

Radioresistance of granulation tissue-derived cells from skin wounds combined with total body irradiation

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Abstract. Combined radiation and wound injury (CRWI) occurs following nuclear explosions and accidents, radiological or nuclear terrorism, and radiation therapy combined with surgery. CRWI is complicated and more difficult to heal than single injuries. Stem cell-based therapy is a promising treatment strategy for CRWI, however, sourcing stem cells remains a challenge. In the present study, the granulation tissue-derived cells (GTCs) from the skin wounds (SWs) of CRWI mice (C-GTCs) demonstrated a higher radioresistance to the damage caused by combined injury, and were easier to isolate and harvest when compared with bone marrow-derived mesenchymal stromal cells (BMSCs). Furthermore, the C-GTCs exhibited similar stem cell-associated properties, such as self-renewal and multilineage differentiation capacity, when compared with neonatal dermal stromal cells (DSCs) and GTCs from unirradiated SWs. Granulation tissue, which is easy to access, may present as an optimal autologous source of stem/progenitor cells for therapeutic applications in CRWI.

Introduction

Combined radiation and wound injury (CRWI) is characterized as radiation injury coupled with wounds, and is expected to occur following nuclear explosions and accidents, radiological or nuclear terrorism, and radiation therapy in combination with surgery or other modalities (1). Wound healing is a complicated process, and exposure to radiation may significantly aggravate the degree of damage and prolong healing time (2,3). Currently,

there is no effective medical countermeasure for the management of CRWI. Emerging stem cell-based therapy is considered to be promising for the treatment of CRWI; however, the cell source remains a challenging issue (4). Among various stem cell populations, bone marrow-derived mesenchymal stromal cells (BMSCs) are commonly applied in experimental models and clinical trials (5-8). However, the lymphohematopoietic system is particularly sensitive and vulnerable to radiation exposure (9), which indicates that bone marrow is not a good source for autologous cell therapy in CRWI, and there is an urgent requirement to develop alternative cell sources.

Skin is the largest organ in the body and during the past decade the importance of the dermis as an easily accessible source of stem cell populations, and their promising significance in wound repair and other diseases has been established (10-12). Recently, the granulation tissue-derived cells (GTCs) were further characterized as an abundant cell source for their important therapeutic efficacy in wound healing and tissue repair (13). As skin is relatively insensitive to radiation, the present study hypothesized that the GTCs from CRWI may represent an alternative source of adult stem cells for transplantation. The aim of the present study was to investigate the biological features of GTCs from the skin wounds (SWs) of CRWI mice (C-GTCs). Multiple biological characteristics, including the radiation sensitivity of C-GTCs, were investigated and compared with BMSCs from CRWI mice, dermal stem cells (DSCs) from neonatal C57BL/6 mice and GTCs from unirradiated SWs.

Materials and methods

Animals and wound model. A total of 14 female C57/BL mice (age, 6 weeks; weight, 20-22 g) were purchased from the Center of Experimental Animals at the Third Military Medical University (Chongqing, China). The mice were randomly divided into two groups (7 mice/group): CRWI group and SW group. Neonatal mice (age, 1 day) were raised and used for neonatal DSC isolation.

Total-body irradiation was delivered at a rate of 0.70 Gy/min from a ^{60}Co gamma-ray source at the Radiation Center of the Third Military Medical University. The mice from the CRWI group were exposed to a total of 6 Gy in a single dose, and an SW was created 30 min after irradiation. In each group, the

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SW was implemented as described previously (14). The mice were anaesthetized by intraperitoneal injection with 1% pentobarbital (30 mg/kg; Merck Millipore, Darmstadt, Germany) and the back hair was shaved. A circular, full-thickness SW (~1.5 cm in diameter) was made in the center of the back using sterilized ophthalmic scissors and forceps following disinfection of the mouse skin with iodophor (Jinshan Co., Ltd., Chengdu, China). The mice were group-housed under standard conditions throughout the study, under a 12-h light/dark cycle with *ad libitum* access to food and water. All procedures on the mice were approved by the ethics committee of the Third Military Medical University.

Cell isolation and culture. To obtain GTCs from the CRWI and SW mice, the mice were sacrificed by cervical dislocation 7 days after the SW was created and the granulation tissues were acquired. The tissues were washed twice with 75% ethanol and phosphate-buffered saline (PBS) and sliced into small sections (~1 mm³). The sections were digested with 0.25% collagenase I (Worthington Biochemical Corp., Lakewood, NJ, USA) and the cells were agitated into cell suspension for 2 h at 37°C, then cultured in Iscove's modified Dulbecco's medium (IMDM; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with Gibco 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1% penicillin/streptomycin (1 ml/100 ml; Beyotime Institute of Biotechnology, Shanghai, China) at 37°C under an atmosphere of 5% CO₂.

BMSCs were obtained from the femurs and tibiae of CRWI mice. The cells were flushed out using a 1 ml syringe and filtered with a 200 Mesh CellCribble (Sangon Biotech Co., Ltd., Shanghai, China). A single-cell suspension was created as described above.

To obtain DSCs, isolation was performed as described previously (15). Full-thickness skin tissue was obtained from four neonatal C57BL/6 mice (age, 1 day), according to a previous study (16). The tissue was washed in 75% ethanol and PBS and the subcutaneous tissue was removed. The skin tissue was sliced into small sections (~4 mm²), transferred to 0.25% trypsin (HyClone; GE Healthcare Life Sciences) and digested overnight at 4°C. The epidermis was discarded and the dermal layer was sliced into smaller sections (~0.5 mm²). The tissue sections were flushed into a cell suspension and cultured as described above. Passage 0-3 cells were used in the further experiments.

Cell attachment and proliferation. To investigate cell adhesion, a cell attachment assay was performed as described previously (17). Resuspended cells from the different populations were seeded into 24-well plates at a density of 1.0x10⁴ cells/well. At 0.5, 1, 2 and 4 h after inoculation, the cells were washed twice with PBS, fixed with 4% paraformaldehyde (Wuhan Boster Biological Technology, Ltd., Wuhan, China) and stained with DAPI (Beyotime Institute of Biotechnology) for 10 min. The cell number in 10 randomly selected fields was counted under a fluorescence microscope (BX51TRF; Olympus Corporation, Tokyo, Japan) and cell adhesion was presented as the mean cell number. A cell proliferation assay was performed as described previously (18). Cells were seeded in 96-well plates at a density of 3,000 cells/well and cultured at 37°C. At 0, 1, 2, 3, 4 and 5 days after seeding, the media was replaced with 100 µl PBS and 10 µl Cell Counting Kit-8 (CCK-8; Dojindo Molecular

Technologies, Inc., Kumamoto, Japan) for each well, and cells were incubated for another 2 h at 37°C. The absorbance was measured at 450 nm using a Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Colony formation assay. To detect the single-cell colony formation ability of the different cell types, a colony formation assay was performed as described previously (19). Cells were plated into 6-well plates (3,000 cells/well) with IMDM supplemented with 10% FBS and cultured at 37°C. After 12 days, cells were fixed with 4% paraformaldehyde, stained using a Wright-Giemsa staining kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and washed twice with distilled water. The number of colonies that contained >50 cells was counted and images of the colonies were randomly captured using a light microscope (CK40-F200; Olympus Corporation). The colony area was measured using ImageJ software 1.48 (NIH, Bethesda, MA, USA) and the mean area of the colonies was calculated.

Detection of senescence-associated β -galactosidase (SA- β -gal) activity. To establish the senescence state of the cells, SA- β -gal activity was determined as described previously (20). Briefly, passage 2 cells were harvested and plated into 6-well plates (1.0x10⁴ cells/well), and 24 h after incubation the SA- β -gal activity was detected using an SA- β -gal staining kit (C0602; Beyotime Institute of Biotechnology) according to the manufacturer's instruction. The numbers of SA- β -gal-positive and total cells in 10 randomly selected fields were counted under a light microscope (CK40-F200), and the percentage of senescent cells was displayed as the ratio of the number of SA- β -gal-positive cells to total cells.

Cell migration assay. To observe the migration ability of the cells, a scratch-wound assay was performed as described previously (21). Confluent cells were continuously scratched through the entire monolayer using a sterile P200 pipette tip (Axygen Scientific Inc., Union City, CA, USA). After washing with PBS, the wells were cultured with fresh medium at 37°C. Images of the wounds were captured (magnification, x400) 0, 12, 24 and 36 h after scratching. The rate of wound closure (%) at various points was calculated as follows: [(Original wound area - residual wound area) / original wound area] x 100.

Examination of cell differentiation. To investigate the differentiation ability of the cells, the cells were incubated in osteogenic and adipogenic differentiation media [Cyagen Biosciences (Guangzhou) Inc., Guangzhou, China]. After a 3-week incubation at 37°C, the cells were stained with Alizarin Red (EMD Millipore, Billerica, MA, USA) and quantitative analysis of osteogenic differentiation was performed using an osteogenesis assay kit (MUBMX-90021; EMD Millipore), according to the manufacturer's instruction. For adipogenic differentiation, induced lipid droplets were visualized with Oil Red O (Sigma-Aldrich, St. Louis, MO, USA). Quantitative analyses of the lipids were performed by spectrophotometry (1510; Thermo Fisher Scientific, Inc.) of isopropanol-extracted Oil Red O staining.

Statistical analysis. All data were analyzed by SPSS 13.0 and expressed as the mean \pm standard error of the mean. Statistical

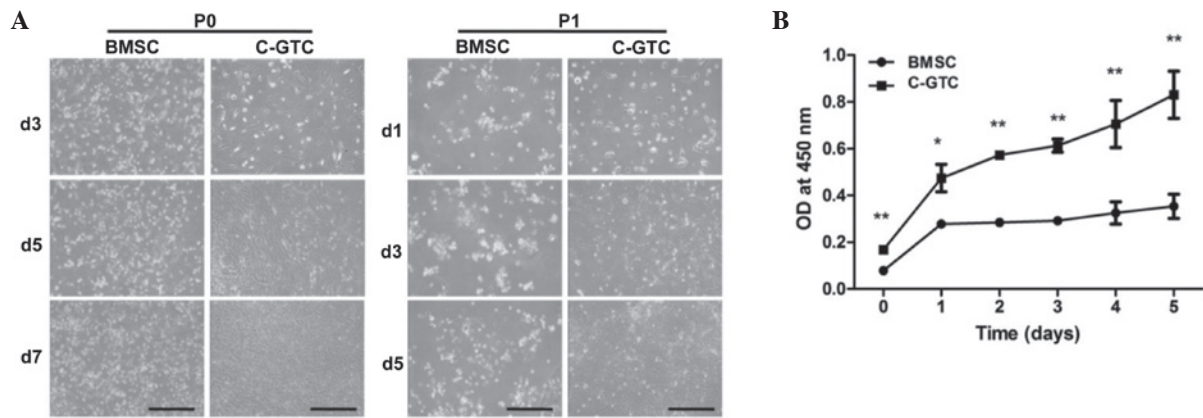


Figure 1. Comparison of proliferative capacity between C-GTCs and BMSCs in the CRWI group. (A) The growth of cells was observed at days 3, 5 and 7 for P0, and at days 1, 3 and 5 for P1 (scale bar, 500 μm). (B) Quantitative analysis of C-GTCs and BMSCs using the Cell Counting Kit-8. *P<0.05, **P<0.01 vs. BMSC group. BMSC, bone marrow-derived mesenchymal stromal cells; C-GTC, granulation tissue-derived cells from the skin wounds of CRWI mice; CRWI, combined radiation and wound injury; P0, primary passage; P1, subculture; d, day; OD, optical density.

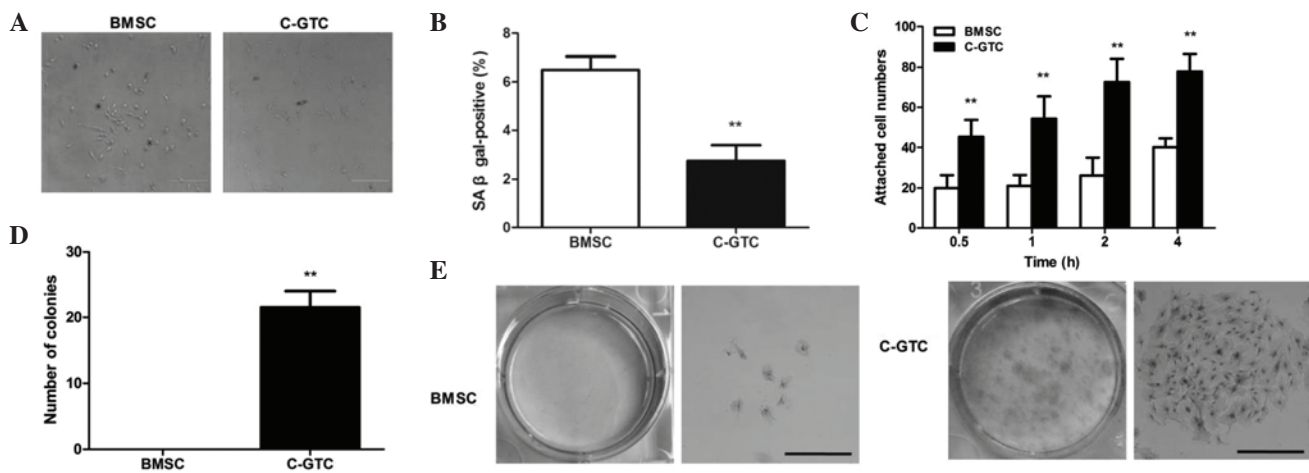


Figure 2. Senescence, adhesion and colony-formation of C-GTCs and BMSCs. (A) SA- β -gal activity analysis in which positive cells were stained blue (dark stain in the figure). (B) Percentage of SA- β -gal-positive cells in a high power field (x200) was calculated. (C) Cell adhesion assay. At the time-point of 0.5, 1, 2 and 4 h after plating, the cells were stained and cell attachment was measured by the number of cells per field. (D) Quantitative analysis of colony formation ability. (E) Colonies were cultured for 12 days and stained with Wright-Giemsa [left: Visual observation; right: Microscopic image (magnification, x200)]; scale bar, 500 μm . **P<0.01 vs. BMSC group. BMSC, bone marrow-derived mesenchymal stromal cells; C-GTC, granulation tissue-derived cells from the skin wounds of CRWI mice; CRWI, combined radiation and wound injury; SA- β -gal, senescence-associated β -galactosidase.

significance was examined with an independent-samples *t* test for comparison of C-GTCs and BMSCs from mice with CRWI, and by one-way analysis of variance for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

C-GTCs and BMSCs exhibit different sensitivities to radiation. The proliferative capacity of BMSCs and C-GTCs derived from mice with CRWI were compared. The primary passage (P0) C-GTCs demonstrated comparable proliferation ability following subculture (P1). However, the BMSCs presented markedly inhibited growth following subculture (Fig. 1A). To further determine the difference, the proliferative ability of the P1 of the two populations was examined; the result indicated marked growth suppression in the BMSCs (Fig. 1B). Furthermore, the biomarker, SA- β -gal was used to

evaluate cellular senescence 24 h after incubation. A higher percentage of blue-stained BMSCs was detected (Fig. 2A and B), indicating a greater quantity of aging cells in BMSCs. These experiments indicate that the BMSCs exhibited a higher sensitivity to damage by CRWI.

To further elucidate the biological characteristics of C-GTCs and BMSCs, the cell adherence and colony formation abilities were evaluated. C-GTCs and BMSCs adhered to plastic surfaces; however, C-GTCs demonstrated more rapid and greater attachment than BMSCs at 30 mins after cell plating, and the significant difference persisted throughout the 4 h of culture (Fig. 2C). With regard to the colony forming assay, no colonies were observed in the BMSC group, while C-GTCs demonstrated a significantly enhanced colony forming capability (P<0.01; Fig. 2D and E).

Morphology, colony formation and proliferation of C-GTCs. C-GTCs were demonstrated to be more radioresistant and

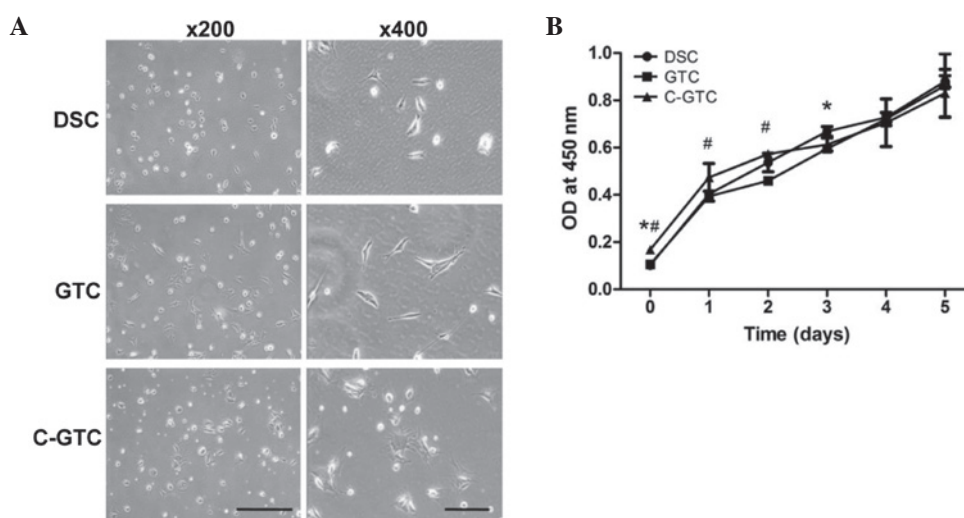


Figure 3. Morphological observation and proliferation of DSCs, GTCs and C-GTCs. (A) Unirradiated DSCs and GTCs demonstrated typical elongated, fibroblast-like morphology, however the C-GTCs exhibited a flattened phenotype. Scale bar, 500 μ m with magnification, x200; scale bar, 200 μ m with magnification, x400. (B) Quantitative analysis using the Cell Counting Kit-8. * $P < 0.05$ vs. DSCs and # $P < 0.05$ vs. GTCs. DSC, dermal stromal cells; GTC, granulation tissue-derived cells; C-GTCs, GTCs from the skin wounds of CRWI mice; CRWI, combined radiation and wound injury; OD, optical density.

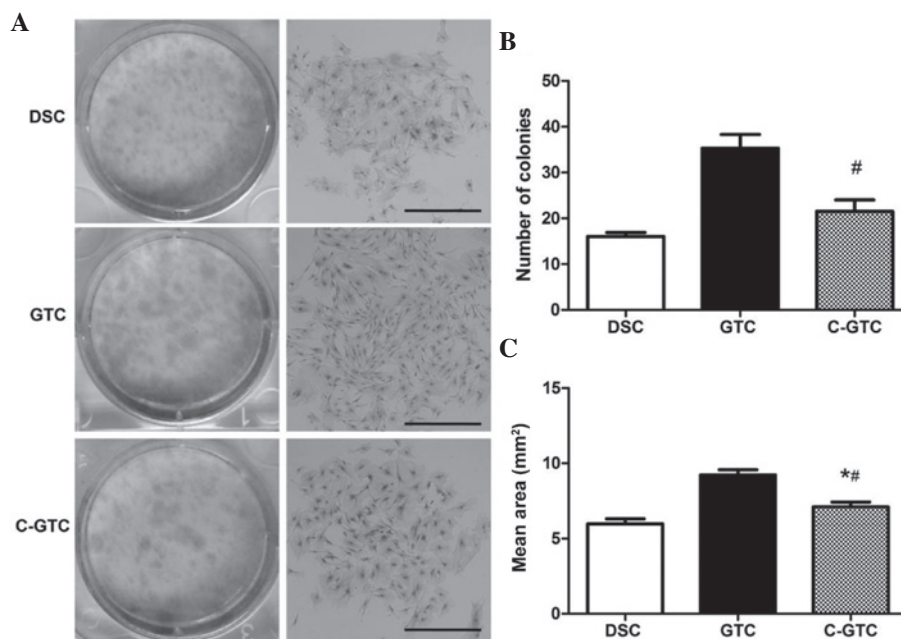


Figure 4. Colony formation assay. (A) Colonies were cultured for 12 days and stained with Wright-Giemsa [left: Visual observation; right: Microscopic images (magnification, x200); scale bar, 500 μ m]. (B and C) Quantitative analysis of colony forming ability. * $P < 0.05$ vs. DSCs, and # $P < 0.05$ vs. GTCs. DSC, dermal stromal cells; GTC, granulation tissue-derived cells; C-GTCs, GTCs from the skin wounds of combined radiation and wound injury mice.

easily accessible when compared with BMSCs from mice with CRWI. To further investigate the biological features of C-GTCs, neonatal DSCs and GTCs from wounds without irradiation (GTCs) were used as control cells.

The morphologies of cells from the three groups (C-GTC, GTC and DSC) were analyzed under a light microscope using unstained cells. GTCs and DSC had an elongated, fibroblast-like morphology, whereas C-GTCs were larger, irregularly-shaped and appeared flattened (Fig. 3A). The CCK-8 assay result suggested that the patterns of cell proliferation exhibited by C-GTCs, GTCs and DSCs were comparable (Fig. 3B). Furthermore, the colony forming capacity, which is a

feature associated with stem cells, was determined. The colony forming experiments demonstrated that GTCs formed the most colonies, followed by C-GTCs and finally DSCs (Fig. 4). Accordingly, the mean area of the colonies from large to small was also in the order GTCs, C-GTCs and DSCs (Fig. 4).

Migration and adhesion of C-GTCs. To examine whether CRWI affects cell migration, the cell migration ability of C-GTCs were evaluated using a scratch wound assay. Images of scratch wounds were captured at 0, 12, 24 and 36 h after treatment, and the wounds in C-GTCs, GTCs and DSCs were completely closed by 36 h. The wound area was then quantified

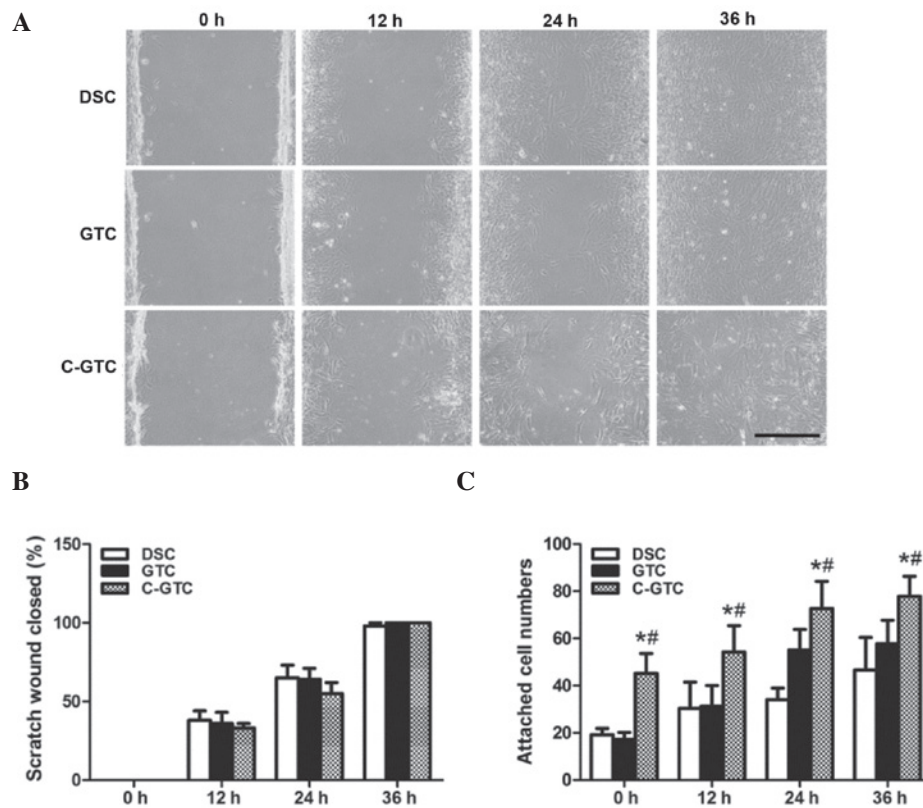


Figure 5. Cell migration and adhesion ability. (A) Images of each group were captured at 0, 12, 24 and 36 h. Scale bar, 500 μ m. (B) The rate of scratch wound closure compared with the primary area. (C) Quantitative analysis of the cell adhesion assay. * $P < 0.01$ vs. DSC and $^{\#}P < 0.01$ vs. GTCs. DSC, dermal stromal cells; GTC, granulation tissue-derived cells; C-GTCs, GTCs from the skin wounds of combined radiation and wound injury.

using Image J software and the healing rate was calculated. The data indicated that there was no significant difference in the rate of C-GTCs when compared with that of GTCs and DSCs ($P > 0.05$; Fig. 5A and B).

To investigate the effect of radiation on cell adhesion, equal numbers of C-GTCs, GTCs and DSCs were plated into 24-well plates and attachment was monitored over 4 h. The result demonstrated that C-GTCs had a marked attachment ability and exhibited processes and flattened shapes (Fig. 5C), indicating that irradiation may improve the adhesive capacity of mesenchymal stem cells (MSCs).

Differentiation potential of C-GTCs. The osteogenic and adipogenic differentiation potential is a unique characteristic of MSCs. Therefore, the differentiation capability of C-GTCs were evaluated, with DSCs and GTCs serving as controls. Fig. 6A demonstrates that C-GTCs were able to differentiate into osteocytes (with the mineralized nodules highlighted by Alizarin Red staining). However, quantitative analysis indicated that C-GTCs formed fewer mineralized extracellular matrices than GTCs ($P < 0.01$; Fig. 6B). In addition, when cultured in adipo-inductive media for 7 days, all three populations were able to form lipid globules; the lipid droplets were stained with Oil Red O (Fig. 6A). The results indicate that all three cell populations displayed the potential to differentiate into adipocytes, including C-GTCs, although absorbance of the Oil Red O extract was lower in the C-GTC group than those of the control groups (GTCs and DSCs; Fig. 6C). These results indicated that the differentiation ability of GTCs was not abrogated by CRWI.

Discussion

Adult stem cell-based therapy presents as a promising treatment strategy for diseases and injuries, including CRWI (22). Stem cells are isolated from different types of tissue, including bone marrow, adipose tissue, the skin and umbilical cords (23). However, the cell source is a challenge for the management of wounds that are difficult to heal, such as CRWI. In the current study, the effects of radiation on the isolation and proliferation of C-GTCs and BMSCs in mice with CRWI were investigated. Although it has been reported that BMSCs exhibit a certain quantity of radioresistance in order to retain their stem cell characteristics, including proliferation, adherence, colony formation ability and differentiation potential (24), the present results demonstrated that BMSCs were more sensitive to the damage caused by CRWI. In addition, it was particularly difficult to harvest ideal cells from the bone marrow of mice with CRWI. Notably, the isolated C-GTCs demonstrated higher resistance to CRWI and exhibited a significantly lower level of senescence when compared with BMSCs, and preserved their self-renewal and multilineage differentiation capacities as effectively as neonatal DSCs and GTCs from unirradiated SWs.

Skin is the largest organ of the body and the dermis has been shown to contain various stem cells populations (25). It has been established that stem cells are important in wound healing and that, following wounding, newly formed granulation tissue is enriched in cells that express stem cell surface markers (26). Previous studies have reported the therapeutic implications of GTCs in different animal models (26,27). The

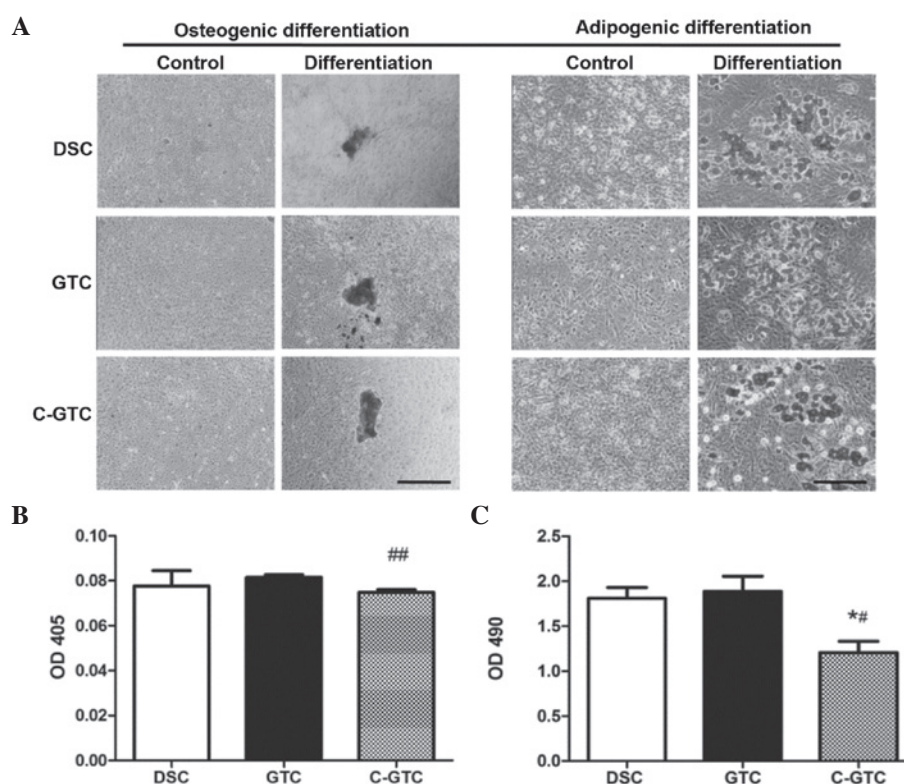


Figure 6. Osteogenic and adipogenic differentiation in DSCs, GTCs and C-GTCs. (A) Representative images of control and induced differentiated cells. Cells that underwent osteogenic differentiation were stained with Alizarin Red and cells that underwent adipogenic differentiation were stained with Oil Red O. Scale bar, 500 μ m. Quantitative analysis of (B) mineralized nodules and (C) Oil Red O-positive staining. ^{*}P<0.05 vs. DSCs, [#]P<0.05, ^{##}P<0.01 vs. GTCs. DSC, dermal stromal cells; GTC, granulation tissue-derived cells; C-GTCs, GTCs from the skin wounds of combined radiation and wound injury mice; OD, optical density.

GTCs are able to secrete important factors, such as vascular endothelial growth factor and maintain their trilineage differentiation ability *in vitro* (28). Furthermore, GTCs may mitigate damage and accelerate repair in liver and kidney injury (28,29). Therefore, granulation tissue is emerging as a potential source of multifunctional cells for transplantation therapy.

Previous studies have demonstrated that transplantation of mesenchymal stem/stromal cells is a potent therapeutic method for radiation-induced damage in various organs and tissues, such as salivary glands (8), lungs (30), the liver (31,32), skin (33), bone marrow (34) and intestines (35,36). Studies have demonstrated that MSCs exert their therapeutic effects via multiple mechanisms, including engraftment and differentiation into target cell types, immunomodulation and anti-inflammation activities [by decreasing the expression levels of inflammatory cytokines, such as interleukin (IL)-1 α , IL- β and tumor necrosis factor- α], and promoting the paracrine action of growth factors associated with neovascularization (33,36). In addition, the transplantation of MSCs was reported to upregulate the expression of cell cycle-associated genes, including cyclin-dependent kinase inhibitor 1A (37). Furthermore, oxidative stress may be reduced following MSC therapy (31). In our previous study, it was reported for the first time, to the best of our knowledge, that systemic transplantation of neonatal dermal multipotent cells significantly promoted survival, and accelerated hematopoietic recovery and wound healing in rats with CRWI. This indicated that stem cell therapy achieves multiple therapeutic effects and provides a potential novel strategy for the treatment of severe

traumatic injuries comprised of multiple tissue/organ damage, such as radiation combined injuries (38). In the present study, it was verified that the isolated C-GTCs possessed comparable stem cell-associated properties with neonatal DSCs and GTCs from normal wounds without irradiation. Considering the ease of accessibility, granulation tissue is proposed to be an optimal, autologous source of stem/progenitor cells for therapeutic applications in CRWI, for the replacement of skin, as well as for tissue repair of other organs.

The source of MSCs for transplantation therapy of CRWI has been widely investigated. Previous research has demonstrated that granulation tissue derived from the dermis is a promising stem cell source and that the GTCs exhibit stem cell-associated properties. In the current study, the characteristics of C-GTCs (obtained from CRWI mice) were investigated, and the C-GTCs displayed advantageous properties when compared with BMSCs, including improved radiation resistance and biological characteristics, such as proliferative, colony formation and adhesion abilities. In addition, C-GTCs better retained their stem cell characteristics when compared with DSCs and GTCs that were obtained from normal granulation tissue. In conclusion, C-GTCs have been demonstrated as a potential autologous source for the treatment of CRWI.

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