Original Article

Irradiation Response of Adipose-derived Stem Cells under Three-dimensional Culture Condition*

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Abstract

Objective Adipose tissue distributes widely in human body. The irradiation response of the adipose cells *in vivo* remains to be investigated. In this study we investigated irradiation response of adipose-derived stem cells (ASCs) under three-dimensional culture condition.

Methods ASCs were isolated and cultured in low attachment dishes to form three-dimensional (3D) spheres *in vitro*. The neuronal differentiation potential and stem-liked characteristics was monitored by using immunofluoresence staining and flow cytometry in monolayer and 3D culture. To investigate the irradiation sensitivity of 3D sphere culture, the fraction of colony survival and micronucleus were detected in monolayer and 3D culture. Soft agar assays were performed for measuring malignant transformation for the irradiated monolayer and 3D culture.

Results The 3D cultured ASCs had higher differentiation potential and an higher stem-like cell percentage. The 3D cultures were more radioresistant after either high linear energy transfer (LET) carbon ion beam or low LET X-ray irradiation compared with the monolayer cell. The ASCs' potential of cellular transformation was lower after irradiation by soft agar assay.

Conclusion These findings suggest that adipose tissue cell are relatively genomic stable and resistant to genotoxic stress.

Key words: Adipose-derived stem cells; Three-dimensional cell culture; Irradiation response; High- and low-LET irradiation

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INTRODUCTION

Cancer or malignant tumors is one of the most serious diseases in human. Even with extensive research in the past 30 years, there is still no effective approach to control and cure it. Radiotherapy is one of the major approaches for cancer therapy. Low LET-γ- or X-ray irradiation treatment has been commonly used in radiotherapy due to its ability in tumor cell killing. However, the problem associated with tumor recurring and lack of specific target, would result in

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normal tissue damage and side effect. Recently, high LET and high-energy particles, such as carbon beam has been used for radiotherapy with high efficacy[1]. High LET and high-energy particles induced DNA lesions are difficult to repair and, therefore, are more effective in killing of tumor cells. Most importantly, the physical characteristics of the charged particles is to allow delivery higher dose and higher energy of particles at targeted tumor region[2]. Therefore, the normal tissue along the path of the particle irradiation would expose to lower irradiation dose, thus the side effect is reduced. However, there are still concerns regarding the exposure of normal tissue to charged particle irradiation during carbon beam therapy[3].

Adipose tissue, distributing widely in human body, is known as the main source of the organism. The ionizing irradiation has inevitable impact on the adipose tissue during the radiotherapy. The response of the adipose tissue to irradiation exposure and whether radiotherapy could induce adipose derived stem cells (ASCs) to transform to cancer stem cell remain unclear. In addition, the sensitivity of ASCs to higher LET irradiation has never been studied.

Cells *in vivo* are three-dimensional (3D), in which cell exhibit considerably difference to the traditional monolayer (2D) cultured cell in cell morphology, cell-cell contact and extracellular-cell matrix (ECM)[4-9]. Therefore, the 2D cell culture mode can not fully exhibit the nature of life in the physiological conditions of microenvironment *in vivo*. To simulate the microenvironment *in vivo*, many 3D cell culture systems have been established, one of which is to take advantage of the natural aggregation tendency of many cell types to culture 3D cell[10]. Natural aggregation 3D spheroids can be commonly obtained through three approaches, i.e. hanging drop method[11], gyratory rotation technique[12], and low-attachment cultivation of cells[10,13]. It has been proved that 3D culture microenvironment plays a vital role in cell reprogramming[14] and determining the properties of stem cells[15].

In this study, we first isolate a type of ASCs from a surgical patient after the approval of the Ethics Committee of Sichuan Cancer Hospital. After cell line identification, we performed 3D sphere culture of human ASCs by using the low attachment surface by coating 0.5% soft agarose on cell culture dish and assessed the irradiation effects of low-LET X-rays and high-LET carbon ions[16] on these 3D cells. Our results showed that 3D cultured cells were relatively more radioresistant than 2D cultured cells.

MATERIALS AND METHODS

Isolation and Characterization of Human Adipose-derived Stem Cells

Subcutaneous adipose tissue was obtained from a patient undergoing surgical procedures after obtaining informed consents and the approval of the Ethics Committee of Sichuan Cancer Hospital. Adipose cells were isolated as previously described[17-18]. Briefly, a small amount of tissue was placed in a 2 mL tube and washed with 300 μL of warmed PBS containing 1% antibiotic until all blood vessels and connective tissues appeared to be liberated (usually 2 washes). The adipose tissue sample was separated and minced into small pieces by using sterilized scissors and digested in DMEM-F12 medium (Invitrogen, USA) supplemented with 1 mg/mL type I collagenase (Sigma, USA) for 45 min at 37 °C in a shaking incubator. After centrifuged at 300 g, the cellular pellet was resuspended and filtered through a 100 mesh filter. The filtrate was centrifuged and the pellet was called processed lipoaspirate (PLA) cells. The PLA cells were washed in PBS solution containing 1% antibiotic and plated into 100-mm cell culture dishes with complete culture medium (DMEM-F12 medium containing 10% fetal bovine serum (FBS), 100 mg/mL streptomycin, 100 U/mL penicillin). The cells were cultured at 37 °C in humidified atmosphere with 5% CO_2. After 24 h non-adherent cells were removed and adherent cells were maintained with a fresh medium. The cells were grown to confluence after the initial plating (Passage 0). The PLA cells in the third passage were used for subsequent experiments.

Immunofluoresence Staining

The adherent cells in passage 3 were washed twice with 1×PBS at room temperature (RT), fixed for 20 min with 4% paraformaldehyde and washed again for three times with 1×PBS (5-10 min interval) at RT. To avoid non-specific binding, the cells were first incubated for 1 h in PBS and 5% goat serum, and then were incubated with the FITC- or PE-conjugated monoclonal antibodies against CD44, CD45, and CD90 for 1 h (Miltenyi Biotec, Germany)[19]. Nuclei were counterstained with 4',6-diamidino--phenylindole (DAPI). Cell staining image was taken under a fluorescence microscope.

Preparation of Low Attachment Dish and Culture of 3D Sphere Cells

Equal volume of pre-warmed 2×DMEM-F12 medium (37 °C) and 1% agarose (42 °C) were mixed and coated on cell culture dish. The mixture were solidified at RT[10]. 1×10^6 ASCs were plated into a 35 mm low attachment culture dish. These cells were cultured at 37 °C with 5% CO_2 and they formed 3D free floating spheres within 24 h. The cellular spheres on day 3 were used for the relevant experiments.

2D and 3D ASCs Neuronal Induction

The 2D and 3D cells were cultured in preinduction medium consisted of DMEM-F12 containing 20% FBS and 1 mmol/L β-mer-onoverlapcaptoethanol (Sigma, USA) for 48 h. After washed with PBS, the 2D and 3D cells were cultured in serum-free induction medium consisted of basic medium DMEM-F12 supplemented with 100 mol/L butylated hydroxyanisole (BHA, Sigma, USA), 10^{-6} mol/L retinoic acid (RA, Sigma, USA), 10 ng/mL epidermal growth factor (EGF, Invitrogen, USA), and 10 ng/mL basic fibroblast growth factor (bFGF, Invitrogen, USA). The cells were incubated with the induction medium, changed twice per week, for 3 weeks[20]. Neuronal differentiation was monitored by detecting the expression of nestin, a protein of intermediate filament expressed by neural stem cell marker[21].

Western Blot

The cells were washed with PBS and lysed in RIPA buffer with Complete Protease Inhibitor Cocktail (Roche, USA) for 10-20 min on ice. The whole cell lysates were harvested and centrifuged at 4 °C. Protein concentrations were measured with Bradford protein assay (BIO-RAD, USA). Equivalent quantity of protein lysates were electrophoresed in 10% SDS-ploy acrylamide gel and transferred to methanol-activated PVDF membrane (GE Healthcare, USA). The membrane was blocked in TBS containing 5% BSA for 1 h. Primary antibody nestin (1:1000, Sigma, USA) were incubated for 2 h at RT. The HRP labeled secondary antibody Goat-Anti Rabbit IgG (1:5000, ZSGB-BIO, China) was incubated for 1 h at RT.

Rhodamine Staining Analysis

The 2D and 3D cultured ASCs were trypsinized and prepared as single cell suspensions in medium. The cells were stained with 5 μg/mL Rhodamine 123 (Invitrogen, USA) and incubated in darkness at 37 °C for 60 min. After staining, the cells were washed with medium twice and incubated again in darkness with medium at 37 °C for 10 min. The Rho fluorescence of stained cells was assayed by using flow cytometer (MACS, Germany).

Radiation Schemes

For low LET X-ray irradiation, a X-ray facility (target: W, Faxitron Bioptics, USA) was used. The dose rate was ~0.8 Gy/min. High LET $^{12}C^{6+}$ ion beams (80-100 MeV/u) were generated by the Heavy Ion Research Facility in Lanzhou (HIRFL, Institute of Modern Physics, Chinese Academy of Sciences, Lanzhou, China). The dose rate was ~0.25 Gy/min.

Colony Survival Assay

After X-ray or $^{12}C^{6+}$ ion irradiation, the 2D and 3D ASCs were trypsinized, counted and reseeded at different densities to yield approximately 50-100 surviving colonies per P-100 dish. After 14 d, seeding colonies were fixed, stained overnight with stain-fixative (0.05% crystal violet+2% methanol in PBS) and scored. Survival curves were generated from three independent experiments with colony numbers normalized to sham-treated controls.

Micronucleus (MN) Assay

The 2D and 3D ASCs irradiated by X-ray or carbon ions beam, as well as their sham irradiated control cells were trypsinized to single cell suspension and then were cultured in monolayer for 48 h. The cells were fixed with Carnoy's solution for 20 min at RT, stained with 20 μL of Acridine Orange in an aqueous solution (10 μg/mL). Analyses were performed with a fluorescence microscope (Axio Imager. Z2) at 20×magnification. At least 1000 cells were scored for each sample.

Soft Agar Colony Assay

Soft agar assays were performed as described[22]. Briefly, the cells were irradiated with 2 Gy X-rays continuously for 5 d and then cultured for one month[23]. Survival cells were seeded in complete media at a density of 5000 cells in 60-mm dishes containing a top layer of 0.35% agarose and a base layer of 0.5% agar. They were incubated at 37 °C for 4 weeks and fed twice a week.

Statistical Analysis

The statistical significance (*P* values) in mean

values of two-sample comparison was determined with Students' *t*-test. A value of $P<0.05$ was considered statistically significant. Values shown on graphs represent the means±sd.

RESULTS

Characterization of Isolated ASCs

Freshly isolated adipocytes were washed with medium 24 h after plating in order to discard unattached cells and then maintained with fresh medium. Adipose cells were cultured to confluence after the initial plating (passage 0). As shown in Figure 1A, the adherent adipose cells demonstrate undifferentiated fibroblast-like morphology growing in directional patterns. At passage 3, PLA cells were identified by cell surface marker immunofluoresence staining ($CD44^+$, $CD45^-$, $CD90^+$)[24] and their differentiation potential were identified by culture with neuronal induced medium. Figure 2 shows that the expression of CD44, CD90 marker is positive (Figure 2A and 2B) while CD45 is negative (Figure 2C) in our isolated adipose stromal cells, demonstrating their characteristics of stem-like cell. The PLA cells expressing several CD marker antigens are similar to those observed on mesenchymal stem cells (MSC) which were termed as adipose-derived stem cells (ASCs) by Mclntosh et al.[24].

Confirmation of Neuronal Differentiation Potential in Monolayer and 3D Culture

To force cells to form 3D sphere morphology, we exploited a low attachment surface approach with a 4 mm layer of 0.5% agarose. When ASCs were cultured

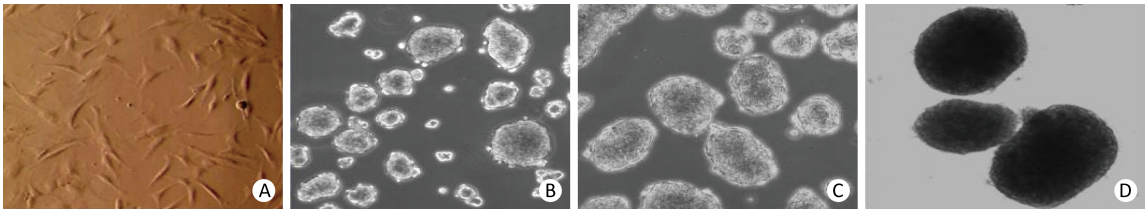

Figure 1. Morphology of monolayer (A) and 3D spheres cultured on day 1 (B), day 2 (C), day 3 (D) of ASCs isolated from subcutaneous fat tissue at passage 3 (10×magnification contrast light microscopy).

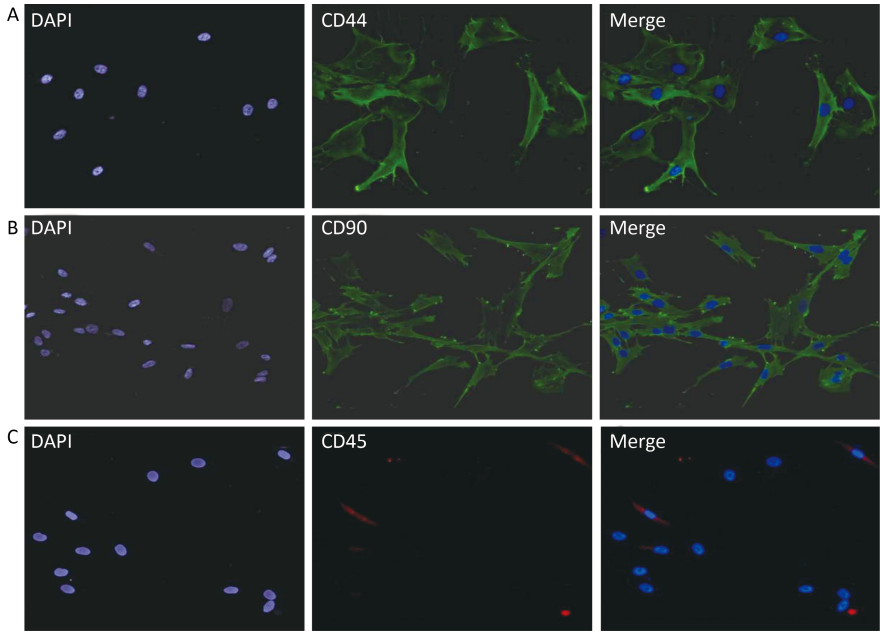

Figure 2. Characteristics of isolated ASCs. Cellular surface CD markers of ASCs were immunostained at passage 3. The representative photomicrographs showed the expression of CD44 (A), CD90 (B) marker and no expression of CD45 (C) in the ASCs (20×magnification fluorescence microscopy).

on low attachment dish, they formed 3D free floating spheres within 24 h (Figure 1B). The cellular spheres became compaction at day 3 (Figure 1D). By means of the immunofluoresence staining, we found that nestin highly expressed in monolayer and 3D sphere cells after culture with neuronal induction medium for 3 weeks (Figure 3B and 3D). Compared to the monolayer cultures, the protein level of nestin in the 3D sphere significantly upregulated (Figure 3E). These results suggested that the isolated ASCs cultured in monolayer and in 3D structure had the neuronal differentiation potential. The differentiation potential of cells in 3D sphere structure were higher than those in the monolayer.

Increasing Population of Rho-low Staining (Rho^{low}) Cells in 3D Cultured Cells

Hoechst was used to identify the stem and non-stem cells due to the fact that hoechst can be excluded out of cells by the ABC transporter, a specific protein in stem cell[25]. Rhodamine 123 (Rho), another fluorescent dye, had been also used to identify the stem cell-like cells by Rho/FACS[26-27]. We determined the stem-like cell percentage within two

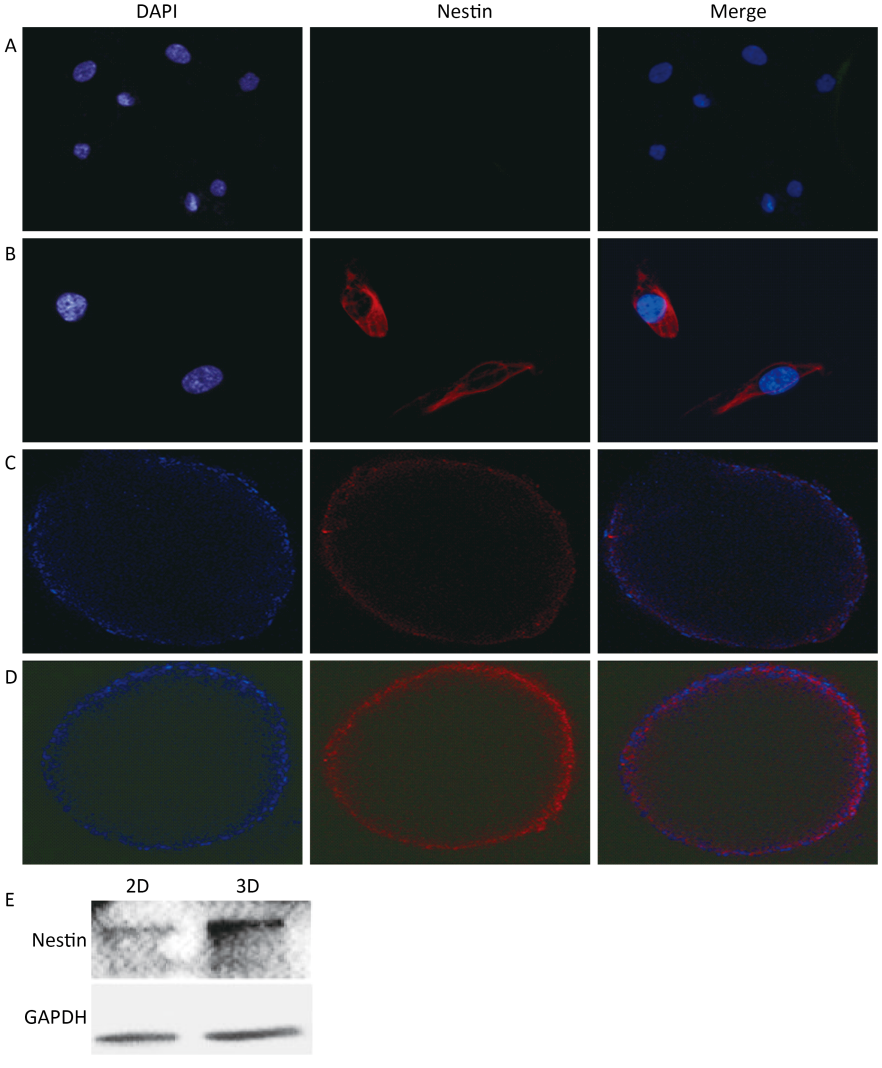

Figure 3. Identification of the differentiation potential of 2D and 3D ASCs induced for three weeks by immunofluoresence staining and western blot assay. (A): Immunostaining of nestin in 2D ASCs without neuronal induction; (B): Immunostaining of nestin in 2D ASCs after neuronal induction; (C): Immunostaining of nestin in 3D ASCs without induction; (D): Immunostaining of nestin in 3D after neuronal induction; (E): Nestin protein expression in 2D monolayer and 3D sphere ASCs after neuronal induction. All the images were taken under 20×magnification fluorescence microscopy.

culture modes of ASCs with the Rho/FACS method. As shown in Figure 4, the percentage of Rho^{low} cells in monolayer grown ASCs cells was 67.49% (Figure 4B), while in 3D grown cells the percentage of Rho^{low} cells was 91.27% (Figure 4C). The result indicated that there was a significant increase of stem-like cells in 3D cultured ASCs spheres.

Radiation Sensitivity of 3D Sphere Culture

To investigate the irradiation sensitivity of 3D sphere culture, which are more similar to the cells normal physiological environment *in vivo*, the constructed 3D ASCs cultures using low attachment dish and the 2D cultures were irradiated by X-rays or $^{12}C^{6+}$ ions beam generated at HIRFL. Figure 5 shows that the clonogenic survival fraction of 3D culture was significantly higher than that of the 2D cells after the irradiations by both X-ray (Figure 5A) and $^{12}C^{6+}$ ion beam (Figure 5B) at high dosage. The survival fraction of 3D and 2D cells after 5 Gy of X-ray irradiation was 4%, 0.3%, respectively. In contrast, the survival fraction of 3D and 2D cells after 5 Gy of $^{12}C^{6+}$ ion beam irradiation was 1.8%, 0%, respectively. However, high LET $^{12}C^{6+}$ irradiation induced more cell killing than low LET X-rays. To further verify irradiation sensitivity of 3D sphere culture, the induced micronucleus frequence was detected. As shown in Figure 6, the micronucleus frequencies of 2D cultures were higher than those of the 3D cultures after the irradiations by both X-ray (Figure 6A) and $^{12}C^{6+}$ ion beam (Figure 6B). These results suggested that 3D culture was resistant to either high-LET or low-LET ion irradiation compared with the 2D culture, which was consistent with the report by Grabham et al.[28].

Tumorigenicity Sphere Formation of 3D and Monolayer Cultures after Irradiation

The soft agar assay is an anchorage independent growth assay in soft agar, which is considered as the most stringent assay for measuring malignant transformation of a single cells *in vitro*[29]. In general, a dose of 2 Gy X-rays/d used for cells for 5 d is equivalent to the fractionated dose of 2 Gy/d for 15 d

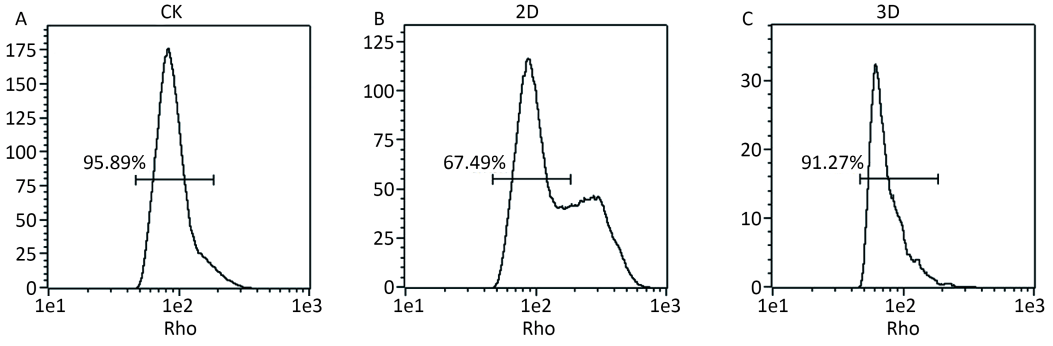

Figure 4. The representative FACS profile for the Rho-low staining cells in 2D monolayer (B) and 3D sphere ASCs (C). (A) represents the negative control cells without Rho staining.

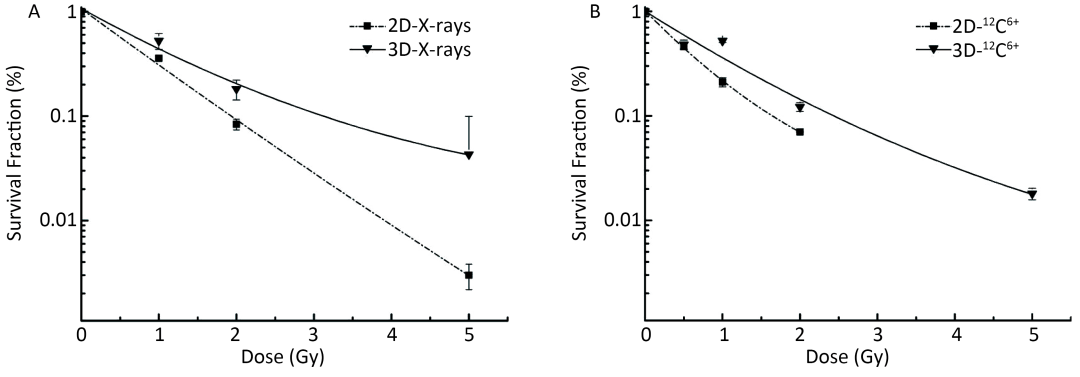

Figure 5. Survival curves of 2D and 3D ASCs after irradiated with X-rays (0-5 Gy) (A) and carbon ion beams (0-5 Gy) (B). Each data point represents the mean of three independent experiments.

used for patient in clinical treatment[23]. To test if ASCs may transform to cancer cell or cancer stem cell after ionizing irradiation for clinical practice, the survival cells of the 2D and 3D cultures irradiated continuously with a fraction dose of 2 Gy/d X-rays for 5 d were plated in double-layered soft agar. It was found that there was no transformation colony 30 d after irradiation by both the 2D and 3D cultured ASCs, suggesting that ASCs were low tumorigenicity under the stress of ionizing irradiation.

DISCUSSION

Adipose tissue originates from mesodermal germ layer and is the most widespread connective tissue in humans. It mainly exists as subcutaneous and visceral fat[30]. The side effects of radiotherapy to treat cancer inevitably include the exposure of adipose tissues, which may increase tissue toxicity. In this study, ASCs were isolated from subcutaneous adipose tissue according to the protocols described in previously published study[31]. To further characterize ASCs derived from subcutaneous adipose tissue, we carried out a series of experiments. Our results suggested that low passage cells line possessed the stemness. In addition, the passage of cell was found to be important for the generation of neuronal induction. The passage less than 5 was perfect for neuronal induction (data not shown).

3D culture microenviroment are more similar to physiological conditions *in vivo* than the 2D[8]. 3D spheres of ASCs formed on low attachment dish, without stimulation with any exogenous agents, acquire *in vivo* adipose tissue-like properties. Biological responses of the 3D cultured cells toward a variety of stresses remain unclear. To investigate these responses of 3D culture may help better understanding of the response of human body to environmental stress. Compared with the 2D cells, the protein level of nestin in the 3D sphere significantly upregulated after neuronal induction (Figure 3E). Neuronal differentiation potential and the percentage of Rho^{low} cells in 3D ASCs spheres were higher than 2D culture mode, suggesting that differentiation potential of ASCs was also correlated with the stem-like cell characteristics.

The potential for adipogenic or chondrogenic differentiation is a hallmark of human mesenchymal stem cells (hMSCs). Some researchers have investigated the influence of the different quality of ionizing irradiation on differentiation potential. The study by Nicolay et al. demonstrated that neither photon irradiation nor high dose carbon ion treatment affect adipogenic differentiation potential of hMSCs[32]. However, Séverine et al. reported that the potential of the chondrogenic differentiation was suppressed in hMSCs after irradiation and fewer chondrocytes was observed[33], suggesting that it is uncertain that whether radiation exposure accelerates or suppresses differentiation of hMSCs. It is interesting for us to study the potential of the ASCs differentiation after irradiation in the future.

Our results showed that the irradiation sensitivity of the 2D culture was significantly higher than that of the 3D cells after either high- or low-LET irradiation. It is reported that hypoxic conditions enhanced the radioresistance of mouse mesenchymal stromal cells[34-36]. We speculated that inner hypoxia of 3D culture might contribute to its radioresistance[37]. Furthermore, cells show round morphology in 3D culture and have distinct gene expression from 2D culture. The interaction between cell-ECM-mediating focal adhesion and actin cytoskeleton is tightly regulated in 3D culture[38], which may have profound impact on radioresistance.

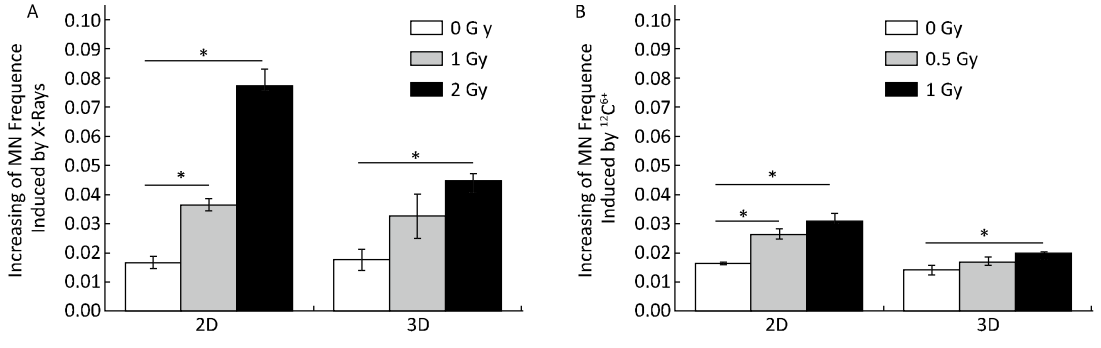

Figure 6. Micronucleus frequency induced in 2D and 3D ASCs by X-ray (A) and carbon ion beam (B). Each data point represents the mean of three independent experiments ($^{*}P<0.05$).

Cell shape is also a potent regulator of physiology, cell growth and stem cell differentiation[39-42].

Colony formation assay in soft agar assays are important for determining oncogenic transformation and correlated with *in vivo* tumor growth of xenografts in mice[43]. Recent studies showed that ASCs at low passage are not tumorigenic[44-46], but the tumorigenesis can be enhanced when tumor cell lines are co-transplanted into nude mice[47]. So far it is not documented that whether ASCs, as pluripotent cells, can be transformed *in vitro* and *in vivo*. In this study, the 2D and 3D cultures were irradiated with fractionated dose of 2 Gy/d X-rays for 5 d and then cultured for one month. The cells were expanded and then plated in double-layered soft agar. There was no colony formed 30 d after incubation, suggesting that the progenies of the irradiated ASCs might have a lower potential of transformation.

CONCLUSIONS

Our results show that, as a widely distributing tissue in human body, the 3D cultured ASCs are radioresistent and both the 2D and 3D cultured ASCs have lower potential of transformation, which might be used as evidence for the irradiation risk assessment and the understanding of irradiation protection from side effects during radiotherapy. Further studies are needed to identify why the ASCs intrinsically are relatively genomic stable and resistant to genotoxic stress.

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