

The effect of noncoherent red light irradiation on proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells

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Abstract Mesenchymal stem cells (MSCs) are promising for use in regenerative medicine. Low-level light irradiation (LLLI) has been shown to modulate various processes in different biological systems. The aim of our study was to investigate the effect of red light emitted from a light-emitting diode (LED) on bone marrow MSCs with or without osteogenic supplements. MSCs both with and without osteogenic supplements were divided into four groups, and each group was irradiated at doses of 0, 1, 2 and 4 J/cm². Cellular proliferation was evaluated using WST-8 and 5-ethynyl-2'-deoxyuridine (EdU) fluorescence staining. The alkaline phosphatase activity, mineralization, and expression of osteoblast master genes (*Collα1*, *Alpl*, *Bglap* and *Runx2*) were monitored as indicators of MSC differentiation towards osteoblasts. In groups without osteogenic supplements, red light at all doses significantly stimulated cellular proliferation, whereas the osteogenic phenotype of the MSCs was not enhanced. In groups with osteogenic supplements, red light increased alkaline phosphatase activity and mineralized nodule formation, and stimulated the expression of *Bglap* and *Runx2*, but

decreased cellular proliferation. In conclusion, noncoherent red light can promote proliferation but cannot induce osteogenic differentiation of MSCs in normal media, while it enhances osteogenic differentiation and decreases proliferation of MSCs in media with osteogenic supplements.

Keywords Low-level light irradiation (LLLI) · Noncoherent light · Proliferation · Osteogenic differentiation · Mesenchymal stem cells (MSCs)

Introduction

Non-union and delayed healing are major complications in orthopedic patients with bone fractures. As a novel reconstructive therapy, bone marrow mesenchymal stem cells (MSCs) are receiving attention because of their easy isolation, expansion, low immunogenicity and potential for long-term generation, making them ideal for repairing bone defects in bone bioengineering. Bone MSCs are derived from bone marrow. They exhibit a fibroblast-like appearance, and can differentiate in vitro into various anchorage-dependent cell types including but not limited to bone, cartilage and adipose tissue [1–5]. Differentiation of MSCs can be induced by the local extracellular matrix [6], growth factors [7] and some physical factors [8].

Low-level light irradiation (LLLI) has been shown to modulate various processes in different biological systems, for example promoting wound healing [9, 10], cellular proliferation [11, 12], collagen synthesis [13] and cytokine production [14, 15]. The absorption of low-intensity laser light by biological systems is of a purely noncoherent nature. The coherent properties of laser light are not manifested at the molecular level [16]. It is considered that laser and LED light produce similar biological responses at

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the cellular level. The effect of photoinduced osteodifferentiation is still controversial, as previous studies have shown different and conflicting results. Oliveira et al. [17] used a diode laser (830 nm, 2.86 J/cm^2) to irradiate odontoblast-like cells. Neither the metabolism (as evaluated using the MTT assay) nor mRNA expression of collagen in the irradiated cells differed significantly from those in the nonirradiated cells. Irradiated cells also showed lower alkaline phosphatase (ALP) activity than the nonirradiated cells. In contrast, Ozawa et al. [18] reported that laser irradiation (830 nm, 3.82 J/cm^2) at an earlier stage of bone formation is more effective than irradiation at a later stage, and that stimulation of bone formation by laser is dependent on the total energy dose. In a recent study, Abramovitch-Gottlieb et al. [19] found that a low-energy laser stimulated the osteogenic phenotype of MSCs in a three-dimensional biomatrix. Kim et al. [20] also combined 647 nm red light and osteogenic differentiation media (ODM) to induce differentiation of MSCs towards osteoblasts. However, the effects of LLLI on osteodifferentiation have not yet been elucidated.

In this work, we investigated the effects of red light emitted from an LED on bone marrow MSCs cultured in either normal medium or ODM.

Materials and methods

MSC isolation and culture

The procedures regarding osteogenic cell isolation and culture have been described by Jiang et al. [21]. Briefly, a 4-week-old Sprague-Dawley rat was killed by neck dislocation according to the Guidelines of the Animal Care and Use Committee for Teaching and Research of Huazhong University of Science and Technology. Bone marrow was collected from the femurs and tibiae with a needle, suspended in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Grand Island, NY), and centrifuged at 2,000 rpm for 5 min. The marrow pellet was washed in phosphate-buffered saline, centrifuged at 1,000 rpm for 10 min, and then resuspended in DMEM. Nucleated cells were isolated with a Percoll density gradient (Invitrogen) by centrifuging at 14,000 rpm for 12 min. The top 60% of the gradient was collected, and then washed with the complete culture medium containing 10% fetal bovine serum (Invitrogen), 100 U/ml penicillin (Sigma-Aldrich, St Louis, MO), 100 mg/ml streptomycin (Sigma-Aldrich), and 0.25 mg/ml amphotericin (Sigma-Aldrich). The cells were then seeded in a tissue culture flask and incubated in the medium as described above with 10 ng/ml leukemia inhibitory factor (Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Nonadherent cells

were removed by changing the medium after 24 h. The culture medium was changed twice a week thereafter. For subculture, cells were detached with 0.25% trypsin (Amresco, Solon, OH) and passaged at a ratio of 1:2 when they had grown to 80–90% confluence. The cells were plated onto 35-mm tissue-culture dishes (Greiner, Frickenhausen, Germany) at a density of 8×10^5 cells/dish, and 96-well ELISA plates (Jet-Biofil, Guangzhou, China) at a density of 3×10^3 cells/well. After incubation for 24 h, the medium in half the dishes/wells was changed to ODM (Cyagen Biosciences, Guangzhou, China) which consisted of low-glucose DMEM supplemented with 50 $\mu\text{g/ml}$ ascorbic acid, 10^{-8} M dexamethasone, and 10 mM β -glycerolphosphate. The medium in the remaining half was not changed from DMEM.

Irradiation procedure

A red-light LED (LL-HP60VC; Jiaguang, Beijing, China) with a continuous output and a wavelength of 620 nm (1 W output) was used in this study. The distance from the LED to the cell layer was 2 cm. The diameter of the light spot was 10 cm. At the cell-layer level, the power density measured using a power meter (Ophir Optronics, Jerusalem, Israel) was 6.67 mW/cm^2 . Because biostimulation after irradiation may continue for 48 h [22], the cultures were irradiated every other day since in half of them the medium had been changed to ODM. The first irradiation day was set as day 0. The duration of each irradiation for red light was calculated as 150 s for 1 J/cm^2 , 300 s for 2 J/cm^2 and 600 s for 4 J/cm^2 . Two cultures were used as controls: MSCs incubated in DMEM without irradiation (control 1), and MSCs incubated in ODM without irradiation (control 2). Nonirradiated cells were maintained outside the incubator under the same conditions as the irradiated cells.

Cell proliferation assays

Since the distribution of the light energy density from a LED is not uniform, the energy dosages absorbed by different wells in 96-well microplates are significantly different [23]. We seeded cells in disassembled wells of ELISA plates, and every well was placed at the same point and irradiated separately. Cell viability was assessed with a WST-8 kit (Beyotime Institute of Biotechnology, Jiangsu, China) at 2, 4, 6 and 8 days, respectively. At the indicated times, WST-8 was added to the cells, according to the manufacturer's instructions, followed by incubation for 1 h. OD_{450} , the absorbance value at a wavelength of 450 nm, was read in an ELX 800 universal microplate reader (Bio-Tek Instruments, Winooski, VT). The value is directly proportional to the number of viable cells in a culture medium and cell proliferation.

According to the results of WST-8 assay, we also investigated the newly synthesized DNA of replicating cells irradiated with 2 J/cm² on day 4 using a combination of Hoechst 33342 staining and a Cell-Light EdU DNA cell proliferation kit [24] according to the manufacturer's instructions (Guangzhou RiboBio, Guangzhou, China). EdU-labeled cells were counted manually in ten fields of view randomly selected from each well, and percentages were calculated.

Alkaline phosphatase activity assay

ALP activity peaks at the end of the proliferative stage and before matrix maturation [25]. According to some studies [8, 26], osteogenically induced cells would show a peak of ALP activity approximately between 7 days and 14 days after they had been seeded in the osteogenic differentiation system. An ALP activity assay was performed at 3, 7 and 10 days after the first irradiation. The cells were rinsed twice with phosphate-buffered saline, then 0.1% Triton X-100 containing 10 mmol/l Tris-HCl, 5 mmol/l MgSO₄ and 0.1% NaNO₃ was added to 500 μl, and the cells were frozen at -80°C three times to disrupt the cell membranes. ALP activity and protein content were measured using an ALP activity kit (Nanjing Jiancheng Biological Engineering Institute, Nanjing, China) and a Micro BCA assay kit (Lipulai, Beijing, China). All results were normalized in relation to protein content [27].

Mineralization assay

For mineralization assay, calcium phosphate hydroxyapatite was detected using a commercial von Kossa staining kit (Genmed Scientifics, Shanghai, China) on day 21. The mineral deposit area of each dish was calculated from the positively stained areas measured in 20 randomly selected microscopic fields (96,467.83 μm²/field) using Image-Pro1 Plus analysis software (Media Cybernetics, Silver Spring, MD) [18].

RNA preparation and RT-PCR analysis

When cells had reached approximately 90% confluence, the medium of half the dishes was changed to ODM. After 3 days of incubation in ODM, the cells were treated with red light. Total RNA of bone marrow MSCs cultured in 35-mm dishes was extracted after irradiation by an acid guanidinium thiocyanate/phenol/chloroform extraction method. Cells were homogenized using TRIZOL reagent (Invitrogen), and total RNA was isolated. RNA was reverse-transcribed (RT) with a reverse transcriptase kit (Toyoba, Osaka, Japan). According to the manufacturer's instructions, RT was performed in a 20-μl reaction mixture. Aliquots of cDNA were amplified in a 25-μl

polymerase chain reaction (PCR) reaction mixture which contained 10 nM 5'- and 3'-oligomers. The expression levels of collagen type I (*Col1α1*, an early marker of osteodifferentiation in proliferative stage), ALP (*Alpl*, a medium-term marker of osteodifferentiation before matrix maturation), osteocalcin (*Bglap*, a late period marker in matrix maturation), and *Runx2* (also called *Cbfa1*, initially expressed in osteoprogenitor cells) [28, 29] were determined. We designed the PCR primers with Primer 3 (<http://frodo.wi.mit.edu/primer3/>). The gene sequences were searched in MEDLINE. The primer sequences are shown in Table 1.

Statistical analysis

All data are expressed as means±standard deviation ($n=3$). The analyses were performed with SPSS 13.0 software (SPSS, Chicago, IL). Statistical analysis was done using a one-way ANOVA. All comparisons were evaluated at a 95% confidence level ($P<0.05$).

Results

Effect of LLLI on cellular proliferation

As shown in Fig. 1, viable cell numbers increased rapidly from day 0 (24 h after cell seeding) to day 4, and then reached a stationary phase by day 6. Similar cell growth curves were observed in every group throughout the cell culture period. At 4, 6 and 8 days, cells cultured in DMEM showed significantly higher viabilities than that cells cultured in ODM. Among the DMEM cultures, red light at all doses significantly stimulated cell viability as compared with the control 1 culture. Cultures irradiated at 1 J/cm² had the fastest proliferation rates. Their OD₄₅₀ was significantly higher than that of the control 1 culture on day 4 ($P=0.0034$, <0.01) and day 6 ($P=0.017$, <0.05). Cultures irradiated at 2 J/cm² also showed a higher OD₄₅₀ on day 4 ($P=0.0067$, <0.01) and day 6 ($P=0.023$, <0.05) than the control 1 culture. Cultures irradiated at 4 J/cm² only showed a significantly higher OD₄₅₀ on day 4 ($P=0.039$, <0.05). Among the ODM cultures, the control 2 culture and those irradiated at 1 J/cm² showed similar proliferation rates. Red light at 2 and 4 J/cm² significantly inhibited cell viability as compared with the control 2 culture. Irradiation at 2 J/cm² resulted in a lower OD₄₅₀ on day 6 ($P=0.036$, <0.05) compared with the control 2 culture. The slowest proliferation rate was induced by irradiation at 4 J/cm², as lower OD₄₅₀ values were observed on day 4 ($P=0.0057$, <0.01) and day 6 ($P=0.028$, <0.05).

EdU labeling was performed on day 4. As shown in Fig. 2, In the DMEM cultures, more irradiated cells showed red fluorescence indicating EdU labeling ($P=0.029$, <0.05).

Table 1 Primers for RT-PCR

No.	Gene	Oligonucleotide	Product length (bp)
NM_053304.1	<i>Collα1</i>	5'-GGTGGTTATGACTTCAGCTTCC-3' 5'-CAGTACTCTCCGCTCTTCCAGT-3'	244
NM_013059	<i>Alpl</i>	5'-CCTGGACCTCATCAGCATTT-3' 5'-AGGGAAGGGTCAGTCAGGTT-3'	180
M25490	<i>Bglap</i>	5'-AGCTGTGCCGTCCTACTTT-3' 5'-CAGTACTCTCCGCTCTTCCAGT-3'	190
XM_001060656	<i>Runx2</i>	5'-CAGACCAGCAGCACTCCATA-3' 5'-CAGCGTCAACACCATCATT-3'	178
NM_031144	<i>β-actin</i>	5'-AGCCATGTACGTAGCCATCC-3' 5'-CTCTCAGCTGTGGTGGTGAA-3'	230

Among the ODM cultures, there were fewer EdU-labeled cells in the control 2 culture ($P=0.021$, <0.05) and irradiated cultures ($P=0.013$, <0.05) than in the control 1 culture. However, red light irradiation did not induce significant differences among the ODM cultures.

Effect of LLLI on cellular differentiation

As shown in Fig. 3, all DMEM cultures showed similar ALP activity levels which were clearly lower than that in the ODM cultures. Their ALP activities did not significantly fluctuate with the changes in incubation time and irradiation dose. Among the ODM cultures, irradiation at 4 J/cm^2 significantly increased ALP activity as compared with the control 2 culture on day 3 (1.26-fold, $P=0.034$) and day 7 (1.32-fold, $P=0.021$). ALP activities of MSCs in ODM reached a peak on day 10, and cultures irradiated at

2 J/cm^2 showed significantly higher ALP activity than the control 2 culture (1.22-fold, $P=0.032$).

A well-delineated, three-dimensional nodular structure of mineralized tissue was formed by MSCs cultured in ODM, while no nodular structure was found in MSCs cultured in DMEM (Fig. 4). The number of bone nodules present in 35-mm dishes with ODM was counted, and significant stimulation by red light irradiation occurred at all energy doses. The staining density in dishes was significantly stimulated by LLLI. The control 2 culture was considered as standard (100%). MSCs in ODM irradiated at doses of 2 and 4 J/cm^2 showed significant increases in staining intensity of 134% ($P=0.024$) and 166% ($P=0.013$), respectively.

As shown in Fig. 5, the mRNA expression levels of *Collα1*, *Alpl*, *Bglap* and *Runx2* in the irradiated cells were compared with those of cells in the control 1 and control 2

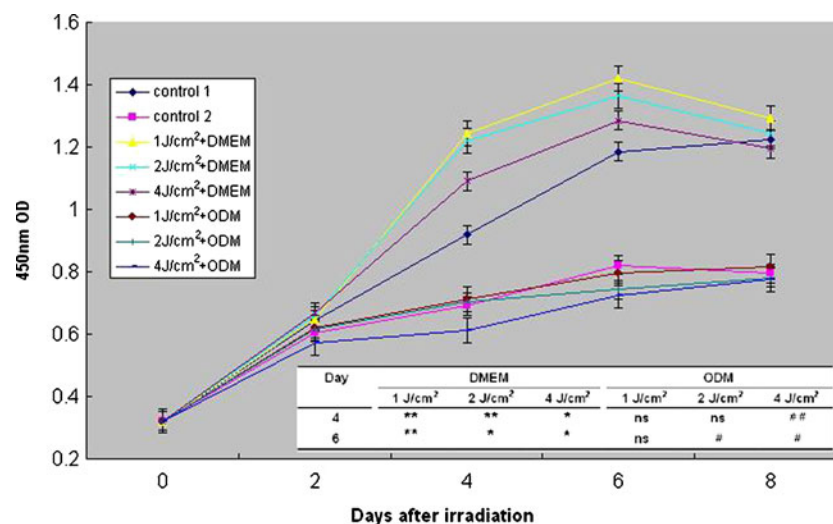
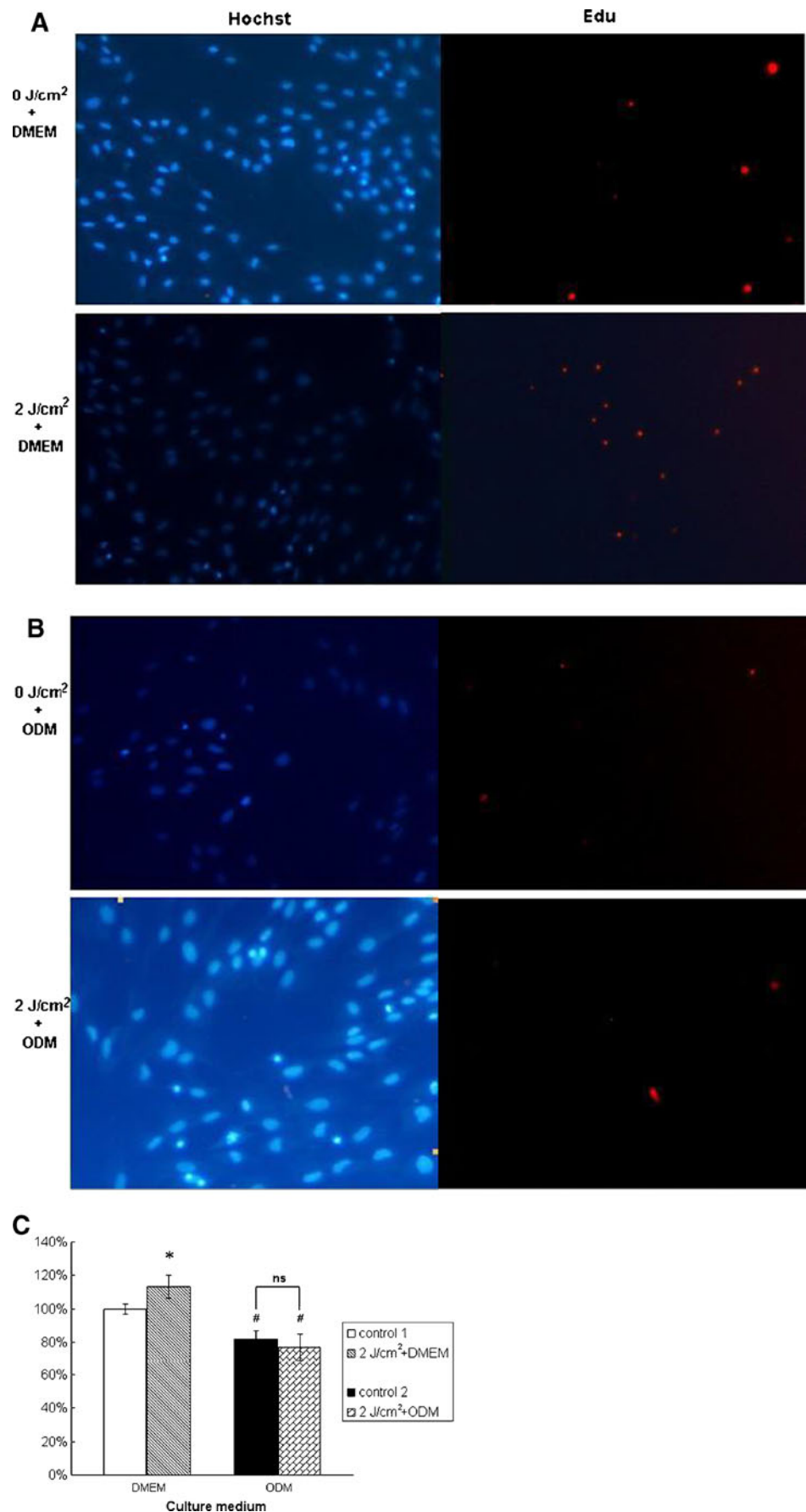


Fig. 1 Effect of LLLI on viability of rat bone marrow MSCs. Irradiated MSCs in DMEM showed a significant increase in viability on days 4 and 6 as compared to those in the control 1 culture. The final saturation densities were not significantly different among the DMEM cultures, whereas they were significantly higher than in the control 2 culture. The final saturation densities were not significantly

different among the ODM cultures. On day 4, cell viability following LLLI at 4 J/cm^2 was significantly lower than in the control 2 culture. On day 6, cell viability following LLLI at 2 and 4 J/cm^2 was significantly lower than in the control 2 culture (** $P<0.01$, * $P<0.05$ vs. control 1; ## $P<0.01$, # $P<0.05$ vs. control 2; $n=3$)

Fig. 2 Effect of LLLI on DNA synthesis in rat bone marrow MSCs. The experiment was performed on day 4. All cell nuclei showed blue fluorescence indicating Hoechst 33342 staining, and EdU labeling showed replicating cells. **a** Among the DMEM cultures, more cells showed red fluorescence indicating EdU labeling following LLLI. **b** Among the ODM cultures, LLLI had no significant effect. **c** Analysis of EdU-labeled cells. Among the DMEM cultures, more irradiated cells showed red fluorescence indicating EdU labeling. Among the ODM cultures, there were significantly fewer EdU-labeled cells in the control 2 culture and irradiated cultures than in the control 1 culture. However, red light did not induce significant difference among the ODM cultures ($*P<0.05$ vs. control 1; $^{\#}P<0.05$ vs. control 1)



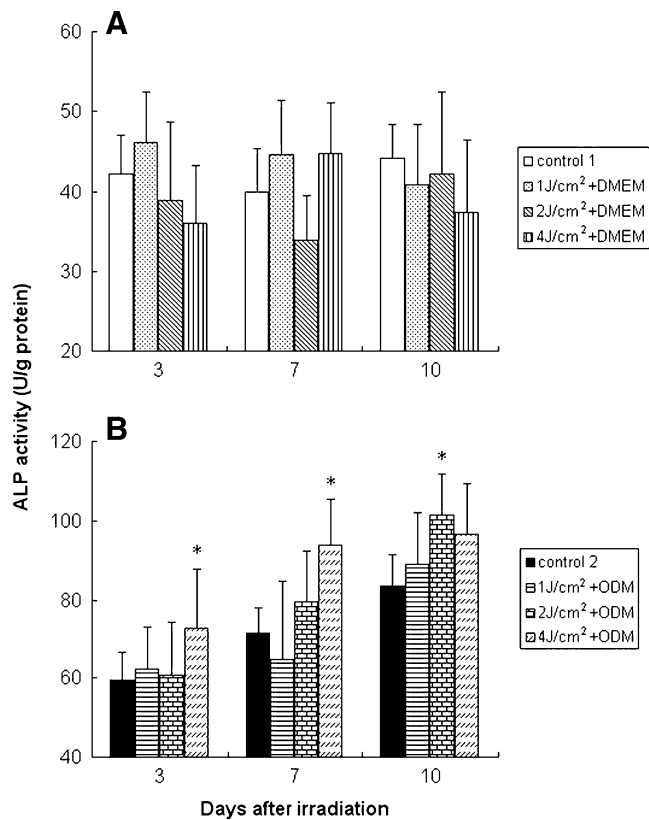


Fig. 3 Effect of LLLI on ALP activity in rat bone marrow MSCs. Analysis was performed on days 7, 10 and 14 after the first irradiation. The data presented are means±standard deviation ($n=3$) ($^{\#}P<0.05$ vs. control 1; $*P<0.05$ vs. control 2)

cultures. In the DMEM cultures, none of the genes showed significant differences in mRNA expression, but the mRNA expression levels of irradiated cells were clearly lower than that in cells of the control 2 culture. In ODM cultures, LLLI had no significant effect on the expression levels of *Collα1* and *Alp1*, but significantly increased the levels of *Bglap* and *Runx2*. The expression levels of *Bglap* mRNA following irradiation at doses of 1, 2 and 4 J/cm² were 97% ($P=0.27$), 153% ($P=0.026$) and 143% ($P=0.037$), respectively, and the levels of *Runx2* were 126% ($P=0.029$), 188% ($P=0.014$) and 129% ($P=0.032$), respectively, versus the expression level in the control 2 culture.

Discussion

In the present study, the effects of 620-nm red light at different energy dosages on bone marrow MSCs were investigated. The major findings were: (1) red light promoted proliferation of MSCs cultured in normal medium, and suppressed proliferation of MSCs cultured in ODM; (2) red light enhanced osteogenic differentiation of MSCs cultured in ODM, but did not induce osteogenic differentiation of MSCs cultured in normal medium.

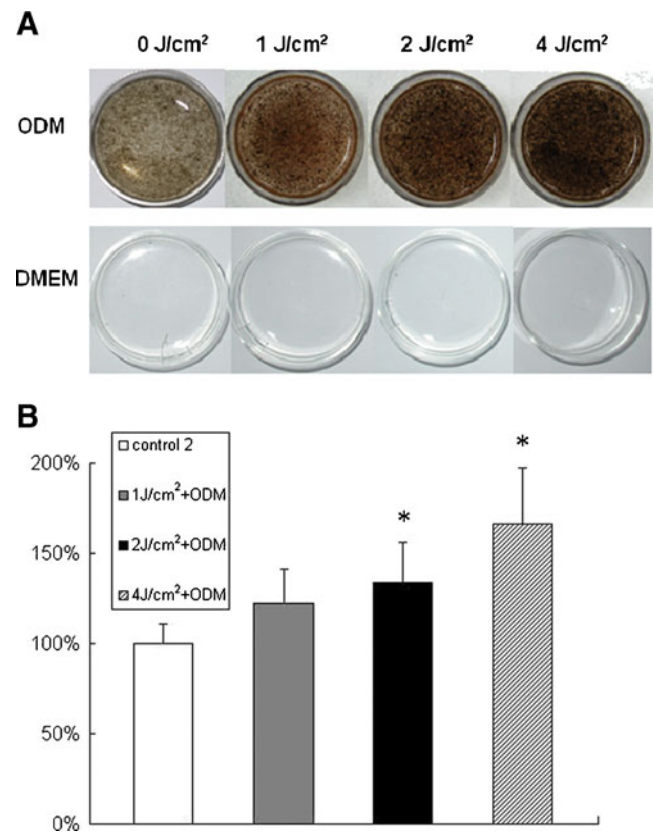
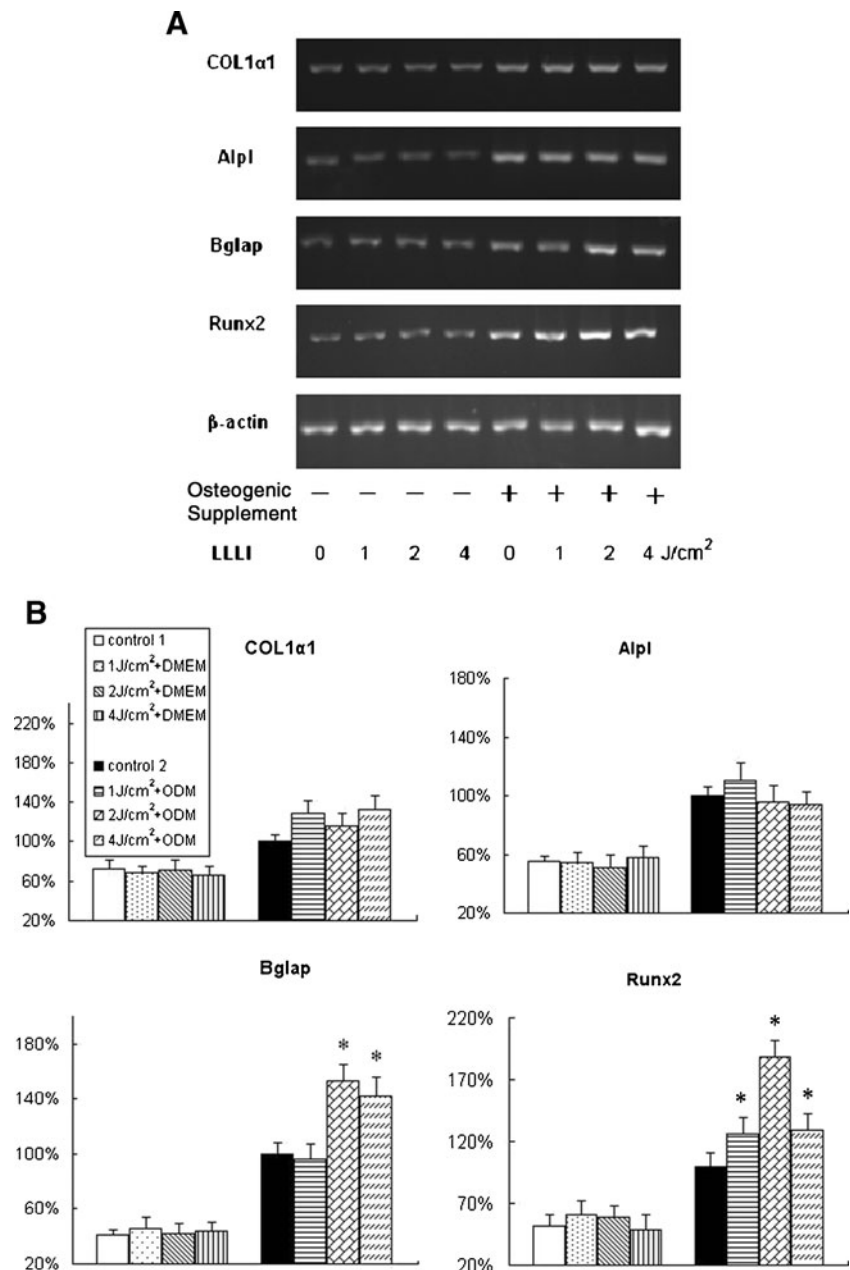


Fig. 4 Effect of LLLI on mineral nodule formation in rat bone marrow MSCs. **a** Von Kossa staining of MSCs on day 21. Mineral nodules formed in ODM cultures, whereas no mineral nodules formed in DMEM cultures. **b** Analysis of mineral nodule formation in ODM cultures. MSCs in ODM irradiated at doses of 2 and 4 J/cm² showed increases in staining intensity of 134% and 166%, respectively, versus control 2 ($*P<0.05$ vs. control 2)

Some studies have shown that proliferation of MSCs can be promoted by laser irradiation [11, 12, 30, 31]. Karu [16] and Posten et al. [32] suggested that coherent and noncoherent light with the same wavelength, intensity and irradiation time provide similar effects. It seems reasonable to assume that LED light would have the ability to affect proliferation of MSCs. In our study, the results of the WST-8 and EdU assays confirmed that noncoherent light is also able to stimulate proliferation of bone marrow MSCs cultured in normal medium. However, irradiation with red light slowed down cellular proliferation of MSCs cultured in ODM. This reduced rate of proliferation was matched by an increased rate of osteodifferentiation of MSCs in ODM. A possible explanation is that there is a reciprocal relationship between growth and osteogenic differentiation in MSCs [28]. Genes involved in the production and deposition of the extracellular matrix are expressed during the proliferative period, and the synthesis of an organized bone-specific extracellular matrix contributes to the shut-down of proliferation [33]. This reciprocal relationship also suggests that the induction of osteogenic differentiation by

Fig. 5 The mRNA expression levels of *Col1 α 1*, *Alp1*, *Bglap* and *Runx2*. Total RNA was isolated on day 4 (* P <0.05 vs. control 2)



red light irradiation occurs during the proliferative stages. Moreover, cultures of irradiated cells showed final saturation densities similar to those in the corresponding ODM control culture, whereas irradiated cultures showed a clear enhancement of the intensity of von Kossa staining compared with that of the control 2 culture. Therefore, it seems that the significant increase in the number of nodules following red light irradiation was not attributable to a general increase in cell number, but rather to an increase in the percentage of cells showing mineralization.

MSCs are pluripotent and can differentiate into multiple cell lineages [1–5], but they do not differentiate spontaneously. Appropriate in vitro conditions is a necessary factor

for osteodifferentiation. In this study, 620-nm red LED light significantly enhanced ALP activity and mineral deposition which correlated with osteodifferentiation of MSCs cultured in ODM. As shown here, red light enhanced the expression of Runx2 and Bglap, which are osteogenesis markers of osteoblast differentiation [34, 35]. Since Runx2 and Osteocalcin can both be expressed at the same time before the mature osteoblast phase [29, 36], the RT-PCR results indicated that the primary induction of osteogenic differentiation by red light irradiation appeared to be commitment to the osteoblast pathway through an early increase in *Runx2* gene expression, rather than at the level of osteoblast maturation. On the other hand, although the

MSCs in DMEM were also irradiated at the same doses, they did not express osteogenic phenotypes, such as ALP activity, mineral matrix formation and upregulation of osteoblast master gene. We speculate that red light alone cannot activate the signaling pathway that controls osteodifferentiation. However, it could intervene in the differentiation signaling pathway by some means still unknown if the signaling pathway had already been activated. In other words, red light irradiation could be an adjunctive tool to increase osteodifferentiation, although it cannot induce osteodifferentiation alone.

It is now well established that osteodifferentiation is marked by sequential stages of cellular proliferation and bone extracellular matrix maturation [25]. ALP activity is a transient early marker of osteodifferentiation in MSCs known to peak at the end of the proliferative stage and before matrix maturation [33]. Early progenitor cells do not express ALP activity, but differentiate through a defined number of cell divisions, ultimately expressing a mature osteoblast phenotype: a postmitotic, osteogenic cell with ALP activity [37]. In the ODM cultures in our experiments, the ALP mRNA expression in all red light irradiated cultures did not show significant difference from that in the control 2 culture, whereas the cultures irradiated at 2 J/cm² and 4 J/cm² showed a significantly higher ALP activity than others. ALP is synthesized as an inactive precursor, pro-ALP, and is processed into an active form by proteolytic cleavage [38]. The ALP mRNA expression corresponds to the amount of pro-ALP, whereas hydrolyzed *p*-nitrophenyl phosphate reflects the amount of activated ALP. Red light irradiation could enhance the proteolytic cleavage of pro-ALP.

To sum up, we investigated the role of noncoherent red light irradiation in photoinduced osteogenic differentiation by determining the cellular proliferation and osteodifferentiation effects of LED light on bone marrow MSCs cultured in two different biological systems. Irradiated MSCs in two different in vitro environments showed different behaviors. Red LED light at different energy densities promoted proliferation but did not induce osteogenic differentiation of MSCs in normal medium, while it enhanced osteogenic differentiation and decreased the rate of proliferation of MSCs in ODM. We conclude that red light can effectively increase the rate of osteodifferentiation despite its inability to induce osteodifferentiation alone. This result will help us to understand the mechanisms of action of light on bone. Our findings may contribute to regenerative medical treatment of bone fractures, since they provide the basis for a cheap and efficient strategy for controlling the expansion and differentiation of MSCs in vitro prior to transplantation.

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