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Original Article

Antioxidant Effect of Annexin A-1 Induced by Low-dose Ionizing Radiation in Adipose-derived Stem Cells

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Radiation therapy is one of the primary options for the treatment of malignant tumors. Even though it is an effective anti-cancer treatment, it can cause serious complications owing to radiation-induced damage to the normal tissue around the tumor. It was recently reported that normal stem cell response to the genotoxic stress of ionizing radiation can boost the therapeutic effectiveness of radiation by repairing damaged cells. Therefore, we focused on annexin A-1 (ANXA1), one of the genes induced by low-dose irradiation, and assessed whether it can protect adipose-derived stem cells (ADSCs) against oxidative stress-induced damage caused by low-dose irradiation and improve effectively cell survival. After confirming ANXA1 expression in ADSCs transfected with an ANXA1 expression vector, exposure to hydrogen peroxide (H_2O_2) was used to mimic cellular damage induced by a chronic oxidative environment to assess cell survival under oxidative conditions. ANXA1-transfected ADSCs demonstrated that increased viability compared with un-transfected cells and exhibited enhanced anti-oxidative properties. Taken together, these results suggest that ANXA1 could be used as a potential therapeutic target to improve the survival of stem cells after low-dose radiation treatment.

Key Words: ADSC, ANXA1, Low-dose radiation, Oxidative stress

INTRODUCTION

Cancer is a disease caused by genetic mutations and is among the leading causes of death worldwide. Surgery, chemotherapy, and radiation therapy are the most common methods used to treat cancer. Surgery is the most preferred for solid cancers, and chemotherapy and radiation therapy are used to treat advanced-stage cancers or to prevent recurrence after surgery. These treatments can be used alone or in combination to treat lesions.

Radiation therapy utilizes high-energy electromagnetic radiation to retard the growth of or kill cancer cells, as rapidly proliferating cancer cells are more sensitive to DNA damage than normal cells (Hur and Yoon, 2017; Liauw et al., 2013). However, during radiation therapy, normal cells neighboring the tumor also receive a considerable dose of ionizing radiation. This exposure can cause damage to the healthy tissues, which may manifest immediately or later, if the patient survives. In addition, radiation therapy also has side effects such as hormonal imbalance and diminished bone marrow function, via damaging healthy cells (Baskar et al., 2012). Low linear energy transfer (LET) radiation (X-rays, -rays, and particles) generate free radicals and reactive oxygen

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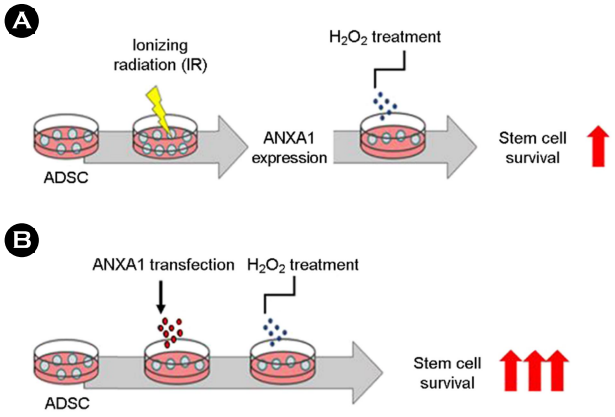

Fig. 1. Schematic diagram of the proposed function of ANXA1 expressing in ADSCs against oxidative stress. (A) ANXA1 function induced by low-dose ionizing radiation and (B) Increased cell survival of ANXA1 overexpressing ADSC under oxidative stimulation conditions.

species (ROS) such as superoxide (O_2) and hydroxyl radical (OH) as well as non-radical molecules such as hydrogen peroxide (H_2O_2) and singlet oxygen (O_2). Intracellular ROS can cause oxidation of biological macromolecules and activation of intracellular signaling pathways, which induce apoptosis and cell cycle arrest (Hur and Yoon, 2017; Seifried et al., 2007; Buldak et al., 2015). ROS induced by low LET radiation is strongly associated with oxidative stress, which is detrimental to human health. Overproduction of H_2O_2, the most common ROS, may lead to oxidative stress, which may in turn result in permanent changes in cells, leading to loss of function of proteins and disease (Onmaz et al., 2011). Thus, intracellular redox potential can influence cellular functions, and its dysregulation is associated with disease (Bentzen, 2006; Beerman, 2017).

Although radiation therapy prolongs patient survival, the damage it causes to normal tissue remains an important clinical concern. In particular, resident stem cells around the lesion can also be damaged. However, recently, it has been reports that low-dose radiation enhances the functioning of stem cells (Mohrin et al., 2010).

Therefore, we assessed how low-dose radiation affects stem cell survival and focused on ANXA1, one of the genes activated by radiation.

ANXA1 is a ubiquitous 37-kDa protein that belongs to the family of calcium/phospholipid-binding proteins (Lim and Pervaiz, 2007). It is expressed in various tissues and is involved in several biological processes such as calcium signaling, membrane aggregation, inflammation, phagocytosis, cell proliferation, apoptosis, and tumor progression (Beerman, 2017). In this study, we evaluated how ANXA1 affects the biological function of adipose-derived stem cells (ADSCs) exposed to low-dose radiation (Fig. 1).

MATERIALS AND METHODS

Cell culture

ADSCs were kindly provided by Dr. Jae-Yeol Cho at Seoul National University in Seoul, Republic of Korea. The ADSCs were cultured in Dulbecco's modified Eagle's medium with high glucose (Hyclone, Logan, UT) containing 10% fetal bovine serum (Hyclone) at 37℃ in a 5% CO_2 incubator.

X-ray irradiation

ADSCs were seeded in a 35 mm dishes (9×10^4 cells) and then cultured for 24 h and irradiated. An X-ray radiographic system (APOLLON, GEMSS, Republic of Korea) was used to irradiate the ADSC cells. The ADSC cells were directly exposed to X-rays at a tube voltage of 120 kVp with a tube current of 320 mA. The radiation dose from a single X-ray exposure was 0.02 mGy, and a total radiation dose of 1 mGy was delivered to the ADSC cells.

RNA extraction and the reverse transcription-PCR (RT-PCR) assay and conventional polymerase chain reaction (PCR)

ADSCs were seeded in 6-well plates (9×10^4 cells/well) and harvested after irradiation (1 mGy) or H_2O_2 (hydrogen peroxide) treatment at 60 mM for 4 h. Total RNA was isolated from the cells using Trizol reagent (Invitrogen, California, USA) following the manufacturer's manual. Complementary DNA (cDNA) was synthesized using the DiaStar 2× RT Pre-Mix (Solgent, Daejeon, Republic of Korea). The synthesized cDNA was subjected to conventional PCR using a Solg 2× Taq PCR Pre-Mix (Solgent) according to the manufacturer's protocols. The PCR products were electrophoresed on 2% agarose gels (Agarose, Molecular Biology

Table 1. List and sequences of primers used

Genes	Forward	Reverse
GAPDH	AGG GCT TTT AAC TCT GGT	CCC CAC TTG ATT TTG GAG GGA
ANXA1	AAT CCA TCC TCG GAT GTC GC	ACA CGT TTA CGT CTG TCC CC
GPX4	GCC TTC CCG TGT AAC CAG T	GCG AAC TCT TTG ATC TCT TCG T

Grade, Vivantis) prepared using 1× TAE buffer (Biosesang). Gel images were obtained using a chemiluminescence analyzer (VilberLourmat, Eberhardzell, Germany). Table 1 shows the primer sequences used in this study.

Generation of ANXA1-expressing vector and its transfection into ADSCs

The ANXA1-expressing vector was cloned to be expressed by the cytomegalovirus promoter. Plasmid construction was confirmed by restriction digestion. The plasmid was then transformed into *Escherichia coli* DH5α cells and purified using the Axygen® AxyPrep Plasmid Mini-prep Kit (Axygen, New York, USA). The purity and concentration of the plasmid was evaluated based on the ratio of absorbance at 260 nm and 280 nm and absorbance at 260 nm wavelength, respectively. ADSCs were seeded in 6-well plates (4×10^4 cells/well) one day before transfection. pcDNA3.1-GFP plasmid containing the ANXA1 expression cassette (Cosmogentech, Seoul, Republic of Korea) was then transfected into the cells using DOTAP (1,2-dioleoyl-3-trimethylammonium-propane), a liposome-based delivery carrier prepared according to a previous protocol established by our laboratory (Koh et al., 2019).

Cell viability assay under oxidative condition

To assess the anti-oxidant effects of ANXA1, H_2O_2 was used to mimic the oxidative stress induced by low-dose radiation. Untreated ADSCs were used as control. First, to determine the optimal concentration of H_2O_2 to treat ADSCs with, the cells were treated with various concentrations of H_2O_2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (2.5 mg/mL; Sigma, UK) was used to determine cell viability after treatment with H_2O_2. Briefly, the medium in each well was removed and replaced with 300 μL MTT reagent and the plates incubated at 37℃ for 4 h. Following this, the supernatant was removed, and the cells were treated with dimethyl sulfoxide (Sigma-Aldrich, USA) for 15 mins at 37℃. Absorbance was measured at 570 nm wavelength on VerxaMax (Associates of Cape Cod Incorporated, MA). Cell viability was normalized as a percentage using the absorbance value for the control.

Statistical analysis

Data were analyzed using paired *t*-test and one-way analysis of variance. $P < 0.01$ was considered statistically significant and is indicated in the figures as ***. Statistical analyses were performed using the Prism software for Windows (ver. 5.01; GraphPad Software, USA).

RESULTS

ANXA1 expression induced at the transcriptomic level by low-dose radiation

To assess whether ANXA1 expression is induced in ADSCs when irradiated with low-dose radiation, cells were seeded in a 35 mm dish at 3×10^5 cells/mL. 24 h after seeding, ADSCs were exposed to low-dose (1 mGy) radiation. The expression of ANXA1 at the transcriptome level was then assessed using cDNA synthesis analysis at 6 h (sample A) and 48 h (sample B) after irradiation. Our data indicated that ANXA1 expression was higher at the 6 h time point compared with that in the control group (0 h). Notably, ANXA1 expression returned to normal levels 48 h post-irradiation (Fig. 2A). These patterns were similarly observed in quantitative data (Fig. 2B). These results indicate that low-dose radiation induces transiently the expression of ANXA1 in ADSCs.

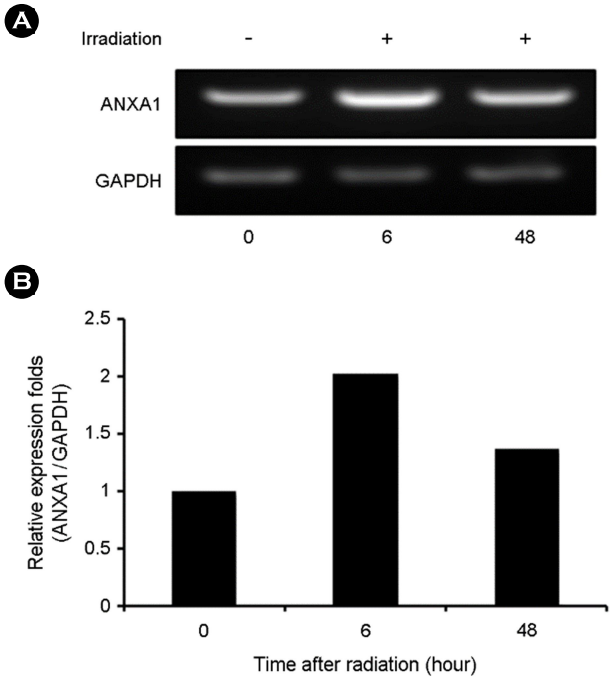

Fig. 2. The confirmation of ANXA1 expression induced by low-dose ionizing radiation. (A) ADSCs were irradiated with low-dose radiation (1 mGy) and then the expression of ANXA1 assessed in transcriptome level at 6 and 48 h post-irradiation. (B) Quantified values of ANXA1 expression were normalized by GAPDH.

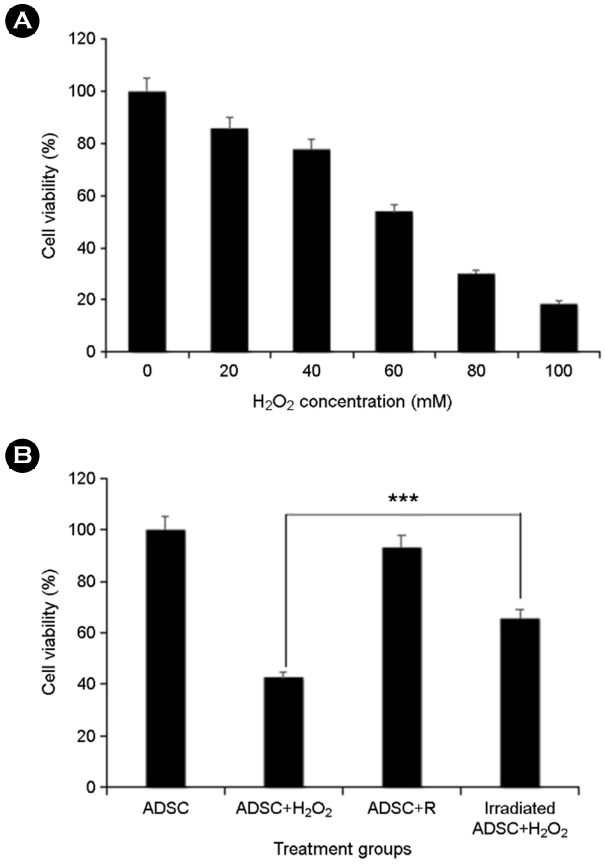

Fig. 3. Anti-oxidant effect of ANXA1 expressed by low-dose radiation under oxidant stress condition. (A) Optimal concentration of H_2O_2 to mimic oxidative environment in ADSC. Stem cells were treated with H_2O_2 at various concentrations for 4 h, and then the IC_{50} of 60 mM concentration was decided. (B) Anti-oxidant effect of ANXA1 expressed by low-dose radiation under oxidative stress condition. ***P<0.01, as compared with control group.

Viability of ADSCs expressing radiation-induced ANXA1 under oxidative conditions

To assess whether ANXA1 induced by low-dose radiation is resistant to oxidative cellular damage, cells were treated with various concentrations of H_2O_2 (from 20 to 100 mM) to mimic the *in vivo* oxidative environment. As shown in Fig. 3A, ADSC viability was inversely proportional to H_2O_2 concentration. The half maximal inhibitory concentration (IC_{50}) of H_2O_2 was 60 mM. To evaluate the antioxidant effects of ANXA1, ADSCs were exposed to low-dose radiation, and then cell viability was determined using MTT assay. Fig. 3B depicts the viability of ADSCs treated with low-dose radiation and H_2O_2. Our data indicated that cells treated with 60 mM H_2O_2 exhibited lower viability compared with controls, consistent with the result in Fig. 3A. Notably, irradiated ADSCs were observed to significantly higher viability compared with those treated with H_2O_2. However, ADSCs treated with H_2O_2 after irradiation exhibited a higher survival rate compared with cells of the H_2O_2-treated group (Fig. 3B). These results indicate that radiation-induced ANXA1 induces antioxidant properties in ADSCs.

Improved antioxidant properties of ANXA1-expressing ADSCs

As shown in the results in Fig. 3, we confirmed that radiation-induced ANXA1 is resistant to oxidative cellular damage. Therefore, we examined whether artificially expressed ANXA1 also exhibits antioxidant properties. Thus, an ANXA1-expressing vector was transfected into ADSCs. Two days after the transfection, the cells were treated with H_2O_2 (Fig. 4A). As shown in Fig. 3B, the viability of cells treated with H_2O_2 or radiation alone was similar to the results

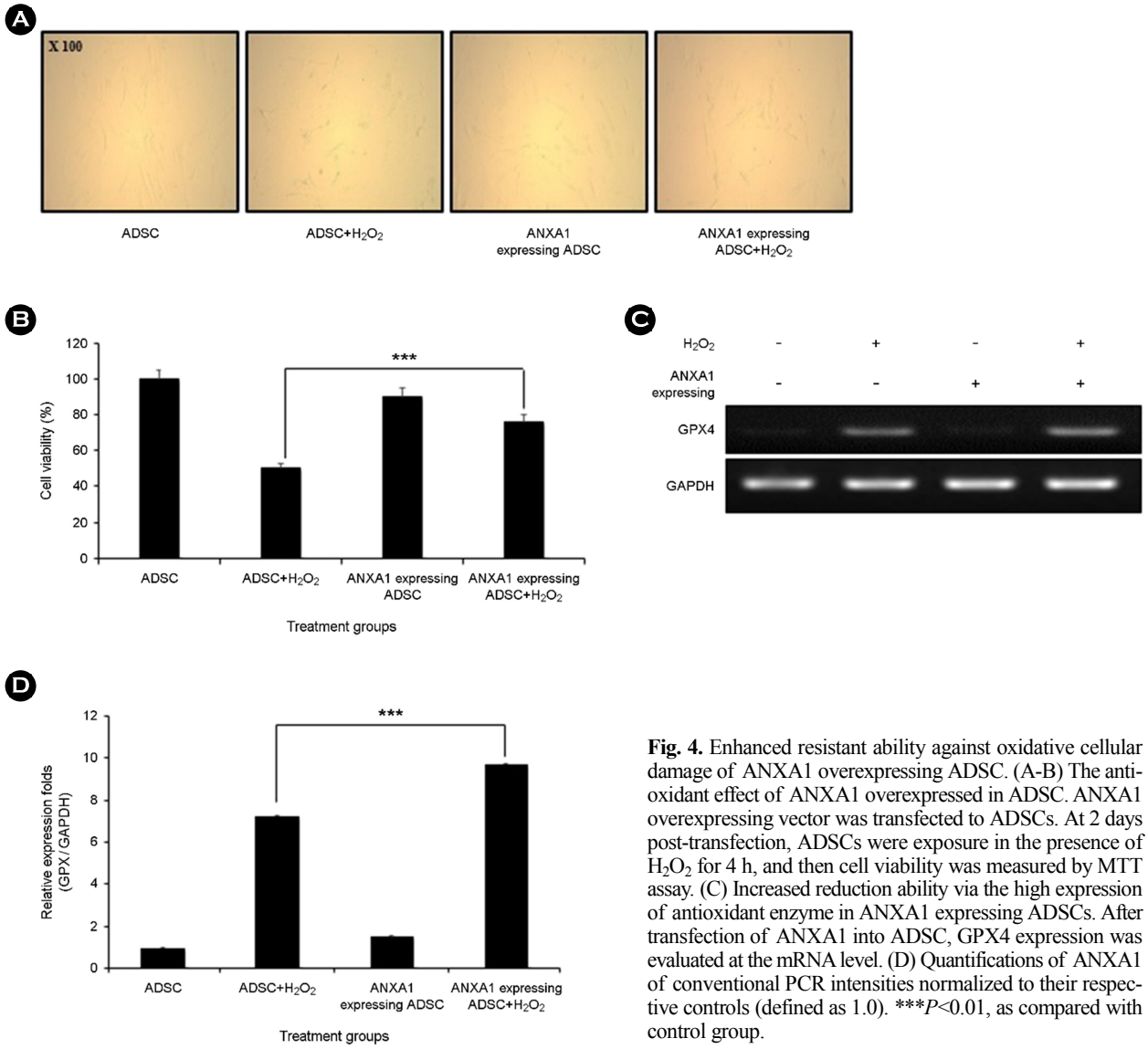

Fig. 4. Enhanced resistant ability against oxidative cellular damage of ANXA1 overexpressing ADSC. (A-B) The antioxidant effect of ANXA1 overexpressed in ADSC. ANXA1 overexpressing vector was transfected to ADSCs. At 2 days post-transfection, ADSCs were exposure in the presence of H_2O_2 for 4 h, and then cell viability was measured by MTT assay. (C) Increased reduction ability via the high expression of antioxidant enzyme in ANXA1 expressing ADSCs. After transfection of ANXA1 into ADSC, GPX4 expression was evaluated at the mRNA level. (D) Quantifications of ANXA1 of conventional PCR intensities normalized to their respective controls (defined as 1.0). ***P<0.01, as compared with control group.

shown in Fig. 3B. However, ADSCs transfected with the ANXA1 expression vector exhibited a higher survival rate compared with un-transfected ADSCs treated with H_2O_2. This indicates that artificially expressed ANXA1 is also resistant to oxidative stress, leading to increased stem cell survival. Next, to confirm the mechanism behind the antioxidant effect shown in Fig. 4B, the expression of the antioxidant enzyme glutathione peroxidase 4 (GPX4) was evaluated. GPX4 catalyzes the reduction of H_2O_2 to protect cells against peroxidation and oxidative stress. As shown in Fig. 4C-D, GPX4 expression exhibited a trend similar to that of cell survival (Fig. 4B). GPX4 expression was lower in H_2O_2-treated cells than in controls. In contrast, ANXA1-overexpressing ADSCs exhibited the highest GPX4 expression level in all groups, causing an enhanced antioxidant effect.

Taken together, the results of this study demonstrate that ANXA1 acts as a potent antioxidant under oxidative stress conditions.

DISCUSSION

Radiation therapy, one of the avenues for treatment of malignant tumors, prolongs patient survival (Baskar et al., 2012; Perez and Mutic, 2013). However, damage to normal

tissue surrounding tumors remains an important clinical concern (Hur and Yoon, 2017). Ionizing radiation, used to treat cancer, affects cancer cells as well as normal cells, including resident stem cells adjacent to the tumor (Narayanan et al., 1997). If normal cells near the tumor are damaged by radiation exposure, serious complications can occur (Bentzen, 2006). It is important to address or prevent radiation-induced cytotoxicity to normal tissue, as the severity of radiation-induced toxicity can be worse than the initial lesions treated. Recent advances in stem cell biology have demonstrated that exposure of stem cells to the genotoxic stress of low-dose ionizing radiation can improve the therapeutic efficacy by repairing damaged cells. However, high-dose radiation can induce death of resident stem cells before they exhibit a positive effect on therapeutic efficacy, and tissue regeneration and recovery (Ciccia and Elledge, 2010; Le et al., 2017).

In this study, we focused on the radiation-induced expression of the ANXA1 gene in ADSCs. This gene is involved in various biological processes, such as inflammation, apoptosis, and calcium transfer signal pathways (Huang et al., 2016). However, its function in stem cells is not well known. Thus, we examined the radiation-induced expression of ANXA1 because no studies have reported its biological function in stem cells.

First, we assessed whether ANXA1 expression is induced by low-dose radiation. We found that ANXA1 levels were upregulated 6 h after ADSCs were irradiated with 1 mGy radiation (Fig. 2). This result indicates that low-dose radiation induced the expression of ANXA1 in ADSC.

Radiation is used primarily in clinical as a method to treat cancer. However, the treatment also affects normal cells, including resident stem cells, around the irradiated site. Thus, it is important to investigate its effect on normal cells to reduce the severity of the side effects of radiation therapy (Baskar et al., 2012; Peters et al., 2000). Exposure to radiation has been documented to increase stress in affected regions (Benitez et al., 2004). Specifically, oxidative stress induced by radiation has been documented to decrease the viability of the exposed cells. Therefore, we examined whether ANXA1 exhibits anti-oxidative properties in stem cells. The oxidative environment was mimicked by treating ADSCs with H_2O_2 (Fig. 3). Our results indicated that radiation-induced expression of ANXA1 improved viability of H_2O_2-treated cells compared with that of untreated controls (Fig. 4). This indicates that ANXA1 acts as an antioxidant.

To further clarify the function of ANXA1, we induced overexpression of ANXA1 by transfecting ADSCs with an ANXA1 expression vector. Furthermore, ADSCs over-expressing ANXA1 not only exhibited higher cell survival under oxidative stress conditions, as shown in Fig. 4, but also exhibited higher expression of GPX4.

Taken together, our study suggests that ANXA1 can be used as a potential therapeutic target to enhance the cell viability of ADSCs after radiation treatment.

Abbreviations

annexin A-1, ANXA1; adipose-derived stem cells, ADSCs; hydrogen peroxide, H_2O_2; Low linear energy transfer, LET; reactive oxygen species, ROS; superoxide, O_2; hydroxyl radical, OH; glutathione peroxidase 4, GPX4.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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