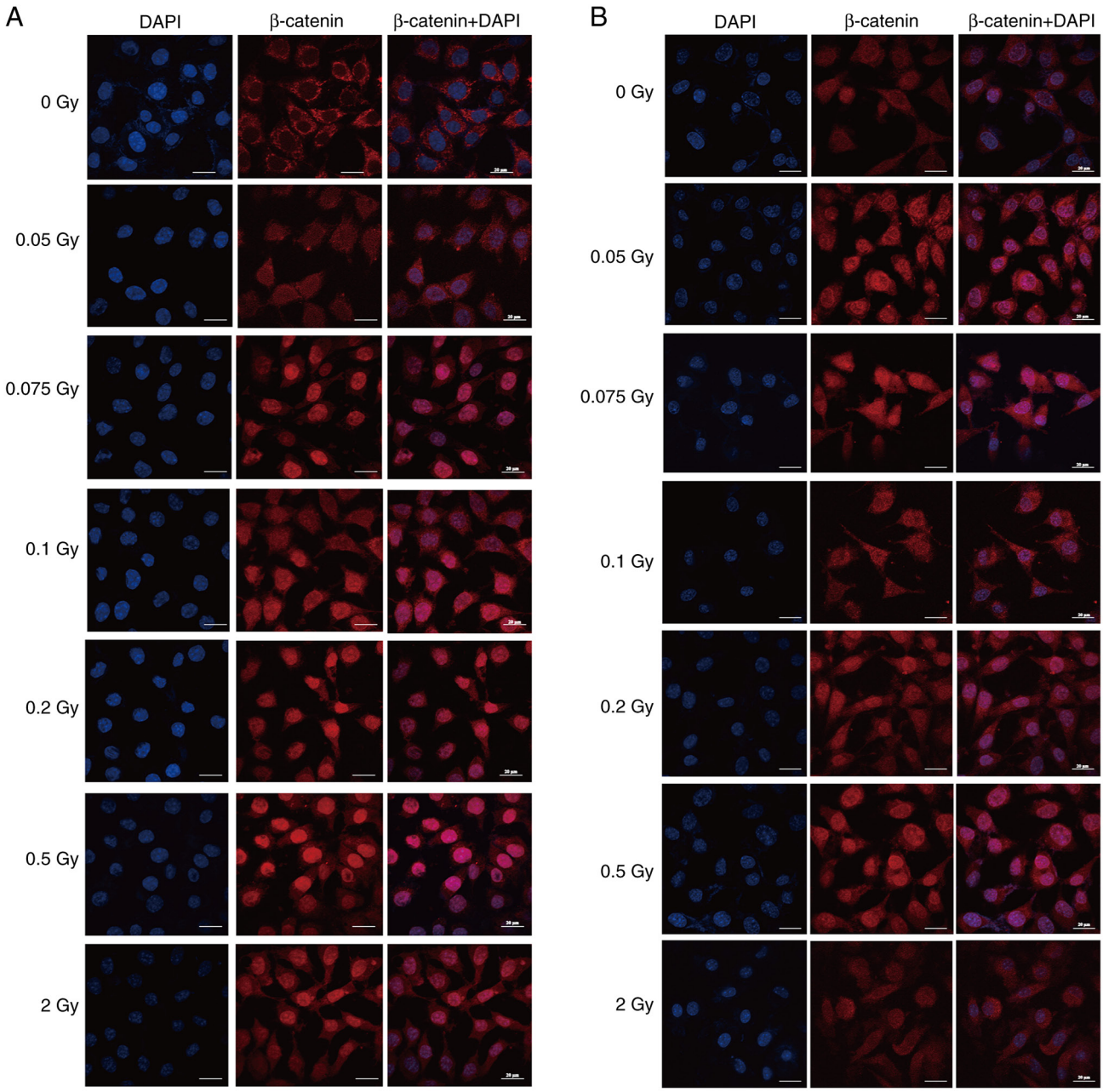

Figure 6. Immunofluorescence microscopy image showing the immunolocalization of β-catenin protein in SRA01/04 cells at 24 (A) and 48 h (B) after irradiation with different doses of γ-rays. This experiment was repeated three times independently. Scale bar, 20 μm.

the wild-type (WT) and NC groups (P<0.01 or P<0.001); conversely, the level of p-β-catenin was significantly higher in the knockdown group than in the WT group (P<0.01), suggesting an inhibition of the Wnt/β-catenin signaling pathway after HMGB1-knockdown (Fig. 8). Using confocal laser scanning microscopy, the localization of β-catenin was examined in HMGB1-knockdown cells exposed to various low doses of γ-radiation. The findings of the present study indicated that in unirradiated cells, β-catenin expression was predominantly confined to the cell membrane and cytoplasm in the WT, NC and HMGB1-knockdown groups, suggesting that the Wnt/β-catenin signaling pathway remained in inactivated state (Fig. 9A). Nevertheless, at 48 h post-exposure to 0.1 and 0.2 Gy radiation, Wnt/β-catenin signaling pathway significantly activated, marked by the expression of β-catenin translocated into the nucleus in the WT and NC groups (Fig. 9B and C). By contrast, in the HMGB1-knockdown group, β-catenin remained predominantly expressed in the cell membrane and cytoplasm, exhibiting minimal nuclear expression and no detectable nuclear translocation (Fig. 9B and C). This observation suggests that low-dose ionizing radiation cannot fully activate the intracellular Wnt/β-catenin signaling pathway in HMGB1-knockdown cells.

Impact of HMGB1 on cell proliferation and migration after low-dose γ-ray irradiation. To investigate the role of HMGB1 in abnormal proliferation and migration of LECs induced by low-dose ionizing radiation, CCK-8 and Transwell migration assays were conducted to assess the

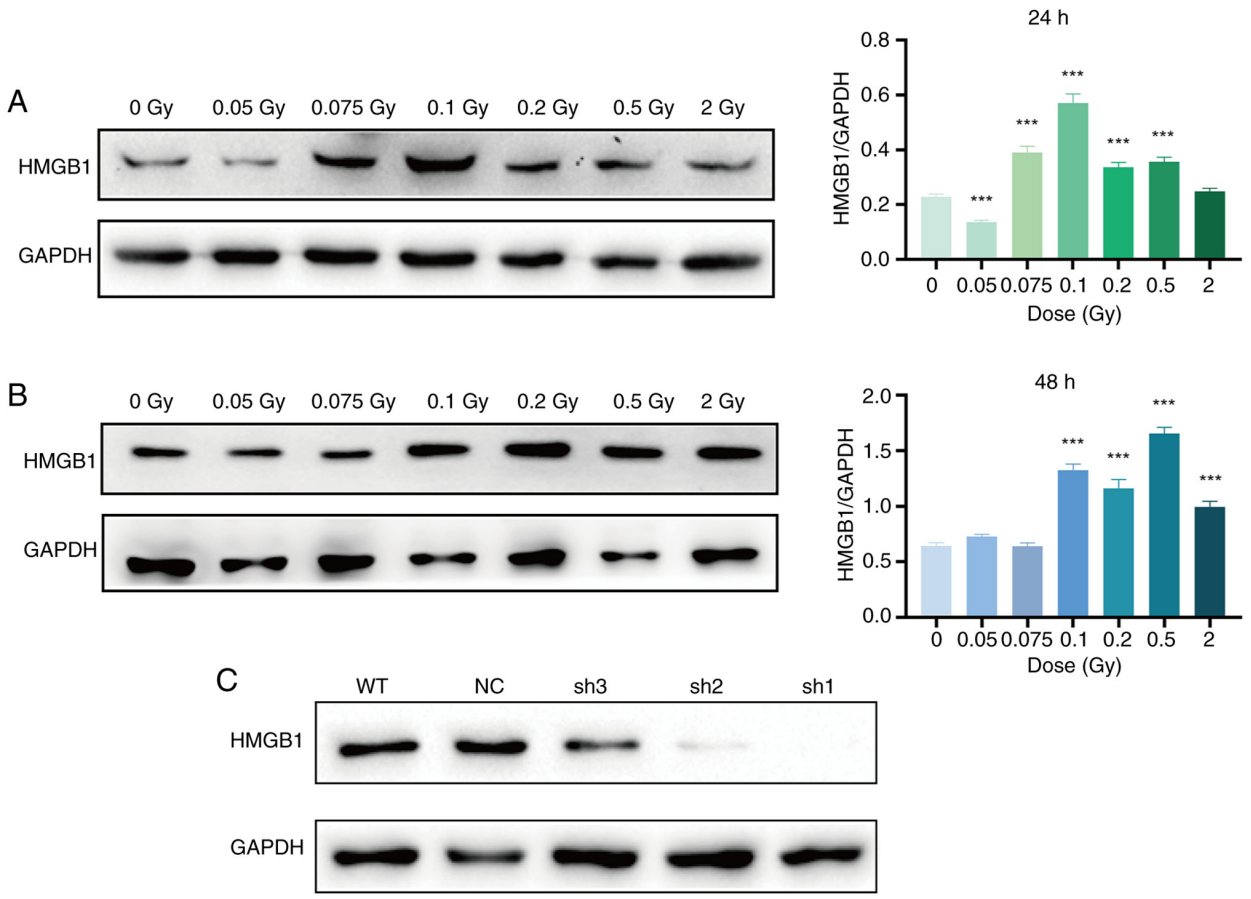

Figure 7. Expression levels and quantitative analysis of HMGB1 in SRA01/04 cells at 24 (A) and 48 h (B) after irradiation with different doses of γ-rays. (C) Identification of lentiviral infected stable HMGB1 knockdown cell lines. GAPDH was used as a loading control. Data represent the mean ± SD of 3 independent experiments. ***P<0.001 compared with the non-irradiated group, One-way ANOVA. HMGB1, High mobility group box protein 1.

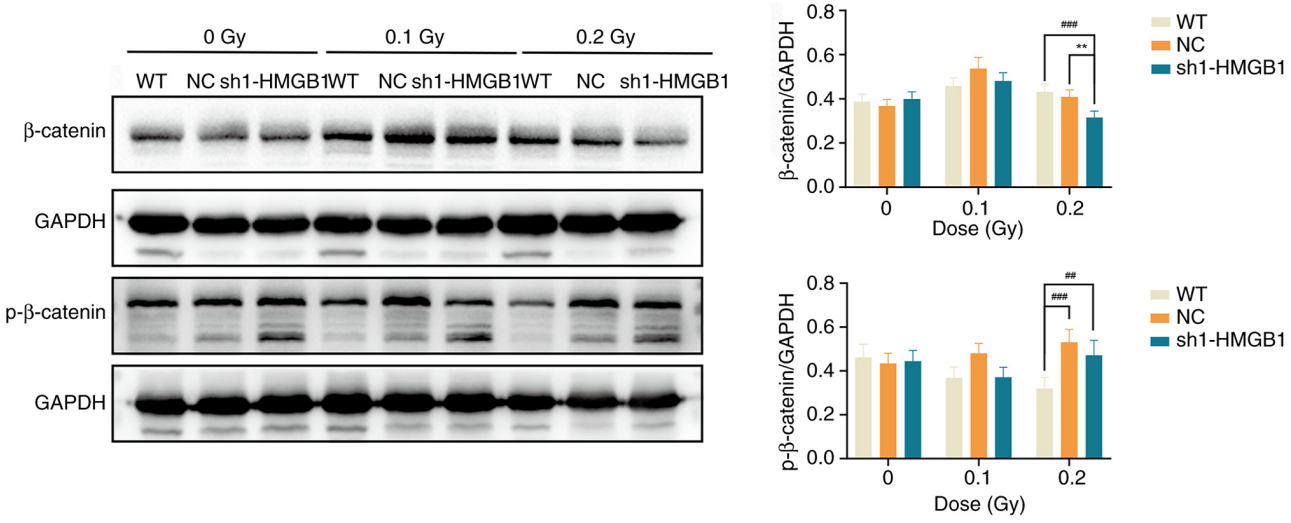

Figure 8. Effect of HMGB1 on the expression of β-catenin in SRA01/04 cells after low-dose γ-ray irradiation. Expression and quantification of total and p-β-catenin in cells at 48 h after irradiation with different doses of γ-rays. GAPDH was used as a loading control. Data represent the mean±SD of 3 independent experiments. **P<0.01, compared with the NC group at the same irradiation dose; ##P<0.01 and ###P<0.001, compared with the WT group at the same irradiation dose, One-way ANOVA. HMGB1, High mobility group box protein 1. p-β-catenin, phosphorylated β-catenin; WT, wild-type; NC, negative control; sh-, short hairpin.

proliferation and migration abilities of HMGB1-knockdown cells at 48 h after 0.1 and 0.2 Gy irradiation. The results of CCK-8 assay indicated that, without radiation, there was no significant difference in OD values between the knockdown group and the NC or WT groups (P>0.05) (Fig. 10A and B), suggesting that cell proliferation was not significantly

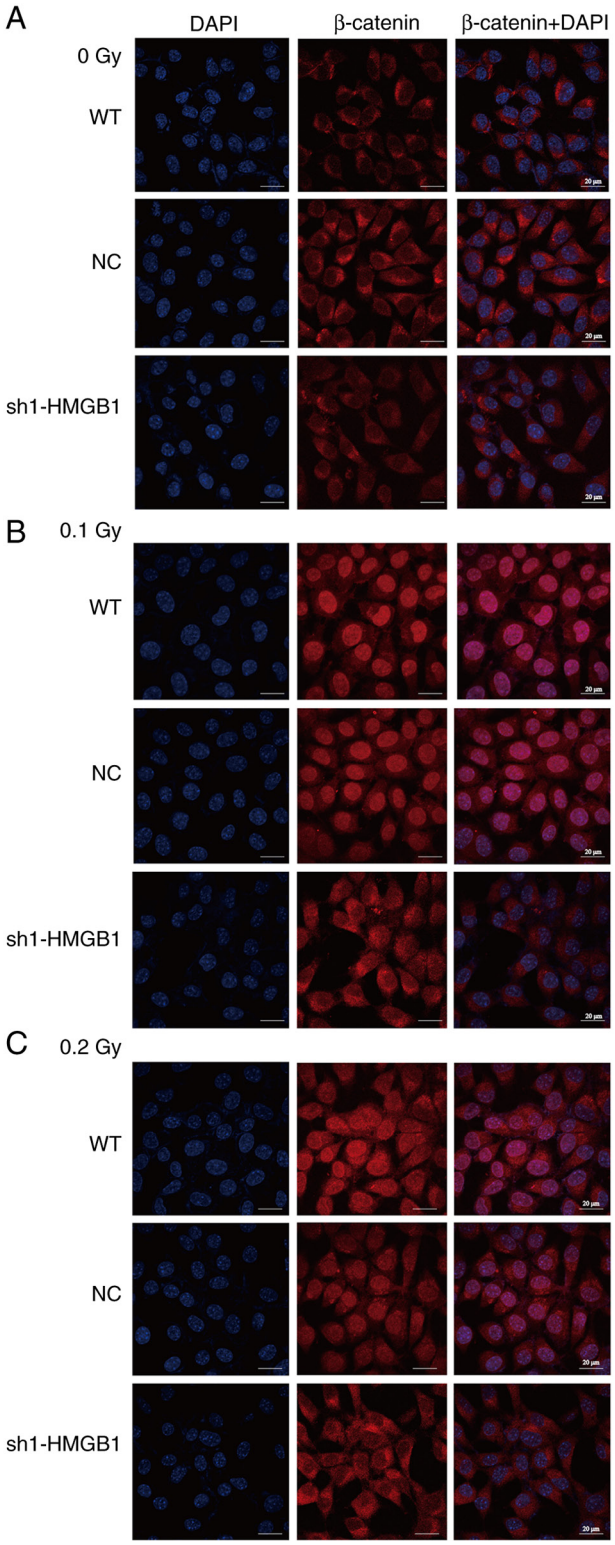

Figure 9. Effect of HMGB1 on the localization of β-catenin in SRA01/04 cells after low-dose γ-ray irradiation. (A) Non-irradiated group. (B) 0.1 Gy irradiation. (C) 0.2 Gy irradiation. This experiment was repeated three times independently. Scale bar, 20 μm. HMGB1, High mobility group box protein 1; WT, wild-type; NC, negative control; sh-, short hairpin.

influenced by HMGB1 knockdown. However, at 48 h after 0.1 Gy irradiation, the cell viability of the knockdown group was significantly diminished compared with the NC group ($P<0.05$) and also demonstrated a reduction compared with the WT group, with lower OD values than both NC and WT groups (Fig. 10A and B). Similarly, at 48 h after 0.2 Gy irradiation, both cell viability and OD values of the knockdown group were significantly lower than those of the NC and WT groups ($P<0.01$) (Fig. 10A and B). These findings indicated that the proliferative ability of the knockdown group is weakened compared with the WT and NC groups after the same low-dose γ-ray exposure.

The results of the Transwell migration assays showed that at 48 h whether the cells were unirradiated or exposed to 0.1 Gy irradiation, the WT group exhibited a significantly stronger cell migration ability compared with both the NC group and the knockdown group ($P<0.05$ or $P<0.01$) (Fig. 10C). This suggested that HMGB1 knockdown diminishes cell migration ability, although it is also possible that the lentivirus itself exerts an influence on the migration of SRA01/04 cells. At 48 h after 0.2 Gy irradiation, the cell migration ability of the knockdown group was significantly weaker than the NC group and the WT group ($P<0.05$ or $P<0.001$) (Fig. 10C). These findings indicated that the migration ability of the knockdown group is weakened compared with the WT groups after the same low-dose γ-ray exposure.

Discussion

The induction of cataracts by ionizing radiation is a complex process likely associated with multiple factors such as DNA damage, oxidative stress and telomere abnormalities (22). Although significant strides have been made in research, the mechanism of opacification in the lens posterior capsule due to low-dose ionizing radiation remains far from fully elucidated.

The present study investigated the potential mechanism by which low-dose ionizing radiation induces abnormal proliferation and migration of LECs, leading to lens opacity. It was initially identified that low-dose ionizing radiation enhanced HMGB1 expression in SRA01/04 cells. Furthermore, the impact of HMGB1 expression on the proliferation and migration of LECs following exposure to low-dose ionizing radiation may be associated with the activation of the Wnt/β-catenin signaling pathway.

It has been previously reported that low-dose ionizing radiation can promote abnormal proliferation and migration of HLE-B3 cells. The HLE-B3 cell line is an immortalized human lens epithelial cell line that maintains proliferation ability, stable epithelial morphology in long-term culture and stable differentiation ability (23). However, a single cell line may not fully replicate the physiological and pathological properties of normal LECs, as these cells may exhibit genetic and phenotypic variations compared with the actual cells. Genetic background plays an important role in low-dose ionizing radiation-induced lens opacity. For instance, Gao *et al* (24) found in a case-control study of individuals from the Yangjiang high-background radiation area that those carrying the allele of ATM rs189037 and the C allele of TP53 rs1042522 have an increased risk of radiation-induced lens opacity. To improve the comprehensiveness of the study, another common human lens epithelial cell line, SRA01/04, was selected. During continuous culture, SRA01/04 cells maintain their characteristics as epithelial cells and do not differentiate into lens fiber cells. The cell proliferation

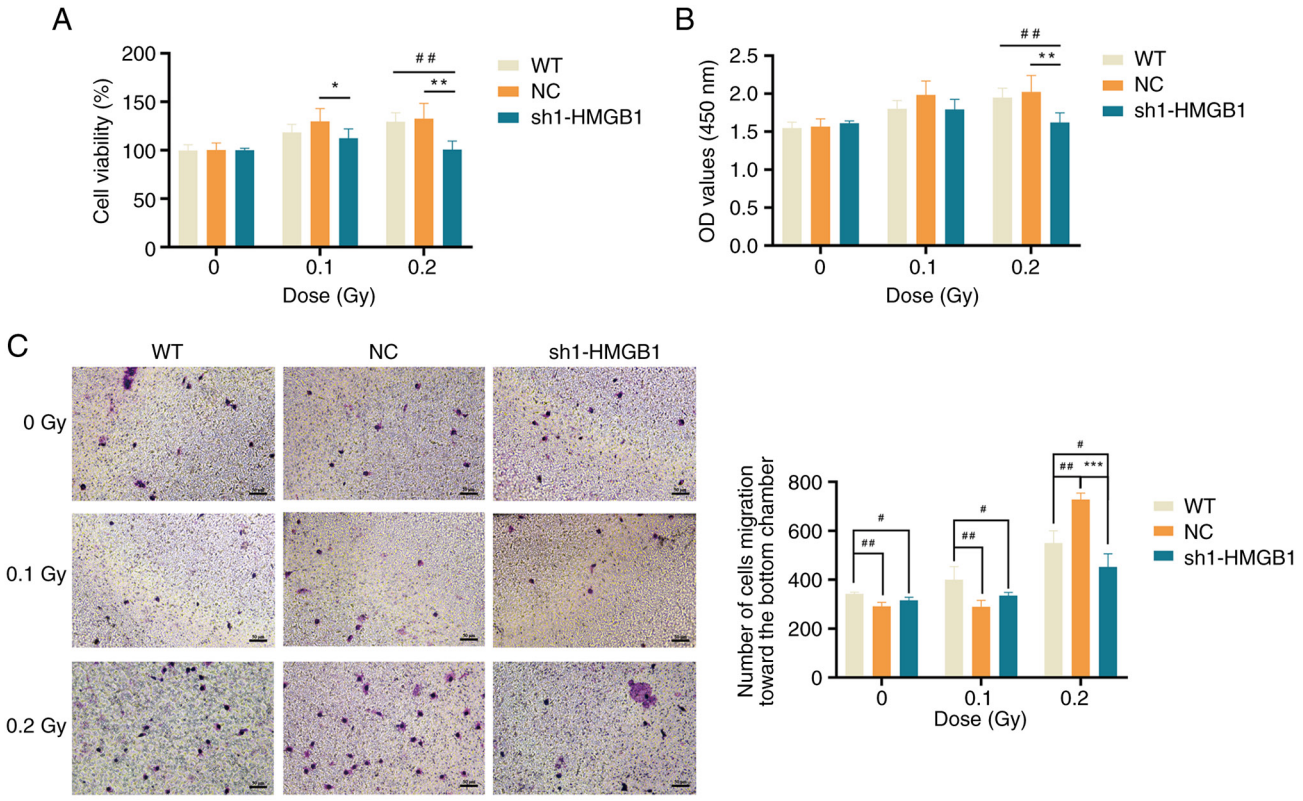

Figure 10. Effects of HMGB1 on the proliferation and migration of SRA01/04 cells after low-dose γ-ray irradiation. (A) Effect of HMGB1 on cell viability (n=6). (B) Effect of HMGB1 on cell proliferative activity (n=6). (C) Representative Giemsa staining and number statistics of cells migrating to the bottom of the chamber at 48 h after 0.1 and 0.2 Gy γ-ray irradiation (n=3). Data represent the mean±SD of 3 independent experiments, Scale bar, 50 μm. *P<0.05, **P<0.01 and ***P<0.001 compared with the NC group at the same irradiation dose; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 compared with the WT group at the same irradiation dose, One-way ANOVA. HMGB1, High mobility group box protein 1; OD, optical density; WT, wild-type; NC, negative control; sh-, short hairpin.

experiment showed that both cell viability and the number of live cells increased after irradiating SRA01/04 cells with 0.05-0.5 Gy γ-rays at 8-72 h. Cell viability remained elevated at 7 days after low-dose irradiation, indicating a lack of significant dose-response relationship. This further supports the idea that low-dose ionizing radiation can promote proliferation in LECs. However, exposure to a higher dose of 2 Gy resulted in a temporary increase in cell proliferative capacity at 24 h post-irradiation. At 72 h and beyond after 2 Gy irradiation, the cell proliferative capacity diminished or showed no significant difference compared with the unirradiated group. In a study by Chen *et al* (25) on the effects of neutron radiation on rat lenses, high-dose ionizing radiation significantly induced apoptosis in LECs, while the low-dose radiation group exhibited less apoptosis. The heightened sensitivity of lens cells to radiation is not necessarily linked to ionizing radiation-induced cell death.

Additionally, results from the migration assay revealed an increase in the number of cells migrating to the bottom of the Transwell chamber at different time points after low-dose ionizing radiation. This was accompanied by an expansion of the gap closure area, indicating that low-dose ionizing radiation enhances the migratory ability of SRA01/04 cells. A colony formation assay demonstrated that low-dose ionizing radiation significantly promoted the proliferation of migratory SRA01/04 cells. It was observed that SRA01/04 cells continued to grow well and maintained their proliferation ability after migrating from serum-free medium to a medium containing 10% FBS following low-dose radiation exposure. The irradiated group exhibited a significantly higher number of colony-forming units compared with the non-irradiated group. Low-dose ionizing radiation also markedly enhanced the proliferation of migratory cells. Meanwhile, the effects of 0.05, 0.1, 0.2 and 2 Gy on the proliferation and density of mouse lens cells over an extended period were assessed, discovering scattered nucleated cells in the posterior capsule of the lens after irradiation. Nucleated cells were also detected in the posterior lens capsule of unirradiated mice, potentially related to a heightened risk of spontaneous cataract development in middle-aged mice. It was hypothesized that extended exposure to low-dose radiation may lead to changes in lens shape and visual impairment over time, which necessitates confirmation through population epidemiological studies.

Precise spatiotemporal regulation of canonical Wnt signaling is crucial for eye development. In a lens injury model simulating cataract surgery, researchers detected Wnt signaling activation in residual LECs within 12 h post-surgery, indicating an upregulation during the development of posterior capsular opacification (26). Numerous studies have implicated the Wnt/β-catenin signaling pathway in the proliferation, migration and EMT of HLE-B3 cells (8,27). It was found that the Wnt/β-catenin signaling pathway also plays an important role in the proliferation and migration of SRA01/04 cells induced by low-dose ionizing radiation. The protein expressions of β-catenin, cyclin D1 and c-Myc

in SRA01/04 cells were increased to varying degrees after different doses of irradiation at various times. At certain time points, the expression of these three proteins exhibited a double-phase reaction; that is, the expression level increased with the dose after low-dose irradiation, usually peaking at 0.1 Gy, and could increase again after higher doses. Similar observations were reported by Chauhan *et al* (28). Whole-genome sequencing was performed on HLE cells 20 h post-exposure to X-ray irradiation at doses of 0, 0.01, 0.05, 0.25, 0.5, 2 and 5 Gy. Some genes demonstrated a non-linear biphasic response in terms of fold change at low doses (<0.5 Gy), reaching a peak at 0.25 Gy. At doses greater than 0.5 Gy, the fold change of these genes gradually increased. Low-dose ionizing radiation significantly reduced the expression of β-catenin protein on the cell membrane and increased the nuclear translocation of β-catenin, suggesting that the proliferation and migration of SRA01/04 cells promoted by low-dose ionizing radiation were related to the activation of the Wnt/β-catenin signaling pathway. The molecular mechanism by which low-dose ionizing radiation activates the Wnt/β-catenin signaling pathway inducing lens opacity will be explored.

HMGB1, one of the most abundant non-histone nuclear proteins, is normally located in the nucleus and plays a role in regulating gene transcription, DNA damage repair and chromosome structure stability (29). It can also be released passively by dead or damaged cells or actively secreted by activated immune cells into the extracellular environment, triggering an inflammatory response (30). Increased expression and secretion of HMGB1 are associated with tumor proliferation, invasion and migration in various cancers, and HMGB1 can serve as a potential biomarker to identify tumors (31). In the present study, HMGB1 expression in SRA01/04 cells was inhibited using lentiviral infection technology. Following 0.2 Gy γ-ray irradiation, total β-catenin expression decreased in the knockdown cells compared with WT cells, while phosphorylated β-catenin expression increased. Immunofluorescence analysis further revealed that after low-dose γ-ray irradiation, β-catenin no longer translocated to the nucleus in HMGB1-knockdown cells. Genetic ablation of HMGB1 abolished radiation-induced β-catenin nuclear translocation, suggesting that HMGB1 may be involved in regulating the Wnt/β-catenin signaling pathway activated by low-dose ionizing radiation. Previous studies also have reported that the Wnt/β-catenin signaling pathway is regulated by HMGB1. Wang *et al* (32) found that miR-665 deactivates the Wnt/β-catenin signaling pathway in retinoblastoma by directly targeting HMGB1, thereby inhibiting tumor development. Zhou *et al* (33) showed that administering exogenous HMGB1 to rats with myocardial infarction improved cardiac function via Wnt/β-catenin signaling pathway activation. In the cell proliferation and migration experiments of the present study, HMGB1-knockdown cells subjected to 0.1 or 0.2 Gy γ-ray irradiation exhibited significantly reduced proliferation and migration capabilities compared with the WT group irradiated with the same dose, resulting in 77% reduction in proliferation rate and 82% suppression of migratory activity. In addition, the proliferation and migration abilities of knockdown cells after low-dose irradiation were still enhanced compared with those of unirradiated knockdown cells, indicating that low-dose ionizing radiation can promote the proliferation and migration of HMGB1 knockdown cells, but the promoting effect was weaker than that of WT cells. Based on *in vitro* research results, future study by the authors will use lens-specific HMGB1-conditional knockout mice to clarify the role of the HMGB1/Wnt-β-catenin signaling axis in mediating low-dose ionizing radiation-induced ocular lens opacity.

In summary, the preliminary findings of the present study indicated that low-dose γ-ray irradiation upregulates HMGB1 expression in SRA01/04 cells, which may promote cell proliferation and migration by activating the Wnt/β-catenin signaling pathway. The activation of the Wnt/β-catenin signaling pathway appears to be partially dependent on HMGB1 expression. Future investigations are needed to further explore the interactions and molecular mechanisms between the two in regulating the functional abnormalities of LECs induced by low-dose ionizing radiation. The present study unravels a novel molecular target for lens opacity induced by low-dose ionizing radiation, aiding in early intervention and treatment of radiation-induced cataracts.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

PW and MT conceived and designed the study. PW and CP conducted experimental research and acquired data. PW, CP and LF performed data analysis, interpretation and visualization. DY, YH and YL prepared the experimental animal model and examined tissue staining. All authors contributed to drafting the manuscript or revising the manuscript. All authors read and approved the final version of the manuscript and confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved [approval no. (2022)005] by the Experimental Animal Welfare and Ethics Committee of the National Institute for Radiological Protection, Chinese Center for Disease Control and Prevention (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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