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Differential Effect of Ionizing Radiation Exposure on Multipotent and Differentiation-Restricted Bone Marrow Mesenchymal Stem Cells

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ABSTRACT

Debilitating effects of bone marrow from ionizing radiation exposure has been well established for hematopoietic stem cells; however, radiation toxicity of mesenchymal stem cells (MSCs) has been controversial. The present study addressed if ionizing radiation exposure differently affected bone marrow MSCs with various differentiation commitments. Mouse bone-marrow-derived MSCs, D1 cells of early passages ($\leq$5 passages; p5) maintained the complete characteristics of multipotent MSCs, whereas, after $\geq$45 passages (p45) the differentiation capability of D1 cells became partially restricted. Both p5 and p45 D1 cells were subjected to single dose irradiation by radioactive isotope ^{137}Cs. Radiation treatment impaired cell renewal and differentiation activities of p5 D1 cells; however, p45 D1 cells were less affected. Radiation treatment upregulated both pro- and anti-apoptotic genes of p5 D1 cells in a dose-dependent manner, potentially resulting in the various apoptosis thresholds. It was found that constitutive as well as radiation-induced phosphorylation levels of histone H2AX was significantly higher in p45 D1 cells than in p5 D1 cells. The increased repair activity of DNA double-strand breakage may play a role for p45 D1 cells to exhibit the relative radioresistance. In conclusion, the radiation toxicity predominantly affecting multipotent MSCs may occur at unexpectedly low doses, which may, in part, contribute to the catabolic pathology of bone tissue. J. Cell. Biochem. 111: 322–332, 2010.

KEY WORDS: IONIZING RADIATION; MESENCHYMAL STEM CELL; DIFFERENTIATION; RADIATION TOXICITY

Extensive research on the health effects of ionizing radiation has been conducted using standard epidemiological and toxicological approaches for decades to characterize responses of populations and individuals to whole body exposure at lethal doses. Radiation exposures associated with current human activities are increasingly expected to be at low or non-lethal dose-rates from medical treatments [Waselenko et al., 2004; Azizova et al., 2008; Kuniak et al., 2008], waste clean up, and environmental isolation of materials associated with nuclear weapons and nuclear power production [Senyuk et al., 2002], and nuclear power plant criticality accidents [Hirama et al., 2003]. It has increasingly recognized that the exposures to non-lethal dose-rate radiation may result in the late onset debilitating disorders. Reports of the LD 50/30 (50% fatality after 30 days) for humans vary between 3 and 7 Gy [Langlois et al., 1987]. The Radiation Effects Research Foundation Life Span Study including a cohort of 86,572 atomic bomb survivors in Hiroshima and Nagasaki reported that while the major cause of death was due to solid cancer, there was a significant trend of increasing incidences of and death by non-cancer diseases [Preston et al., 2003; Zhang et al., 2005]. Accelerated bone dystrophy such as osteoporosis was among those non-cancer diseases experienced by this cohort.

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Bone marrow tissue is highly sensitive to ionizing radiation. It has been extensively investigated on the effect of radiation exposure to bone marrow hematopoietic stem cells (HSCs) that are sensitively affected on viability and function [Dainiak and Sorba, 1997]. The treatment of whole body irradiation of 2–3 Gy and bone marrow transplantation allows the repopulation of donor HSCs in the recipient bone marrow, and has become an established treatment for curative therapy of leukemia. On the contrary, mesenchymal stem cells (MSCs) of these patients long after the total body irradiation and bone marrow transplantation treatment have been found to exhibit a complete host profile [Dickhut et al., 2005; Rieger et al., 2005]. These observations suggest that exogenous MSCs derived from bone marrow transplantation do not repopulate in the recipient bone marrow and that MSCs possess radioresistant characteristics and patients' MSCs have survived the whole body irradiation.

While the recipients survive for longer term in recent years, unexpected skeletal complications of total body irradiation and bone marrow transplantation have become realized such as osteopenia or age-unrelated osteoporosis that cause significant chronic morbidity [Schimmer et al., 2000; Cohen and Shane, 2003]. Adult bone tissue is constantly remodeled through recruitment and guided differentiation of local stem cell populations. The development of late onset bone dystrophy may be caused by radiation-induced injury to bone marrow HSCs and MSCs affecting the replenishment of renewing cells and/or their differentiation capability of osteoclasts and osteoblasts, respectively. Unlike HSCs, the radiation effect on MSCs has not been investigated until recently. Studies using MSCs isolated from bone marrow aspirate of a single human donor [Chen et al., 2006] and three donors [Li et al., 2007], as well as from neonate rat tibia and femur [Schonmeyr et al., 2008] reported different cellular responses to radiation treatments in vitro. These contradicting observations may, in part, be due to significant cellular variations in bone marrow aspirate samples.

The purpose of this study was to characterize the radiation-induced injury of MSCs with clonally defined phenotypes. We selected mouse bone marrow MSCs D1 ORL-UVA cells (D1 cells) as an experimental model. D1 cells from early passages exhibited multipotent MSC phenotype, whereas differentiation capability of those from late passages was found to be restricted. Our data demonstrated that whereas differentiation-restricted MSCs were found less radiosensitive, the significant radiation toxicity of multipotent MSCs was elicited under unexpectedly low doses.

MATERIALS AND METHODS

MOUSE BONE MARROW MSC

Mouse bone marrow derived stromal cells (D1 ORL UVA [D1], ATCC® Number: CRL-12424™) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Cellgro, Mediatech Inc., Manassas, VA) supplemented with 10% Fetal Bovine Serum (FBS; Benchmark, Gemini Bio-Products, West Sacramento, CA), 1% penicillin–streptomycin (MD Biomedicals, Thermo Fisher Scientific, Waltham, MA)—henceforth Control Medium—under a humidified atmosphere of 5% CO_2 in air, 37°C. Cells were always passaged at subconfluency to prevent contact inhibition. D1 cells of early passages (less than 5 passages; p5) and late passages (more than 45 passages; p45) were used in this study.

Separately, primary bone marrow stromal cells (MSCs) were harvested from femurs and tibias of adult mice following a standard method [Wang et al., 2008] and cultured in Control Medium. Primary MSCs of early passages (less than 5 passages; p5) and late passages (more than 15 passages; p15) were used.

COLONY FORMING EFFICIENCY

D1 cells (10^2/ml) were plated in p100 plates and incubated in Control Medium for 14 days. Cells were stained with Crystal Violet in methanol and photographed.

MESENCHYMAL STEM CELL SURFACE MARKER EXPRESSION

The expression of MSC and HSC cell surface markers of in p5 and p45 D1 cells was characterized by immunocytology and fluorescence-activated cell sorting system (FACS). For immunocytology, D1 cells (1×10^4 to 5×10^4 cells) were plated into 8-well Lab-Tek™ II Chamber Slide™ System (Thermo Fisher Scientific) and incubated in Control Medium for overnight. After fixed with 4% paraformaldehyde/PBS and treated with Image-iT™ FX Signal Enhancer (Molecular Probes, Invitrogen, Carlsbad, CA), cells were incubated overnight at 4°C with antibodies for Ly-6A/E (sca-1), CD-73, CD-90 (BD Pharmingen, Franklin Lakes, NJ), CD44 (Calbiochem, Merck KGaA, Darmstadt, Germany) or CD45.1 (Abcam, Cambridge, MA) in dilution buffer (10%FBS, 0.5%BSA, 0.01% Triton X-100 in Calcium and Magnesium free PBS). Some slides were incubated with Phycoerytrin conjugated mouse monoclonal anti-CD11b antibody (Lifespan Biosciences, Seattle, WA) for 1 h in dilution buffer after fixing in 100% ice-cold methanol for 20 min on ice. All slides were further treated with Alexa Fluor® 488 conjugated secondary antibody (Molecular Probes, Invitrogen) followed by DAPI staining. A confocal laser-scanning microscope (Leica SP2 1P-FCS, Leica Camera AG, Solms, Germany) was used to acquire photomicrographs.

For FACS analysis, the same primary and secondary antibodies as above were utilized. Cells were harvested by trypsin treatment, washed in PBS, fixed in ice-cold 70% ethanol and immediately stored at −20°C for at least 1 day. Cells in Flow Cytometry Buffer (FCB: 1% Fetal Bovine Serum, 0.1% Bovine Serum Albumin, 0.01% Triton in PBS) were incubated with primary antibodies for 30 min and with secondary antibodies in ice for 20 min followed by FACS analysis.

ADIPOGENIC AND OSTEOGENIC DIFFERENTIATION

Adipogenic Medium was prepared by supplementing Control Medium with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM Dexamethasone, 10 μM Insulin and 200 μM Indomethacin. Adipogenic differentiation was determined by staining with Oil Red-O solution following an established protocol.

Osteogenic Medium was prepared by supplementing the Control Medium with 50 μM Ascorbic Acid, 10 mM Beta Glycerophosphate, and 100 nM Dexamethasone. An alternative Osteogenic Medium was prepared by supplementing Control Medium with 50 μM Ascorbic Acid, 10 mM Beta Glycerophosphate, 100 ng/ml human recombinant BMP-2 (R&D Systems, Minneapolis, MN) and 50 μg/ml

heparin (H3393-1MU). Osteogenic differentiation was determined by staining for alkaline phosphatase (ALP), picric acid Sirius Red staining and von Kossa staining following the established protocols.

EXPRESSION OF ADIPOGENIC AND OSTEOGENIC DIFFERENTIATION-RELATED MOLECULES

The expression of PPAR γ-2, Runx-2, osteopontin (OPN) and ALP was examined by Western blot analysis. From adipogenic- or osteogenic-differentiated D1 cells, cytoplasmic cell lysates and nuclear extracts were prepared in RIPA Buffer (Sigma–Aldrich, St. Louis, MO) containing protease inhibitor (Thermo Fisher Scientific). Denatured protein samples (30 μg) were subjected to electrophoresis in a 12% Bis Tris Gel and then transferred onto a nitrocellulose membrane (Protan®, Whatman GhbH, Dassel, Germany). After treated with StartingBlock™ T20 (TBS) Blocking Buffer (Thermo Fisher Scientific), nitrocellulose membranes were incubated overnight at 4°C with primary antibodies for PPAR γ-2 (Cell Signaling Technologies, Danvers, MA), Runx-2 (Abcam), OPN (Rockland, Gilbertsville, PA), ALP (Abcam) or β-actin (Abcam). After incubated with HRP linked secondary antibodies in Blocker™ Blotto in TBS, chemiluminescent reaction was developed by an ECL hydrogen peroxide solution (Thermo Fisher Scientific) and detected in an Intelligent Dark Box LAS3000 (FUJIFILM Holdings America Corporation).

IRRADIATION PROCEDURE

Cells were trypsinized at subconfluency and resuspended in Control Medium. A final concentration of 10^6 cells/ml was irradiated. Single doses from 1 to 12 Gy were administered in a ^{137}Cs irradiator (JL Shepard and Associates, San Fernando, CA). Cells were irradiated in suspension to avoid further stress of manipulation such as trypsinization after irradiation in order to minimize interference with cell recovery. Cells were seeded immediately after irradiation into 96-well plates and at the established time points, cells were subjected to the following assays.

TOTAL PROTEIN ASSAY

The number of cells was determined by measuring the total protein content. Briefly, cells were seeded into 96-well plates and cultured. At the established time points, cells were washed twice in PBS and lysed in RIPA buffer for 5 min. The total protein was determined by BCA™ Protein Assay (Thermo Fisher Scientific). Optical density was measured at a wavelength of 570 nm and results adjust to a calibration curve made by known number of cells.

MTT ASSAY

Cell viability was assessed by colorimetric MTT assay (Chemicon International, Billerica, MA) according to the manufacturer's protocol.

BRDU INCORPORATION ASSAY

Incorporation of BrdU into cells was measured by ELISA assay (Cell Proliferation ELISA, BrdU colorimetric, Roche Diagnostic GmbH, Germany) following the manufacturer's protocol.

SENESCENCE-RELATED β-GALACTOSIDASE ASSAY

Replicative senescence was assessed by senescence β-galactosidase staining assay (Pierce Chemical, Rockford, IL) following the manufacturer's protocol.

CASPASE-3 ASSAY

Cell apoptosis was assessed by caspase-3 detection assay (Chemicon International) following the manufacturer's protocol.

EXPRESSION OF ANTI- AND PRO-APOPTOSIS GENES

Total RNA samples isolated from D1 cells 3 days after 0, 1, 3, 5, or 7 Gy irradiation (RNeasy Mini Kit, Quiagen, Valencia, CA) were subjected to Taqman-based reverse transcription real time polymerase chain reaction (RT-PCR) for Bcl2, Mcl1, TIMP1, FasL, BAX, NOXA, PUMA using commercially available primer/probe cocktails (Mm00477631_m1, Mm01257351_g1, Mm00441818_m1, Mm00438864_m1, Mm00432051_m1, Mm00451763_m1, Mm-00519268_m1, respectively, Applied Biosystems, Foster City, CA), following the manufacturer's protocol.

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE dUTP NICK END-LABELING (TUNEL) ASSAY

D1 cells treated with 0, 1, 3, 5, and 7 Gy irradiation were cultured in 8-well Lab-Tek™ II Chamber Slide™ System (Thermo Fisher Scientific) and incubated in Control Medium for 7 days. D1 cells were subjected to TUNEL assay (Click-iT TUNEL Alexa Fluor 488 imaging assay, Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Cell nuclei were stained by DAPI. The percent apoptotic cells were calculated by the number of TUNEL positive cells divided by the number of total cells in each group.

OSTEOGENIC DIFFERENTIATION

The effect of irradiation on osteogenic differentiation of D1 cells was assessed by colorimetric calcium assay and ALP assay in 96-well plates. For assessing in vitro mineralization, the *o*-cresoftalein colorimetric method was used for calcium determination. To measure ALP concentration, cells were lysed with 0.05% Triton X-100 and incubated with the reagent solution containing phosphatase substrate (Sigma–Aldrich) at 37°C for 15 min. Optical density was measured at a wavelength of 405 nm (reference 620 nm). The final alkaline phosphatase concentration was adjusted per total protein content. Separately, D1 cells in osteogenic differentiation medium were cultured in 6-well plates and in vitro mineralization was evaluated by von Kossa staining.

ADIPOGENIC DIFFERENTIATION

The effect of irradiation on adipogenic differentiation of D1 cells was assessed by Oil Red-O staining in cell culture as described above. Total RNA samples prepared from D1 cells were subjected to Taqman-based RT-PCR for PPARγ2 using a commercially available primer/probe cocktail (Mm01184323, Applied Biosystems), following the manufacturer's protocol.

PHOSPHORYLATION OF HISTONE H2A.X

Phosphorylation of histone H2A.X was examined by Western blot analysis and immunocytology following the protocol described

above, with the primary antibody recognizing phosphorylated histone H2A.X (Cell Signaling Technologies). Three independent Western blot experiments were quantified by means of ImageJ software and the average values were obtained.

RESULTS

CHARACTERIZATION OF MOUSE BONE MARROW MSCs D1 CELLS

Mouse bone marrow derived D1 cells showed sufficient colony formation efficiency (Fig. 1A) and exhibited positive immunostaining for mesenchymal markers (CD44, Sca-1, CD73, CD90) and negative for hematopoietic markers (CD11b, CD45) (Fig. 1B). When cultured in the corresponding differentiation medium, D1 cells developed osteogenic, adipogenic and chondrogenic differentiation (Supplemental Fig. 1). These data confirmed that D1 cells possess the MSC characteristics.

RESTRICTED DIFFERENTIATION CAPABILITY OF D1 CELLS WITH LATE PASSAGES

It was found that D1 cells from late passages (45 or more passages; p45) exhibited more spindle-shaped and showed a higher refractory index than the early passages (5 or less passages; p5; Fig. 1C). The FACS analysis indicated that the CD73 expression was significantly decreased in p45 D1 cells compared to p5 D1 cells (Fig. 1C).

The repeated passages of D1 cells were also found to result in a complete loss of adipogenic differentiation. Whereas p5 D1 cells incubated in the adipogenic differentiation medium showed an increase in PPARγ-2 levels examined by Western blot, PPARγ-2 protein expression of this adipogenic master gene was not detected in p45 D1 cells (Fig. 2A). Lipid droplets formed during adipogenic differentiation were depicted by Oil red O staining in p5 D1 cells but not in p45 D1 cells (Fig. 2B). On the contrary, both p5 and p45 D1 cells indicated the presence of Runx-2, alkaline phosphatase and osteopontin following culture in proper osteogenic media (Fig. 2C). These data suggested that long-term passages selectively hindered the adipogenic differentiation of D1 cells, and thus the multipotent differentiation capability of p45 D1 cells was thought to be restricted.

Similar to D1 cells, primary BMSCs of late passage (p15) significantly decreased the ability to develop adipogenic differentiation (Fig. 2C). Primary BMSCs of early passage (p5) and late passage (p15) exhibited equivalent levels of osteogenic differentiation capacity (Fig. 2D).

RADIATION EFFECT ON RENEWAL CAPABILITY OF MSCs

Both p5 and p45 D1 cells were irradiated by single dose of 0, 3, 7, or 12 Gy by radioactive isotope ^{137}Cs. The relative increase of whole protein content was significantly impaired after 7 days of incubation of p5 D1 cells in a dose dependent manner. Throughout the 14-day

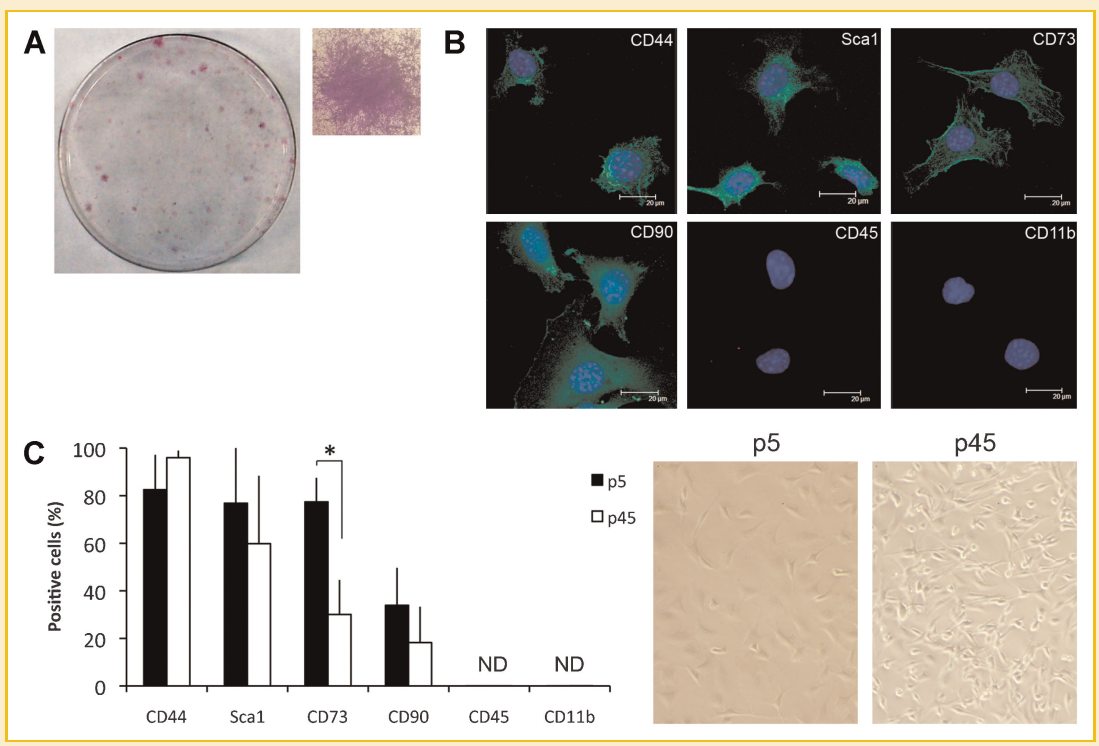

Fig. 1. Characterization of D1 cells. A: A typical crystal violet staining demonstrating the colony formation efficiency of D1 cells. B: Immunocytological staining indicate the positive expression of MSC markers, CD44, Sca1, CD73 and CD90, whereas the negative expression of HSC markers, CD45 and CD11b. C. FACS analyses for MSC and HSC markers of p5 and p45 D1 cells. A significant decrease of CD73 expression was found in p45 D1 cells. Error bars represent standard error. $^{*}P < 0.05$, ND: not detected. Cellular morphology of p5 and p45 D1 cells by phase contract microscopy revealed that narrow spindle-shaped cell morphology was more common in p45 D1 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

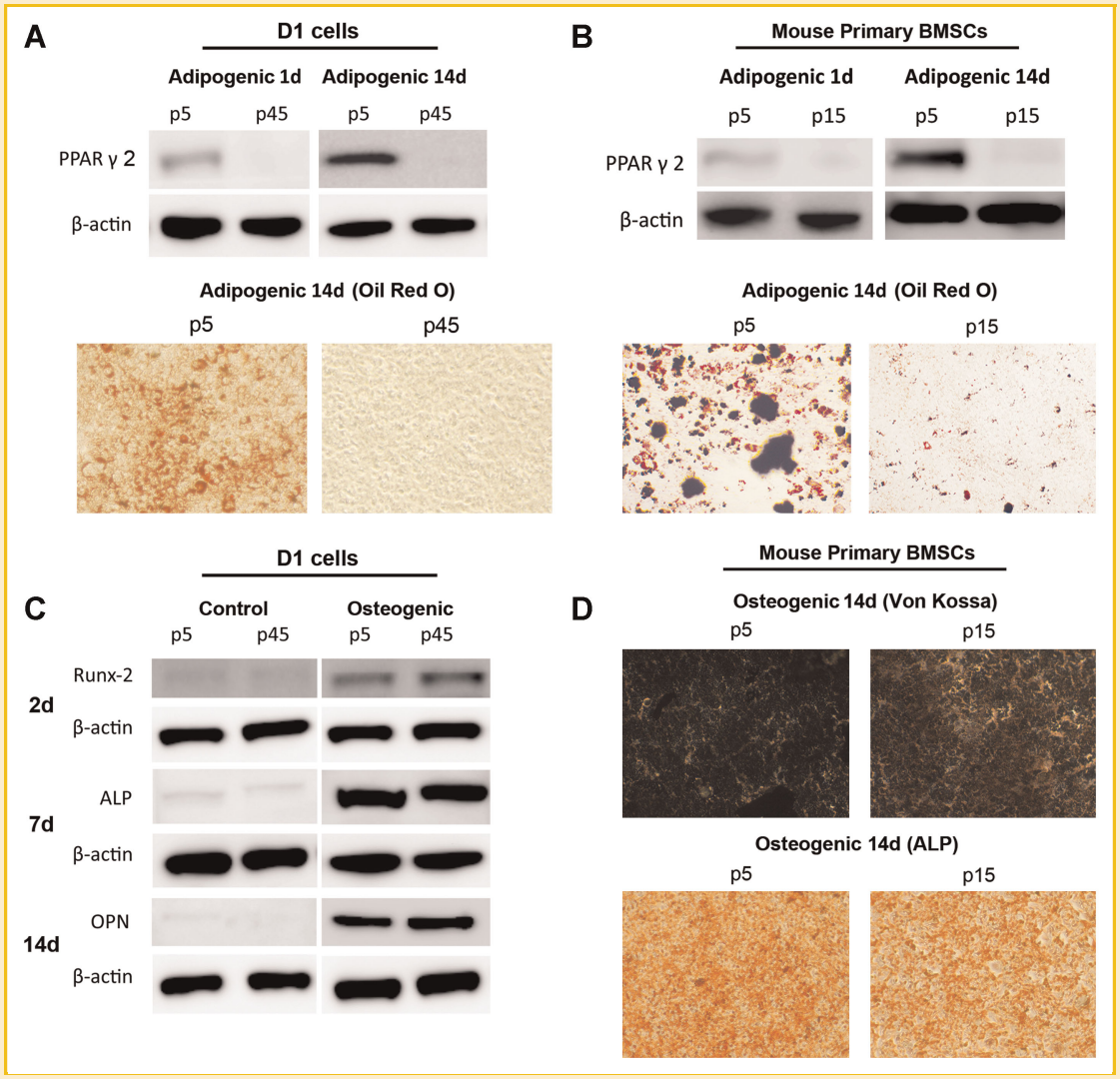

Fig. 2. Differentiation capability of early and late passage MSCs. A: Adipogenic differentiation of early passage (p5) and late passage (p45) D1 cells examined by Western blot analysis of PPARγ-2 expression and Oil Red O staining. B: Osteogenic differentiation of p5 and p45 D1 cells examined by Western blot analysis for Runx-2, ALP and OPN. C: Adipogenic differentiation of early passage (p5) and late passage (p15) mouse primary bone marrow MSCs (BMSCs) examined by Western blot analysis of PPARγ-2 expression and Oil Red O staining. D: Osteogenic differentiation of p5 and p15 BMSCs examined by von Kossa and ALP staining. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

incubation period, p5 D1 cells exposed to the 12 Gy irradiation did not show any increase in the whole protein content, whereas these cells exposed to 3 or 7 Gy irradiation recovered the whole protein increase during the same period (Fig. 3A). On the contrary, p45 D1 cells were less prone to the toxic effects of irradiation than p5 D1 cells, showing substantially uniform increase patterns of the whole protein contents up to 7 Gy of exposure (Fig. 3A).

MTT assay showed that the radiation exposure at 7 and 12 Gy induced the late-onset reduction in cell viability for p5 D1 cells. Such late-onset effect was only achieved at the higher irradiation dose (12 Gy) for p45 D1 cells (Fig. 3B).

The BrdU assay indicated that there was a sharp distinction of radiation effect on cell proliferation between 3 and 7 Gy for p5 D1 cells and between 7 and 12 Gy for p45 D1 cells (Fig. 3C). The senescence-related β-galactosidase assay revealed that radiation treatment did not induce replication senescence for p5 D1 cells (Fig. 3D).

The caspase-3 activity of p5 D1 cells was monitored for 14 days and suggested that 12 Gy irradiation resulted in the late-onset activation of caspase-3; however 3 and 7 Gy irradiations did not have significant effect (Fig. 3E). Furthermore, the effect of irradiation on the expression of anti- and pro-apoptotic genes was examined by RT-PCR (Fig. 3F). While Bcl2 and TIMP1 were not affected, there was dose-dependent increase in Mcl1 expression. At the same time, all pro-apoptotic genes tested such as FasL, BAX, NOXA and PUMA were dose-dependently increased.

Apoptosis-related DNA fragmentation was evaluated by TUNEL assay. There was a mild increase in TUNEL positive cells (arrowheads in Fig. 3G). TUNEL positive cells accounted for $0.24 \pm 0.21\%$, $0.41 \pm 0.39\%$, $2.16 \pm 0.77\%$ $23.29 \pm 10.29\%$, and $16.22 \pm 8.76\%$

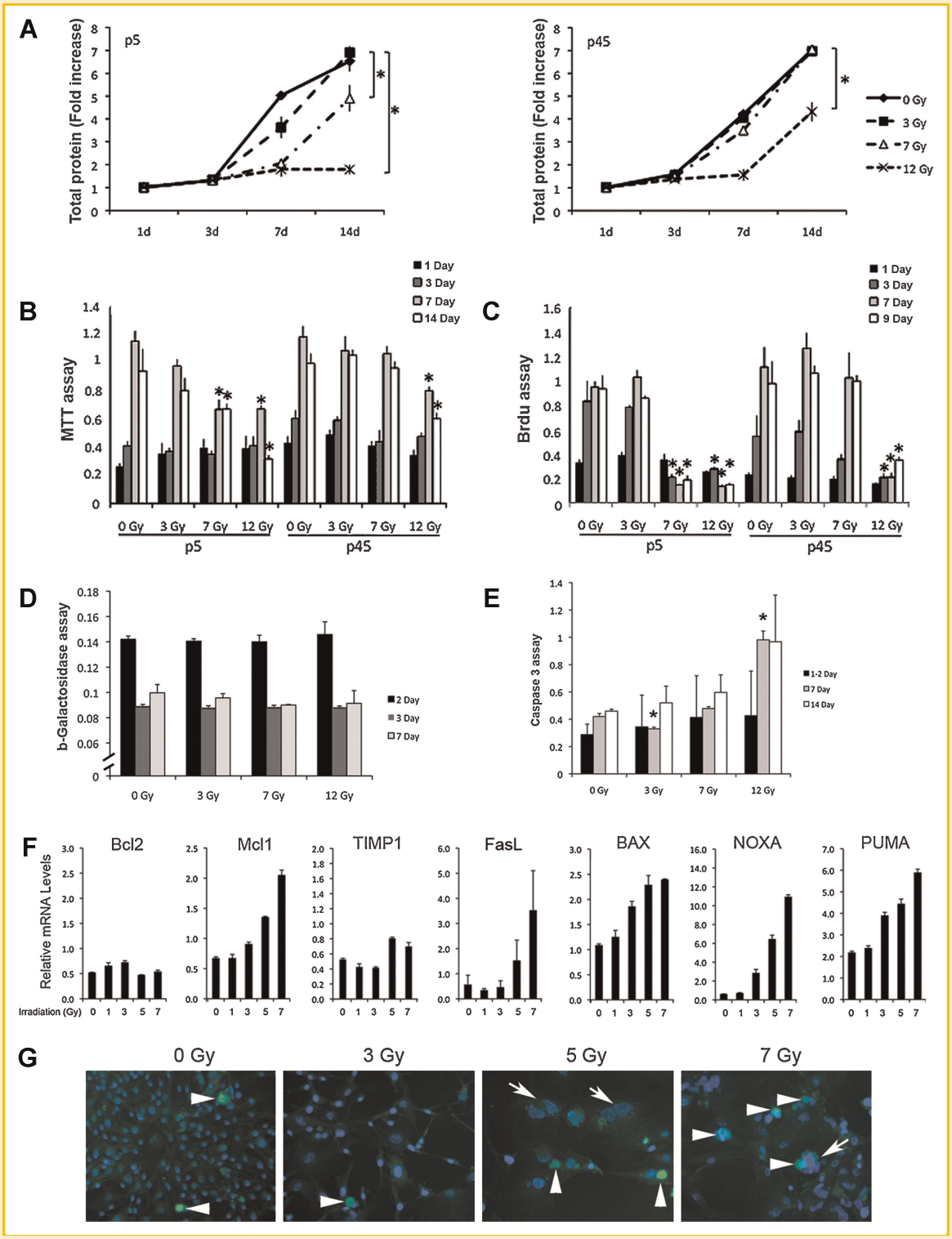

Fig. 3. The effect of 137Cs irradiation on renewal capability of D1 cells. A: Whole protein content measurement of p5 and p45 D1 cells after 0, 3, 7, and 12 Gy of irradiation. B: Post-irradiation cell viability of p5 and p45 D1 cells measured by MTT assay. C: Post-irradiation proliferation activity of p5 and p45 D1 cells measured by BrdU incorporation. D: Post-irradiation replicative senescence of p5 D1 cells measured by β-galactosidase assay. E: Post-irradiation apoptosis of p5 D1 cells measured by caspase-3 expression. F: RT-PCR measurements of steady state mRNA levels of anti-apoptotic molecules; Bcl2, Mcl1 and TIMP1; and pro-apoptotic molecules; FasL, BAX, NOXA and PUMA. D1 cells were harvested 3 days after irradiation. G: TUNEL staining (green) and nuclei (blue) of D1 cells 7 days after irradiation. Error bars represent standard deviation. Student's *t*-test was conducted by comparing the corresponding control in the cells treated with 0 Gy irradiation. $^{*}P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

after 7 days of irradiation at 0, 1, 3, 5, or 7 Gy, respectively. Student's *t*-test revealed that the increased TUNEL positive cells by 3, 5, or 7 Gy irradiation were statistically significant with $P < 0.05$. D1 cells irradiated at 5 and 7 Gy induced unusually large cells with seemingly double nuclei (arrows in Fig. 3G). In a separate Live/Dead assay, polykaryon cells were labeled as live cells (data not shown).

RADIATION EFFECT ON DIFFERENTIATION CAPABILITY OF MSCs

The irradiated p5 D1 cells were examined for osteogenic and adipogenic differentiation. The calcium assay (Fig. 4A) and von Kossa staining (Fig. 4B) concordantly indicate that the osteogenic differentiation was significantly reduced with radiation treatments as low as 3 Gy. The impairment of adipogenic differentiation was also shown by the decreased steady state PPARγ-2 mRNA expression (Fig. 4C) and the decreased lipid formation identified by Oil Red-O staining (Fig. 4D) with irradiation treatment with as low as 3 Gy (Fig. 4B). The ''cut-line'' dose of irradiation for osteogenic differentiation was determined for both p5 and p45 D1 cells using ALP assay (Fig. 4E). For p5 D1 cells, the dose dependent reduction of ALP expression was observed from 1 to 5 Gy; and thereafter, the radiation-induced impairment of ALP expression reached plateau. On the contrary, the osteogenic differentiation of p45 D1 cells was less affected. The significant effect of the single radiation dose in p5 D1 cells was observed at 2–3 Gy.

HISTONE H2AX PHOSPHORYLATION (γH2AX)

H2A.X phosphorylation on Serine 139 was assessed by Western blot analysis. After irradiation, there was a transient increase of γH2AX at day 2 of culture followed by normalization by day 7 (Fig. 5A). For p5 D1 cells, the significant transient increase of γH2AX was evident after the radiation treatment of 12 Gy, and a slight but significant increase was noted after the radiation treatment of 7 Gy. On the contrary, for p45 D1 cells, the radiation treatment of 3 Gy and greater induced the significant transient increase of γH2AX. It was noted that the presence of γH2AX was not completely normalized at day 7 when p45 D1 Cells were exposed to the 12 Gy radiation treatment (Fig. 5B). Immunocytological evaluation of untreated p5 and p45 D1 cells revealed that γH2AX foci were only sporadically found in p5 D1 cells, whereas nearly all p45 D1 cells exhibited γH2AX foci at various levels (Fig. 5C).

DISCUSSION

This study demonstrated that multipotent MSCs; p5 D1 cells, and differentiation-restricted MSCs; p45 D1 cells, exhibited different radiation toxicity, in which the former cells were more radio-sensitive at unexpectedly low doses. Ionizing radiation treatment has been shown to induce apoptosis of HSCs causing acute myelosuppression [Domen et al., 1998; Meng et al., 2003a], as well as replicative senescence of the surviving HSCs [Meng et al., 2003b] resulting in the latent damage of radiation exposure. On the contrary, ionizing radiation has been reported to induce limited toxicities in MSCs. When treated with 12 Gy irradiation, the growth of human bone marrow mesenchymal stromal cells were shown to decrease up to 2-week post-irradiation; but thereafter, those cells regained the normal proliferation rate [Li et al., 2007]. In the present study, p45 D1 cells with 12 Gy irradiation appeared to behave in a similar fashion and the cell growth was regained 1 week after the radiation treatment (Fig. 3A). However, p5 D1 cells did not recover from 12 Gy irradiation (Fig. 3A) and further the differentiation capability appeared to be more sensitively affected at unexpectedly low doses (Fig. 4).

Mouse MSC D1 cells were originally isolated as a clonal cell line from bone marrow, exhibiting osteogenic phenotypes [Diduch et al., 1993]. A recent study comparing the response to biomaterial containing arginine-glycine-aspartate indicated that D1 cells shared similar characteristics with human bone marrow stromal cells, both of which were distinct from committed MC3T3-E1 mouse preosteoblasts [Hsiong et al., 2009]. The present study further demonstrated that D1 cells possessed the specific characteristics as multipotent MSCs (Fig. 1A,B and Supplemental Fig. 1). A novel observation in this study was that when allowed 45 or more passages, D1 cells modified the phenotype highlighted by reduction of the CD73 expression and the complete loss of adipogenic differentiation capability (Figs. 1C and 2). Albeit incapable of adipogenic differentiation, p45 D1 appeared to be functional cells able to properly respond to osteogenic stimuli and endowed with growth rate similar to p5 D1 cells.

Unlike bone marrow mesenchymal stromal cells (BMSCs), which represent diverse cell populations, the clonally derived D1 cells may present only an isolated phenotype, which may make these observations biologically irrelevant. To address this issue, primary BMSCs from mouse bones were applied to the duplicated experiments. The data indicate that the repeated passages of both D1 cells and primary BMSCs similarly resulted in the partially limited differentiation capability. Therefore, this phenomenon should not represent the case of such an extreme change in cell line characteristics [Chang-Liu and Woloschak, 1997]. Neither crisis occurred during the cell culturing, nor the number of passages was so high. Instead, the loss of PPARγ-2 expression in p45 D1 cells and p15 BMSCs may indicate the activation of osteogenic differentiation signals such as bone morphogenetic protein 2 and Wnt/β-catenin [Takada et al., 2009] during cellular aging. Under adverse culturing conditions, BMSCs have a tendency to default towards osteogenic differentiation in vitro. This natural tendency may also be considered when the fate of differentiation was under evaluation, and explain, in part, why adipogenic differentiating ability decreased with passage.

Different radiation sensitivity exhibited by p5 D1 and p45 D1 cells, particularly for the impairment of their differentiation capabilities (Fig. 4), may shed a light on some of the inconsistencies found in literature on the radiation effect of human mesenchymal stromal cells from bone marrow. One study reported that the 9 Gy irradiation did not affect the MSCs' adipogenic, osteogenic and hepatogenic differentiation capabilities [Chen et al., 2006], while the other study reported that the 8 Gy irradiation suppressed adipogenic differentiation and the 4 Gy irradiation decreased osteogenic differentiation [Li et al., 2007]. Most studies utilize primary cells obtained by means of common isolation techniques from bone marrow, which do not generally allow purified cell populations to be

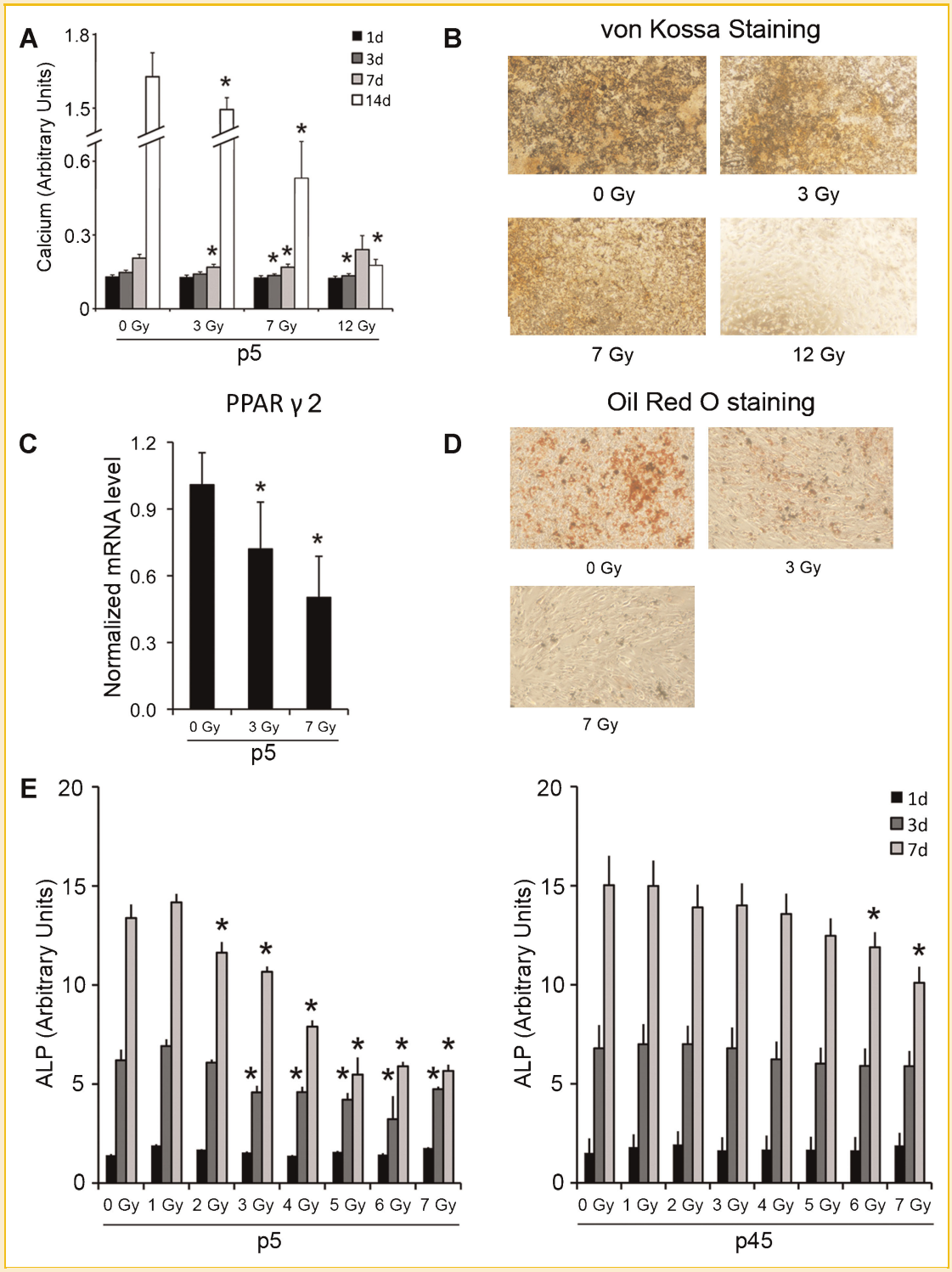

Fig. 4. Irradiation and osteogenic and adipogenic differentiation capabilities of D1 cells. A: Calcium accumulation normalized by whole protein content of post-irradiation p5 D1 cells maintained in osteogenic medium. B: Von Kossa staining of post-irradiation p5 D1 cells maintained in osteogenic medium. C: The steady state mRNA expression of PPARγ-2 of post-irradiation p5 D1 cells maintained in adipogenic medium. D: Oil Red O staining of post-irradiation p5 D1 cells maintained in adipogenic medium. E: Post-irradiation p5 and p45 D1 cells were cultured in osteogenic medium containing BMP2 and measure for ALP expression. Error bars represent standard deviation. Student's *t*-test was conducted by comparing the corresponding control in the cells treated with 0 Gy irradiation. $^{*}P < 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

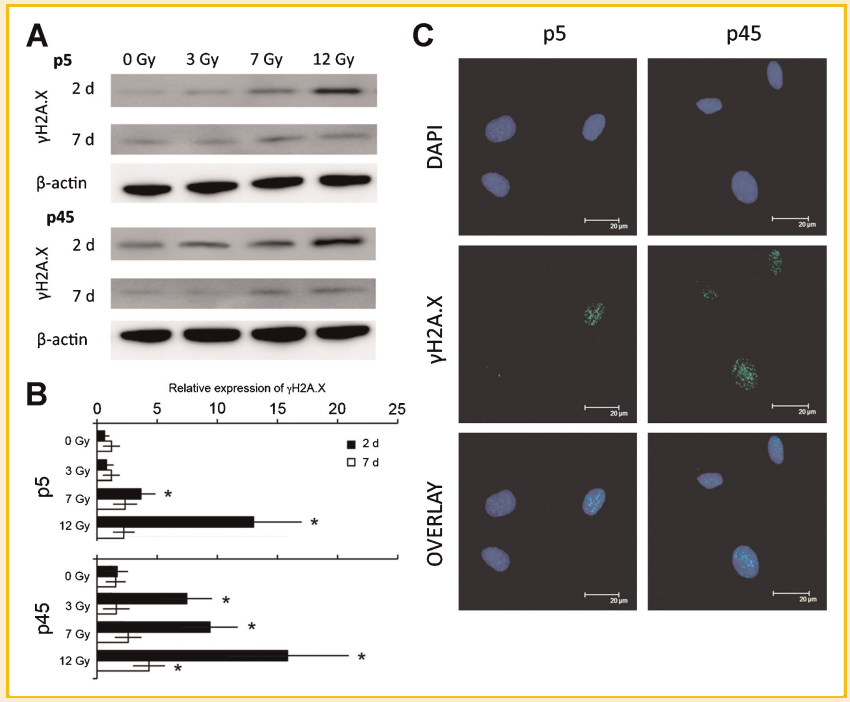

Fig. 5. Histone H2AX phosphorylation on serine 139. A: Western blot analysis of post-irradiated p5 and p45 D1 cells depicted dose dependent increase in γH2AX. B: The expression of γH2AX relative to β-actin from three independent Western blots. Error bars represent standard error. $^{*}P < 0.05$ against 0 Gy control. C: Immunocytological staining for γH2AX in untreated p5 and p45 D1 cells. The constitutive expression of γH2AX appeared to be higher in p45 D1 cells than p5 D1 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

selected, but rather a mixture of progenitors and more committed cells. The presence of more differentiated cells, possibly represented in our simplified in-vitro model by the differentiation-restricted p45 D1 cells, could have hampered the characterization of the truly multipotent subfamily of cells normally described as MSCs. Whereas the differentiation capabilities of p45 D1 cells were not sensitively affected by irradiation, the multipotent p5 D1 cells in our study exhibited significant reduction of adipogenic differentiation as well as osteogenic differentiation measured at as low as 3 Gy of radiation treatments (Fig. 4). To our best knowledge, this study for the first time demonstrated that radiation sensitivity varied significantly among MSCs with different differentiation stages. Our data further suggest that multipotent MSCs are more radiosensitive and the toxic radiation doses may be smaller than previously believed. MSCs appear to survive at the low dose range of 3–5 Gy; however, the reduced differentiation capabilities may contribute to the pathological development of osteoporosis reported in the nuclear bomb survivors [Preston et al., 2003; Zhang et al., 2005], and the long-term surviving patients treated with total radiation and bone marrow transplantation [Schimmer et al., 2000; Cohen and Shane, 2003].

The present study further addressed the mechanism of radiation toxicity for MSCs. The panel of mesenchymal cell surface markers was examined 1 day after irradiation. The FACS analysis revealed that the expression profiles of CD44, Sca-1, CD73 and CD90 were unaffected by the radiation treatment in both p5 and p45 D1 cells (Supplemental Fig. 2A and B, respectively). It was noted that there was a tendency to increase CD44 expression in p5 D1 cells after the radiation treatment of 7 Gy and 12 Gy; however, these increases were not statistically significant, suggesting that the radiation effect might not affect the stem cell characteristics of MSCs.

PPARγ-2 is a critical nuclear receptor protein regulating osteogenic and adipogenic differentiation of bone marrow MSCs. It has been reported that synthetic agonists of PPARγ-2 increase the level of bone marrow adipogenesis, and at the same time, decreased osteogenesis [Schwartz et al., 2006]. The reduction of the PPARγ-2 expression together with simultaneous reduction of osteogenic and adipogenic differentiation under low dose irradiation may indicate that the radiation toxicity on MSCs may not be simply explained by the shift of differentiation commitment.

Ionizing radiation induces DNA damage, premature senescence and apoptosis. The multipotent p5 D1 cells exhibited late-onset radiation toxicity when treated with higher radiation doses; however, there was a sign of recovery from toxicity at lower radiation doses. DNA damages caused by ionizing radiation may be repaired, which allows cells to resume proliferation. Initially, we interpreted the resumed increase of whole protein contents as the recovery of cell renewal activity (Fig. 3A); however, MTT assay did not support the postulated phenomenon (Fig. 3B). Furthermore, DNA synthesis depicted by BrdU incorporation continued to be impaired in radiation-treated p5 D1 cells (Fig. 3C), leaving contradicting results on the effect of irradiation on cell renewal activity of MSCs.

While caspase-3 activities were not significantly affected by low doses (Fig. 3E), there were modest but distinct increase in TUNEL positive cells received 5 and 7 Gy irradiation. RT-PCR

demonstrated that the dose-dependent expression increase of all tested pro-apoptotic molecules such as FasL, BAX, NOXA and PUMA (Fig. 3F). Irradiation has been shown to upregulate FasL [Belka et al., 1998]. BMSC-derived FasL has been linked to the decreased functionality of lymphocytes [Gao et al., 2008], suggesting that the radiation-induced FasL expression may have wider implications in the bone marrow niche. Radiation-induced BAX has been shown to increase permeability of mitochondria, which may be depicted by MTT assay [Lei et al., 2006]. Mice carrying null mutations of p53-dependent factors such as NOXA and PUMA have been shown to exhibit a radioresistant phenotype [Shibue et al., 2003; Erlacher et al., 2005]. Therefore, the data in the present study indicate the activation of apoptotic mechanisms, whereas MSCs exhibited radioresistance to low irradiation doses.

There was a dose-dependent increase in the expression of anti-apoptotic molecule, Mcl1. The knockdown treatment of Mcl1 has been shown to increase the radiation-induced apoptosis [Skvara et al., 2005] and radiation-induced apoptosis has been linked to depletion of Mcl1 but not Bcl2 [Kubota et al., 2007], suggesting that the increased Mcl1 expression may be able to alleviate MSC apoptosis. Strikingly, MSCs treated with 5 and 7 Gy irradiation appeared to undergo morphological changes resulting in the formation of large polykaryon cells with negative TUNEL staining (Fig. 3G). We postulate that the discrepancy between the whole protein content and BrdU may, in part, due to the formation of polykaryon cells. The radiation-induced large polykaryon cells may contribute to the relative increase in the whole protein measurement (Fig. 3A). Furthermore, the formation of polykaryons may be due to the lack of cytokinesis, and the arrest of the succeeding S phase DNA synthesis in polykaryons may largely reflect the lack of increase in BrdU incorporation during later culture days (Fig. 3C).

In response to the generation of DNA double strand breaks, histone H2AX is phosphorylated on serine 139 by members of PI3 kinase family; that is, ATM, DNA-PK and ATR, and forms proteinaceous repair foci at the DNA damages [Rogakou et al., 1998]. Therefore, γH2AX has been used as a surrogate marker of DNA double-strand breakage. Indeed, our data showed a dose dependent increase in γH2AX after irradiation in both p5 and p45 D1 cells (Fig. 5A,B). However, the constitutive levels of γH2AX appeared to be different between p5 and p45 D1 cells (Fig. 5C). It has been reported that the amount of γH2AX produced in response to a given dose of radiation varies significantly in human population [Ismail et al., 2007]. Furthermore, cisplatin has been shown to decrease the number of irradiation-induced γH2AX foci in lymphocytes in cancer patients; and the variation of the cisplatin effect among patients has been found significant [Sak et al., 2009]. Genetically modified mice with the ablation of functional H2AX allele are highly sensitive to ionizing radiation and exhibit elevated levels of radiation-induced genetic instability [Bassing et al., 2002]. Therefore, we postulate that the elevated expression level of γH2AX in differentiation-restricted MSCs may, in part, facilitate the relatively high tolerance to radiation toxicity, suggesting a novel role of epigenetic regulation.

In conclusion, the radiation toxicity on multipotent MSCs in bone marrow may occur at unexpectedly low doses, which may contribute to the long-term catabolic pathology of bone tissue. The mechanism of radiation toxicity in MSCs is still elusive; however, we postulate that the epigenetic modulation associated with differentiation-restriction may play a role in regulating radiation sensitivity.

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