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Original Article

Low-dose radiation modulates human mesenchymal stem cell proliferation through regulating CDK and Rb

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Abstract: Low-dose radiation (LDR) has been known to stimulate cell proliferation. The effect of LDR on human bone marrow mesenchymal stem cells (BMSCs), however, remains to be determined. The current study, therefore, aimed to investigate the effect of LDR on human BMSC proliferation and its mechanisms. To accomplish this, human BMSCs were isolated from ribs and cultured with or without exposition to LDR (75 mGy) for 24 h. Cell proliferation was assessed by MTT assay, the cytokines secreted by the BMSCs were quantified by ELISA, and the proteins associated with cell proliferation and cell cycle were evaluated by immunoblot analysis. BMSCs isolated from human ribs were capable of differentiating into osteoblasts and adipocytes. LDR stimulated human BMSC proliferation (0.580 ± 0.106 vs 0.419 ± 0.026 on day 4, $P < 0.05$; 0.794 ± 0.025 vs 0.689 ± 0.047 on day 7, $P < 0.05$) and increased S-phase proportion. LDR significantly enhanced the production of SCF, GM-CSF, and IL-11. Moreover, BMSCs modulated T-cell proliferation, and LDR further augmented the modulatory effect of BMSCs on T-cell proliferation. Cell cycle-associated proteins, such as Rb, CDK1, and CDC25B, appeared to mediate the stimulatory effect of LDR on BMSC proliferation. The findings of the current study indicate that physical stimulants, such as LDR, could be used for the large-scale expansion of human BMSCs, and thus may be used for MSC cellular therapy in clinic.

Keywords: Low-dose radiation, bone marrow mesenchymal stem cells, CDK, Rb

Introduction

Low-dose radiation (LDR)-induced hormesis has been extensively studied in a variety of biological systems, including the immune, hematopoietic and reproductive systems [1-3]. Bone marrow mesenchymal stem cells (BMSCs) play an important role in maintaining hematopoietic homeostasis induced in response to radiation (hormesis). BMSCs have been considered not only as an attractive cell source for tissue-engineering application, but also as offering other biological significance, including anti-inflammatory and immunomodulatory functions. In this regard, MSCs have recently been pre-clinically or clinically used to treat a wide range of diseases, including stroke, myocardial disorders, spinal cord injury, graft versus host disease (GVHD), and chronic obstructive pulmonary disease (COPD) [4-7]. However, only a small amount of MSCs are present in the adult bone marrow, and their proliferation potential is weak. Therefore, it is crucial to develop a new strategy to stimulate and expand on a large scale the growth of BMSCs *in vitro* [4, 5]. While it has been reported that LDR stimulated the proliferation of several cell types, including normal lung fibroblasts and rat mesenchymal stem cells [8, 9], the effect of LDR on human bone marrow stem cells remains to be determined. The current study was, therefore, designed to investigate the potential mechanisms of action and the effect of LDR on the proliferation of human BMSCs isolated from ribs.

Materials and methods

Materials

Cell culture medium, fetal calf serum (Invitrogen), and basic fibroblast growth factor (bFGF) were purchased from PeproTech. Dexamethasone, ß-glycerophosphate, 2-ascorbic acid phosphate, indomethacin, isobutyle-methylxanthine, and insulin were purchased from Sigma (Sigma, St. Louise, USA).

Isolation and culture of human BMSCs

Bone marrow was derived from human rib after obtaining a consent form from the patient. The study protocol was approved by The Ethic Committee of Jilin University (Changchun, Jilin, China). Mononuclear cells were isolated from the bone marrow using Percoll (Pharmacia, Solarbio) following the manufacturer's instruction. Cells were pelleted by spinning at 900 g × 30 min followed by washing twice with serum-free DMEM. The cells were then plated at a density of 5×10^7 cells/25 cm^2 in DMEM supplemented with 20% fetal calf serum (FCS, Invitrogen, Gibco). Further, the cells were re-fed every four days and passaged after reaching 90% confluence.

Differentiation and characterization of BMSCs

Cells were plated into 24-well tissue culture plates at a density of 10^4/well. Osteogenic differentiation of the cells was induced in low-glucose DMEM supplemented with 10% FCS, 2.0 ng/mL basic fibroblasts growth factor (bFGF), 100 nM dexamethasone, 10 mM ß-glycerophosphate, and 0.05 mM 2-ascorbic acid phosphate. Osteogenesis was induced for three weeks with the changing medium every two days. Cells were then fixed with 90% ethanol, and von Kossa staining was performed to confirm osteogenic differentiation.

For the adipogenic differentiation, cells were plated into 24-well tissue culture plates at a density of 4×10^4/well and induced in high-glucose DMEM supplemented with 10% FCS, 0.5 mM isobutyle-methylxanthine, 200 μM indomethacin, 1 μM dexamethasone, and 10 mg/mL insulin. Adipogenesis was induced for two weeks with changing the medium every two days. Cells were then fixed with 10% formalin and stained with Oil Red to visualize the adipocytes.

Cell proliferation and cell cycle assessment

Cell proliferation was assessed by MTT assay. After the treatment with or without low-dose radiation, cells were plated into a 96-well plate at a density of 10^3/well/100 μL and were then treated with or without 75 mGy for 24 h. MTT assay was performed on day 0, 1, 2, 4, 7, and 10. Briefly, 20 μL of MTT (5 μg/mL) was added into each well of the 96-well plate. After four hours of incubation, 150 μL/well of dimethyl sulfoxide (DMSO) was added and allowed to dissolve the MTT under shaking for 10 min. Then, the optical density at a wavelength of 490 nm was measured.

Lymphocyte proliferation assay

BMSCs were plated into a 96-well plate in triplicate for each treatment condition, at a density of 5×10^4/well, 1×10^5/well, and 2×10^5/well. After 72 h of culture, the cells were treated with mitomycin (20 μg/well/) for 30 min.

T lymphocytes were purified from peripheral blood mononuclear cells (PBMC) by the nylon wool fiber column purification method. The flow cytometry assay results revealed that $CD3^+$ T cells constituted 70-80% of the cells. Next, the T cells were plated into the 96-well plates (1×10^6 cells/well), in which BMSCs were grown, followed by mitomycin treatment as aforementioned. Further, the cells were treated with Con A (5 ug/mL) for 72 h, after which, MTT (20 μL/well, 5 mg/mL) was added and allowed to be absorbed for 4 h. After spinning at 900 g × 5 min, the supernatant was discarded, and 150 μL/well DMSO was added by shaking for 10 min. Then, the optical density was measured at a wavelength of 492 nm.

Immunoblotting analysis

BMSCs were treated with or without 75 mGy for 24 h. After washing twice with cold PBS, the cells were harvested with lysis buffer containing a cocktail of a phosphatase inhibitor, proteinase inhibitor, and phenylmethane sulfonyl fluoride (PMSF) [10, 11]. After brief sonication, the protein concentration was determined using a BCA protein Assay Kit (PIERCE, Rockford, IL, USA). A total of 300 μg ligated protein was loaded in one well across the entire width of 10% SDS polyacrylamide and electrophoresed. After transferring to a PVDF membrane, Rb, phosph-Rb, p21, and ß-actin were blotted with primary antibodies overnight at 4°C. After washing and incubation with HRP-conjugated secondary antibody, the band was visualized with ECL reagents.

Results

Primary culture and directed differentiation

After 48 h of plating, a population of cells was adherent to the culture plate and grown for

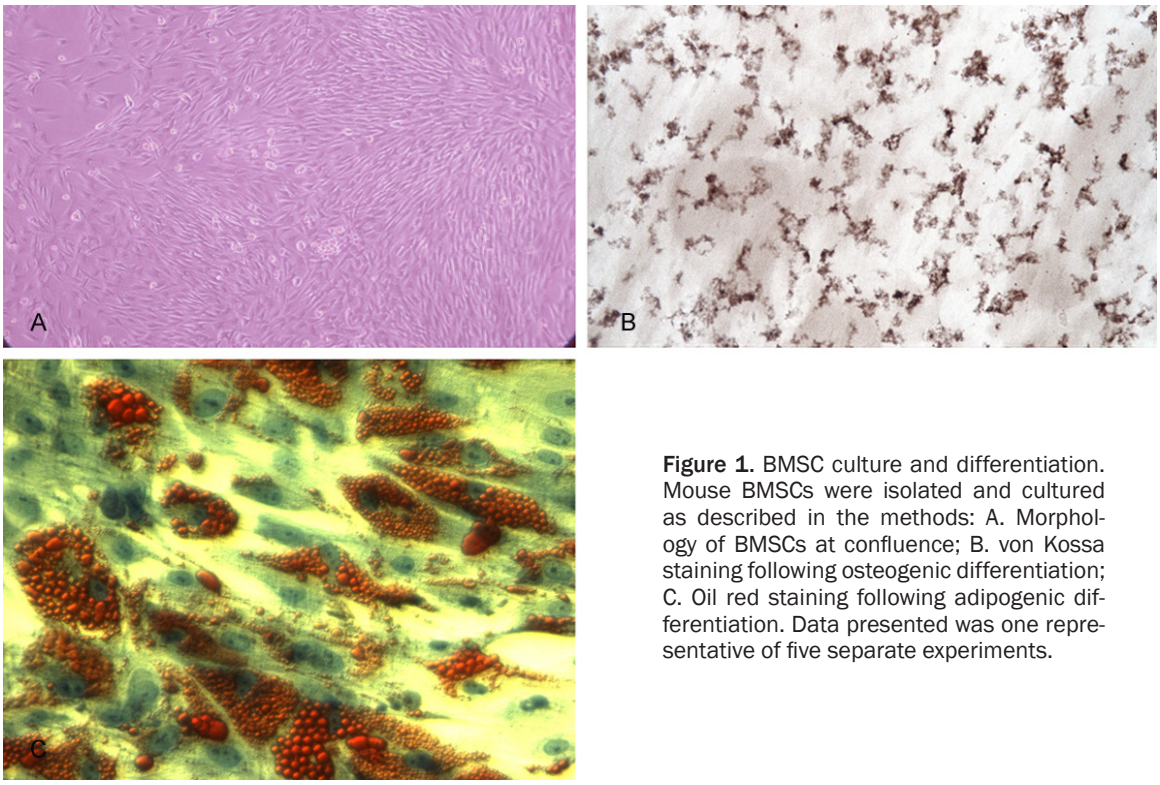

Figure 1. BMSC culture and differentiation. Mouse BMSCs were isolated and cultured as described in the methods: A. Morphology of BMSCs at confluence; B. von Kossa staining following osteogenic differentiation; C. Oil red staining following adipogenic differentiation. Data presented was one representative of five separate experiments.

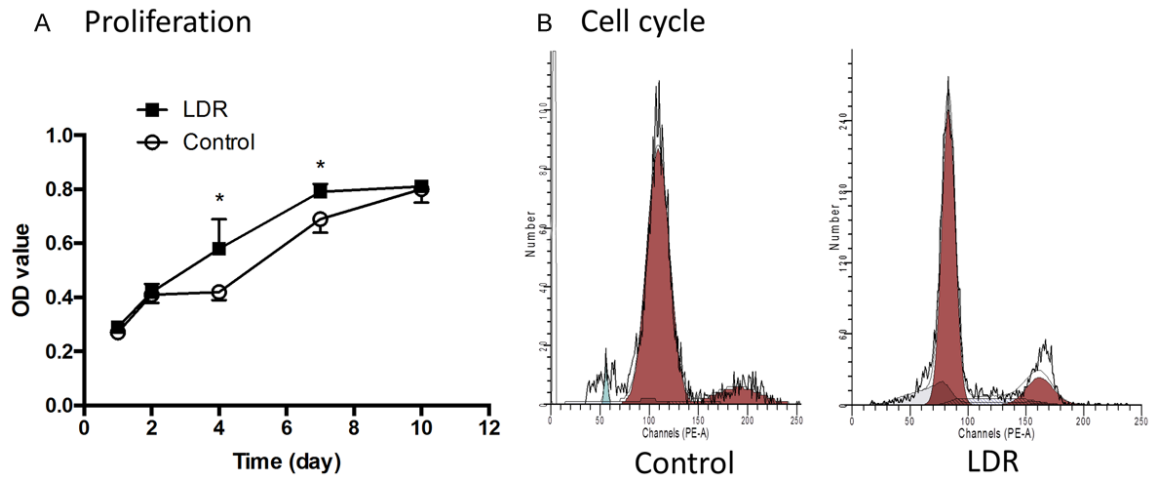

Figure 2. Effect of low-dose radiation on BMSC proliferation and cell cycle. BMSCs were exposed to low-dose radiation (LDR), and cell proliferation was measured by MTT as described in the methods. A. Cell proliferation assessed by MTT. Vertical axis: OD value; horizontal axis: time (days). Open circle: non-treated cells (control); slide square: low-dose radiation-treated cells; B. Cell cycle assessment by flow cytometry. Vertical axis: percentage; horizontal axis: cell cycles.

12-14 days until confluence. These cells were fibroblast-like-shaped (**Figure 1A**) and ready to be detached by trypsin.

The fibroblast-like cells were induced to differentiation into osteoblasts or adipocytes. After three weeks of culture in the osteogenic differentiation, multilayer colonies started to form that were stained positively by von Kossa staining (**Figure 1B**). The BMSCs also differentiated into adipocytes after two weeks of culture in the adipogenic differentiation medium. These adipocytes were positively stained by Oil Red staining (**Figure 1C**).

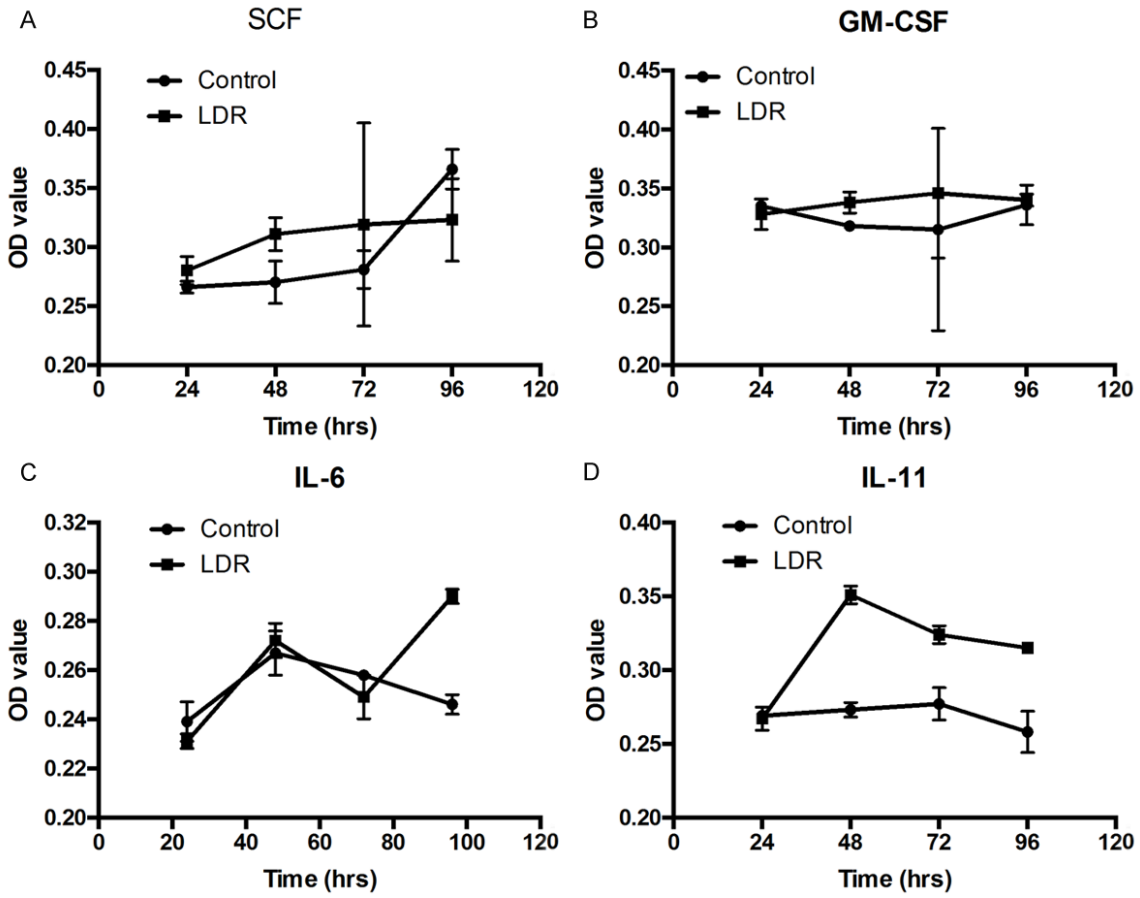

Figure 3. Effect of low-dose radiation on cytokine release. BMSCs were exposed to low-dose radiation for 24 h, and the medium was harvested for quantification of the cytokines by ELISA as described in the methods. A. Stem-cell factor (SFC); B. Granulocyte macrophage colony-stimulating factor (GM-CSF); C. Interleukin-6 (IL-6); D. Interleukin-11 (IL-11). Vertical axes: OD value; horizontal axes: time (day).

Low-dose radiation stimulated BMSC proliferation

Cell proliferation was assessed by MTT assay. As shown in **Figure 2A**, from day 4 to day 7, the proliferation of the BMSCs exposed to low-dose radiation was significantly faster than that of the cells without exposure to low-dose radiation (0.580 ± 0.106 vs 0.419 ± 0.026 on day 4, $P < 0.05$; 0.794 ± 0.025 vs 0.689 ± 0.047 on day 7, $P < 0.05$).

To further confirm the effect of low-dose radiation on cell proliferation, the cell cycle was assessed by flow cytometry after 24 h of low-dose radiation treatment. As illustrated in **Figure 2B**, the S phase increased by 6.98 ± 0.25%, while the quantity of G1 phase cells decreased by 10.22 ± 1.02% in the BMSCs exposed to low-dose radiation.

Effect of low-dose radiation on cytokine release and T-lymphocyte proliferation

As depicted in **Figure 3**, low-dose radiation stimulated the stem-cell factor (SCF) release by BMSCs after 48 h (0.311 ± 0.014 vs 0.270 ± 0.018, $P < 0.05$) and 72 h (0.319 ± 0.086 vs 0.281 ± 0.016, $P < 0.05$, **Figure 3A**) of exposure; GM-CSF release after 48 h (0.338 ± 0.009 vs 0.318 ± 0.004, $P < 0.05$) and 72 h (0.346 ± 0.315 ± 0.086, $P < 0.05$, **Figure 3B**) exposure; and IL-11 release after 48 h (0.351 ± 0.006 vs 0.273 ± 0.005, $P < 0.05$), 72 h (0.324 ± 0.006 vs 0.277 ± 0.011, $P < 0.05$) and 96 h (0.315 ± 0.002 vs 0.258 ± 0.014, $P < 0.05$, **Figure 3D**). Low-dose radiation, however, had no effect on IL-6 release (**Figure 3C**).

BMSCs inhibited T-lymphocyte proliferation in response to the Con A stimulation in a cell

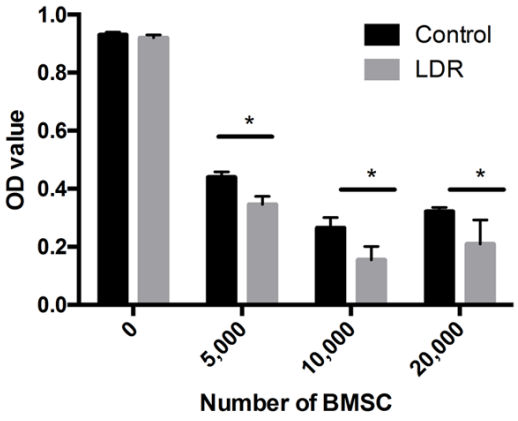

Figure 4. Modulation of lymphocyte proliferation by BMSCs exposed to low-dose radiation. BMSCs were treated with or without low-dose radiation followed by plating in 96-well plates at a density of 5×10^4/well, 1×10^5/well, and 2×10^5/well and allowed to attach for 72 h. T lymphocytes were then plated in the 96-well plates and treated with Con A as described in the methods. The number of T lymphocytes was determined by MTT assay. Vertical axis: OD value; horizontal axes: BMSC cell density. Data presented are the average of five separate experiments.

density-dependent manner (**Figure 4**). Low-dose radiation-treated BMSCs further significantly suppressed T-lymphocyte proliferation ($P < 0.05$).

Mechanism of low-dose radiation stimulation on BMSC proliferation

To investigate the mechanism of action of low-dose radiation on cell proliferation, the effects of phospho-Rb, phospho-CDK, cyclin B1, cyclin E, and CDC25 were assessed by immunoblotting. Low-dose radiation not only induced the phosphorylation of Rb (**Figure 5A**) and CDK1 (**Figure 5C**), but also upregulated the protein levels of α-cyclin E (**Figure 5B**), α-cyclin B1 (**Figure 5C**), and CDC25 (**Figure 5D**). However, the radiation, did not affect α-CDK2 expression (**Figure 5B**).

Discussion

It has been hypothesized that low-dose radiation is beneficial through its stimulatory effect on the activation of repair mechanisms that protect against disease. Previous studies have demonstrated that LDR stimulated the proliferation of several cell types, including normal lung fibroblasts and Chinese hamster fibroblasts [8, 12]. Consistently, the current study demonstrated that LDR stimulated the proliferation of BMSCs, increased the S-phase proportion, and stimulated the secretion of SCF and IL-11. The underlying mechanisms of the stimulatory influence of LDR on BMSC proliferation seem to be mediated by proteins and molecules associated with cell-cycle arrests, such as Rb, cyclin E, CDK1, and CDC25B.

While the effects of high and acute doses of ionizing radiation in humans are easily observed and understood, the impact of low-dose radiation is highly controversial and exceedingly difficult to observe. In this regard, several studies have reported the observation of LDR-induced cell proliferation in certain types of cells, including normal human diploid cells, 2BS cell line, lung fibroblasts, and rat mesenchymal stem cells [8, 9, 12, 13]. In contrast, it has also been reported that LDR induces apoptosis in other cell types, including tumor cells, such HeLa cells, and other human cells [14, 15]. Here, we report that LDR stimulated the proliferation and increase of the S-phase proportion in BMSCs, suggesting that the effect of LDR on cell proliferation or death may vary in different cell types.

A large number of researchers have used the immune system to evaluate the effects of LDR since it is one of the biological systems that are most sensitive to radiation. Moreover, LDR has been reported to induce hematopoietic hormesis, such as hematopoietic progenitor cell proliferation, peripheral blood mobilization, and hematopoietic reconstitution [16, 17]. Thus, it may be interesting to determine if LDR affects the capacity of bone marrow mesenchymal stem cell proliferation and differentiation. Since previous studies have reported that LDR stimulated cell proliferation in a variety of cell types, including rat bone marrow mesenchymal stem cells [9], and that low-dose radiation activates several types of immune cells, including T cells, dendritic cells, natural killer cells, and macrophages [18, 19], the current examination was designed to investigate the LDR-induced effect of BMSC proliferation and modulation on T-cell proliferation. We found that LDR stimulated the proliferation, increased the S-phase proportion of BMSC, and modulated the proliferation of T cells, suggesting that through modulating BMSCs, LDR may be beneficial in modulating the immune response to foreign pathogens and autoimmune diseases.

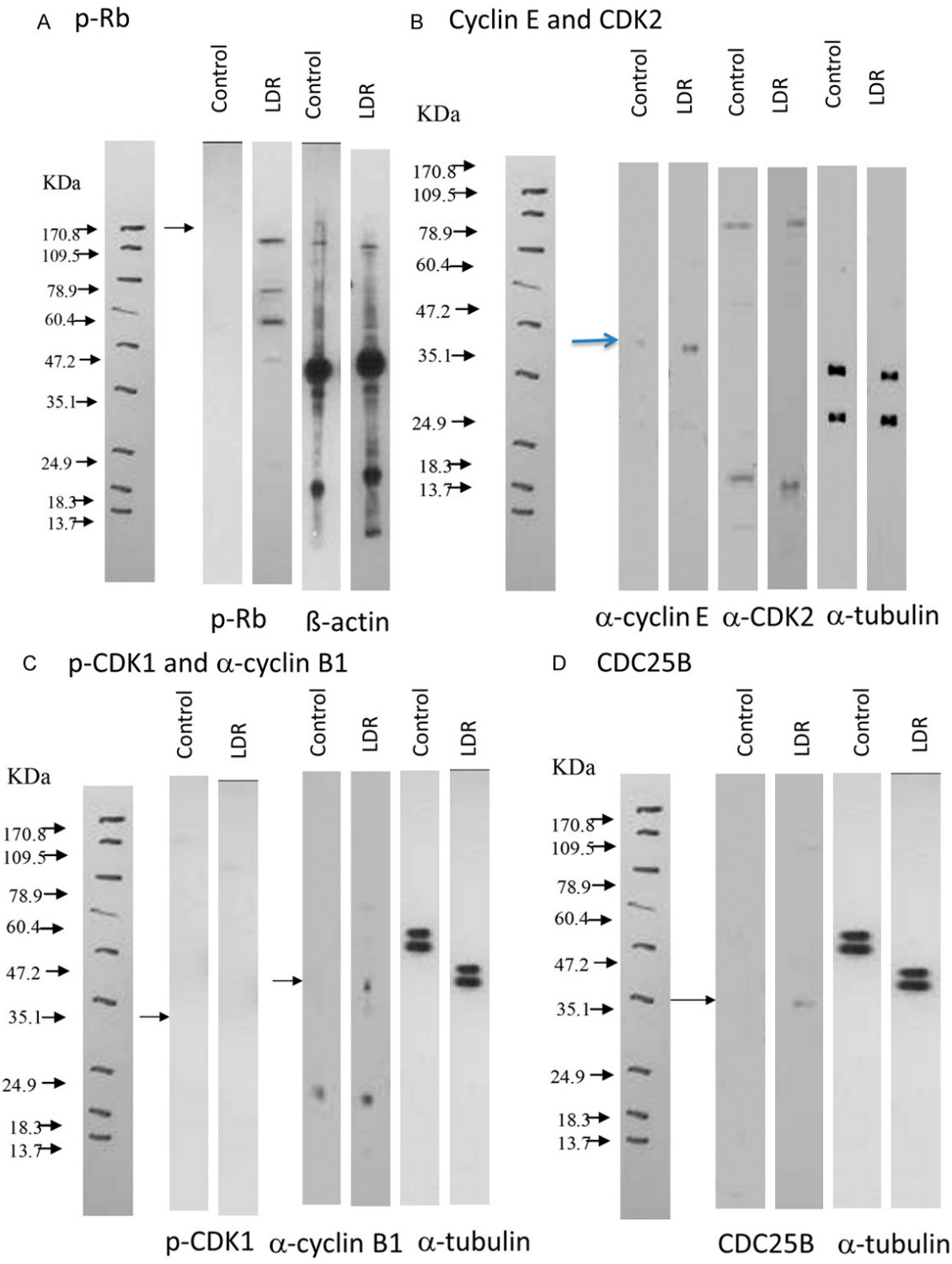

Figure 5. Effect of low-dose radiation on the phosphorylation of Rb and CDK1, and protein levels of cyclin E, CDK2, and CDC25B. BMSCs were treated with or without low-dose radiation as described in the methods. Total cell lysates were subjected for immunoblotting as described in the methods. ß-actin or α-tubulin was used as an internal control. A. Phospho-Rb; B. α-cyclin E and α-CDK2; C. Phospho-CDK1, and α-cyclin B1; D. CDC25B. Data presented are representative of five separate experiments.

BMSCs are capable of differentiating into a wide range of cell types including osteoblasts, adipocytes, and chondrocytes. Initially, the initial interest in the application of MSC therapy for the treatment of a great variety of diseases was focused on the capacity for multilineage differentiation of this cell type. However, recently, BMSCs have been considered as potent modulators of disease-associated tissue microenvironments [20], taking into account their valuable anti-inflammatory and immunomodulatory properties. Thus, in the past decade, studies on BMSCs have been centered on not only the direct tissue and organ regeneration, but also on the modulatory effects on chronic inflammation and autoimmune diseases [21]. The reports on the anti-inflammatory and immunomodulatory properties of MSCs have been prevalent in the recently published literature sources [22-24]. These findings indicate that BMSCs are a potential candidate for effective cellular therapy in a variety of chronic diseases.

To obtain clinically meaningful numbers of MSCs, approaches such as genetic immortalization and formulation of growth media have been used to maintain the' lifespan of MSC and ensure expansion that is sufficient for their clinical use. Few investigations have used physical stimulation to enhance the proliferation of stem cells. In the present study, we demonstrated that 75-mGy X-ray irradiation significantly stimulated MSC proliferation, indicated by the increased total cell numbers and S-phase cells in the cycling cells. Compared to other methods for stem cell expansion, LDR is more convenient to use, cheaper, and with a low risk of contamination of stem cells with the residuals of the reagents used to stimulate cell proliferation. The findings of the current study further support the conception of physical stimulation of BMSCs *in vitro*, which may be used for the application of MSC therapy in clinical settings.

In the current investigation, we also found that LDR induced changes in the secretion of SCF, GM-CSF, and IL-11. These factors may influence the hematopoietic and immune systems since they are generally regulated by cytokines, chemokines, and growth factors. Although other factors were not examined in our study, it is still possible that low-dose radiation may modulate the activities of other cytokines, chemokines, and growth factors, inducing effects on the hematopoietic and immune systems. Nevertheless, this may need to be further elucidated in the future.

Taken together, we conclude that BMSCs isolated from human ribs are capable of differentiating into osteoblasts and adipocytes. Low-dose radiation stimulated BMSC proliferation and increased the S-phase proportion of BMSCs. LDR modulated cytokine and growth factor secretion through the activities of BMSCs. LDR further augmented the inhibition of T-cell proliferation by BMSCs. These findings suggest that LDR may be beneficial to patients with chronic inflammation or autoimmune diseases through modulation of BMSC proliferation and immune modulation.

Disclosure of conflict of interest

None.

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