INFLUENCE OF DONOR AGE ON OSTEOGENIC DIFFERENTIATION AND CELL SHEET FORMATION IN BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

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ABSTRACT

This study aimed to compare the *in vitro* osteogenic differentiation potential and cell sheet-forming capacity of rat bone marrow-derived mesenchymal stem cells (rBMSCs) isolated from 4-week-old (wk-old) and 14-wk-old Wistar rats. rBMSCs were obtained from tibias and femurs and characterized by flow cytometry. Osteogenic differentiation potential was assessed by alkaline phosphatase (ALP) activity and Alizarin Red staining. rBMSCs from both groups were cultured on temperature-responsive plates to develop cell sheets. Sheet structure and detachment were assessed. Over 95% of adherent rBMSCs were positive for CD29, CD44, and CD90, and negative for CD45. rBMSCs from 4-wk-old rats exhibited significantly higher osteogenic differentiation potential, as indicated by increased ALP activity ($P < 0.05$) and mineralized nodules formation, whereas rBMSCs from 14-wk-old rats showed no mineralization. In contrast, only 14-wk-old rBMSCs successfully formed intact and detachable cell sheets, while 4-wk-old rBMSCs did not form cohesive sheets. In conclusion, donor age significantly influences rBMSC behavior. 4-wk-old rBMSCs exhibit enhanced osteogenic differentiation potential, whereas 14-wk-old rBMSCs demonstrate superior cell sheet formation efficacy. Further studies are needed to optimize sheet formation of younger donors.

Keywords: Bone Marrow Mesenchymal Stem Cells, Cell Sheet, Osteogenic Differentiation, Rat Donor Age

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INTRODUCTION

Stem cell-based therapy has emerged as a key strategy in regenerative medicine, offering new avenues to repair, replace, or regenerate damaged tissues and organs (Hoang et al., 2022). Nevertheless, conventional approaches have limitations. Cell suspensions are typically harvested by enzymatic detachment, which can damage essential adhesion molecules (Lu et al., 2019). Scaffold-based systems may cause cell loss, yield poor seeding efficiency, and leave residual materials that trigger inflammation (Taraballi et al., 2016).

By contrast, cell-sheet engineering harvests intact, extracellular-matrix (ECM)-rich layers without proteolytic digestion, thereby preserving tissue architecture, intercellular junctions, and surface proteins while improving host integration (Yamato & Okano, 2004). Owing to these advantages, cell sheets have been used in preclinical calvarial models and show utility in bone regeneration (Cao et al., 2024; Lu et al., 2019).

Among available stem-cell sources, bone marrow-derived mesenchymal stem cells (BMSCs) are attractive for bone regeneration because of their self-renewal, multipotency, and strong osteogenic potential (Banimohamad-Shotorbani et al., 2023). In our previous work using Wistar rats, rBMSCs from 14-wk-old donors showed markedly reduced ALP activity and failed to form mineralized nodules as determined by Alizarin Red staining (Surisaeng et al., 2025). Although 14-wk-old rats are considered young adults (Ghasemi et al., 2021), this observation likely reflects age-associated declines in osteogenic capacity (Fafián-Labora et al., 2015; Li et al., 2015; Zhang et al., 2008). Therefore, this present study aimed to compare the *in vitro* osteogenic differentiation potential and cell sheet-forming ability of rBMSCs isolated from 4-wk-old and 14-wk-old Wistar rats to clarify the influence of donor age on their regenerative properties.

LITERATURE REVIEWS

Mesenchymal stem cells are the most investigated cell types in tissue engineering because they self-renew and differentiate into multiple cell types. Clinically relevant sources include bone marrow, adipose tissue, and perinatal tissues such as the umbilical cord. Among these, BMSCs are particularly attractive for bone repair owing to robust osteogenic potential (Banimohamad-Shotorbani et al., 2023). Under specific conditions, BMSCs can differentiate into osteoblasts, chondrocytes, myoblasts, tenocytes, adipocytes, stromal cells, and other mesodermal phenotypes (Oryan et al., 2017). However, BMSCs reparative function depends on the physiological and pathological context. Aging, in particular, is associated with reduced proliferation and osteogenic capacity of BMSCs. (Fafián-Labora et al., 2015; Li et al., 2015; Zhang et al., 2008). A comparative analysis across different rat age groups showed that the osteogenic differentiation potential declined with age, with the two-wk-old group showing highest potency relative to the five-, six-, and eight-wk groups (Fafián-Labora et al., 2015). These emphasizes the need to account for donor age when developing effective cell-based strategies.

Cell-sheet engineering was introduced to overcome the limitations of cell suspensions and scaffolded constructs, which often result in loss of adhesion molecules and removal of ECM. This scaffold-free approach harvests confluent and cohesive layers without proteolytic enzymes, thereby preserving ECM, adhesion molecules, growth-factor receptors, ion channels, and cell-cell junctions (Yamato & Okano, 2004). Sheets can be obtained using stimuli-responsive surfaces that change their physicochemical state in response to temperature, pH, light, or electrochemical cues, or by physical assistance such as gentle peeling, magnetic capture, or ultrasonic vibration (Cao et al., 2024). Although physical methods are economical, they can damage cells and disrupt sheet continuity. Consequently, responsive surfaces have been adopted. The most commonly used platform employs temperature-responsive plates coated with poly(N-isopropylacrylamide). At 37 °C the surface is hydrophobic and supports

adhesion, spreading, and proliferation. Cooling below approximately 20 °C renders the surface hydrophilic and the confluent monolayer detaches as an intact sheet that remains rich in ECM and maintains cell-cell contacts (Yamato & Okano, 2004). Across previous studies, rBMSC sheets were typically harvested after 2-7 days of culture (Imamura et al., 2015; Ito et al., 2017; Maruyama et al., 2020; Mito et al., 2024). Together, these findings highlight the dual importance of donor age and scaffold-free cell-sheet strategies in optimizing the osteogenic potential of BMSCs, thereby providing a robust foundation for investigating age-related differences in rBMSC sheet fabrication and regenerative capacity.

MATERIALS AND METHODS

Study Animals

All procedures were approved by the Institute of Animal Care and Use Committee (FTM-IACUC 011/2025), Faculty of Tropical Medicine, Mahidol University. All methods were performed in accordance with relevant guidelines and regulations. This study was reported in accordance with the ARRIVE 2.0 guidelines. Wistar rats aged 3 and 13 wks were obtained from Nomura Siam International Co. Ltd. (Bangkok, Thailand) and acclimated to the facility for 5-7 days. Experiments were performed when the rats reached 4 wk and 14 wk of age. Before each procedure, all animals underwent a general health assessment and were weighed.

Isolation and culture of rBMSCs

rBMSCs were isolated from the tibias and femurs. Rats were humanely euthanized by CO_2 inhalation followed by cervical dislocation. Tibias and femurs were harvested, muscle and connective tissue were removed. Bones were briefly sterilized in 70% ethanol, and the ends were cut with sterile scissors. Bone marrow was flushed using a 21-gauge needle with alpha-minimal essential medium (α-MEM; Thermo Fisher Scientific, Waltham, MA, USA). The flushed contents were collected in 15-ml tubes and passed through a 70-µm cell strainer to obtain a uniform suspension. Bone marrow cells were washed twice and cultured in completed medium including α-MEM supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution, and L-glutamine (all from Gibco, Waltham, MA, USA). Cultures were maintained in 75 cm^2 tissue culture flasks and incubated at 37°C in 5% CO_2. Cells were allowed to attach for 24 hours. Non-adherent cells were then removed, and the medium was replaced with fresh α-MEM. The culture medium was changed every 2-3 days. When cultures reached 70-80% confluence, cell-surface markers were analyzed by flow cytometry for CD29 (anti-rat CD29 PE mAb), CD44 (anti-rat CD44H Alexa Fluor 647 mAb), CD45 (anti-rat CD45 FITC mAb), and CD90 (anti-rat CD90 PerCP mAb) (all from BioLegend, San Diego, CA, USA).

Comparison of Osteogenic Differentiation Potential

Induction of Osteogenic Differentiation

To evaluate osteogenic differentiation, rBMSCs were cultured in osteoinductive medium (OIM) containing 10% FBS, 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 0.05 mM ascorbic acid (Sigma-Aldrich), and 0.10 µM dexamethasone (Sigma-Aldrich). Medium was refreshed every 2-3 days. Control cells were maintained in α-MEM containing 10% FBS, 1% antibiotic-antimycotic, and L-glutamine. All experiments were performed in three independent experiments (n = 3). Osteogenic differentiation was assessed by ALP activity and Alizarin Red S staining.

ALP activity

After 7 days of induction, cells were washed with phosphate-buffered saline (PBS) and ALP activity was measured using the Lab Assay™ ALP kit (Fujifilm Wako Pure Chemical Corp, Chuo-Ku, Osaka, Japan). Absorbance was read at 405 nm on a microplate reader (Epoch, BioTek, Winooski, VT, USA).

Alizarin Red S staining

After 21 days of induction, mineralization was evaluated by Alizarin Red S staining. Cells were washed twice with PBS, fixed with 10% formalin for 10 minutes, rinsed, and stained with 2% Alizarin Red S (pH 4.2) (Sigma-Aldrich, St. Louis, MO, USA) for 3 minutes. Excess stain was removed with three PBS washes, and mineralized nodules were imaged.

Fabrication of rBMSC sheets

rBMSCs from 4- and 14-wk-old rats were seeded at 5×10^5 cells/well onto temperature-responsive 6-well plates (Thermo Fisher Scientific, Waltham, MA, USA) and cultured for 3 days. Culture conditions were predetermined from previous experiment with 14-wk-old rBMSCs (data not shown). Cell sheets were harvested by lowering the temperature to 20°C. Detachment time and sheet integrity were recorded.

Statistical analysis

The statistical analyses were conducted using GraphPad Prism Version 7.00 (GraphPad Software, CA, USA). For comparisons between two groups, data were analyzed with t-test. Data were presented as mean ± SE of independent experiments. P-value < 0.05 was considered statistically significant.

RESULTS

Characterization of rBMSCs

In this study, rBMSCs from passage 3-8 were used. Cells derived from both 4- and 14-wk-old rats exhibited a typical spindle-shaped, fibroblast-like morphology (Figure 1A). Flow cytometric analysis demonstrated that more than 95% of adherent rBMSCs in all age groups expressed the mesenchymal stem cell markers CD29, CD44, and CD90, while being negative for CD45, a hematopoietic lineage marker (Figure 1B).

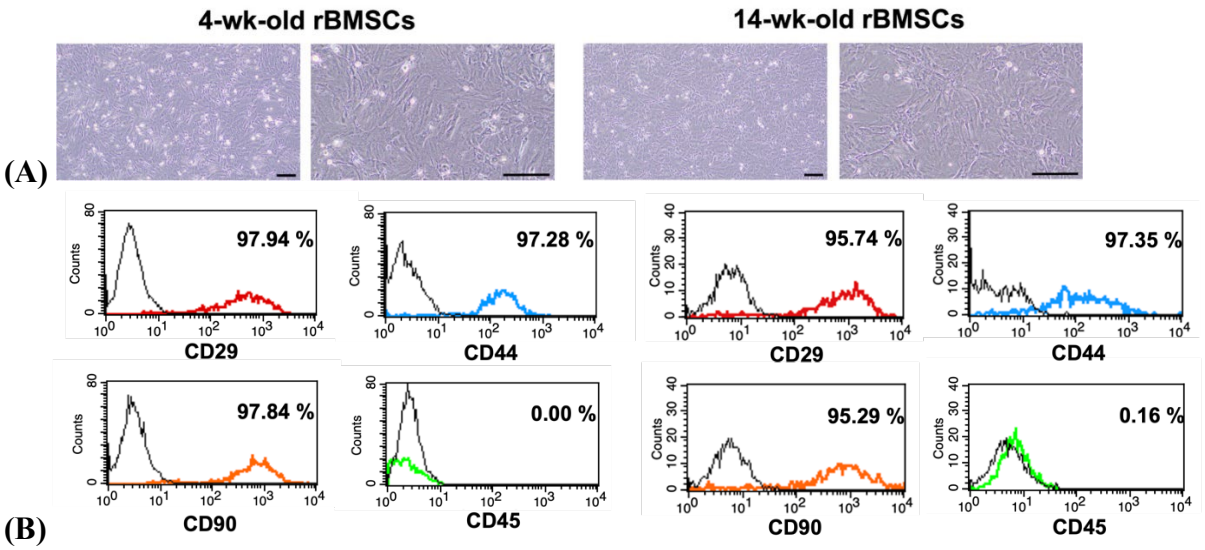

Figure 1 Characterization of rBMSCs. (A) Representative microscopic image showed the spindle-shaped, fibroblast-like morphology of rBMSCs. Scale bar: 200 µm. (B) Flow cytometric analysis demonstrated that rBMSCs were positive for CD29, CD44, and CD90, and negative for CD45.

Osteogenic differentiation potential by donor age

ALP activity measured on day 7 was significantly upregulated in 4-wk-old rBMSCs culture in OIM (2.341 ± 0.054) compared with control medium (1.474 ± 0.100; $P < 0.005$, *t*-test). While 14-wk-old rBMSCs cultured in OIM (0.099 ± 0.007) showed no significant difference from their control (0.101 ± 0.0099) (Figure 2A). Consistently, Alizarin Red S staining on day 21

revealed extensive mineralized nodules in 4-wk-old cultures, most prominently in OIM. In contrast, no mineralized nodules were detected in 14-wk-old cultures under either condition (Figure 2B).

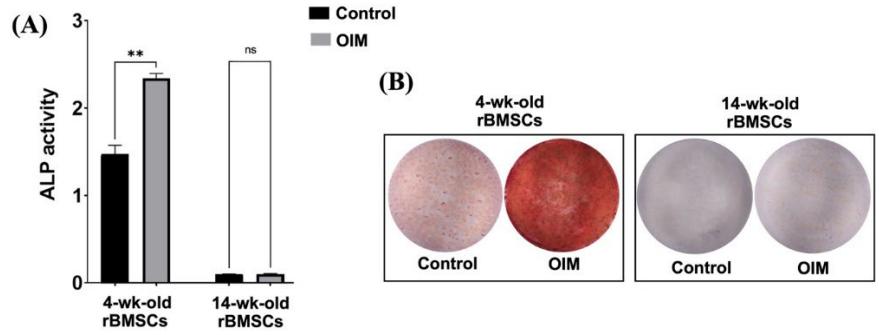

Figure 2 Osteogenic differentiation of rBMSCs by donor age. rBMSCs from 4- and 14-wk-old donors were cultured in control medium or OIM. (A) ALP activity. Data were presented as mean ± SE of three independent experiments (n = 3); $^{**}P < 0.005$; *t*-test. (B) Representative images of Alizarin Red S staining showed mineralized deposits.

Morphology of rBMSC sheets by donor age

The structure, quality, and detachment properties of rBMSC sheets were compared between the two age groups. Microscopic examination showed that 4-wk-old rBMSCs formed fragmented, loosely arranged layers with gaps (Figure 3A) and were unable to form an intact sheet (image not shown). Conversely, rBMSC sheets derived from 14-wk-old rats formed well-organized, densely packed cellular layers with abundant ECM deposition (Figure 3B). These sheets were continuous, free of holes, structurally intact (Figure 4A). They detached from the culture surface within 20-25 minutes and could be handled with forceps (Figure 4B). Results were consistent across all experiments.

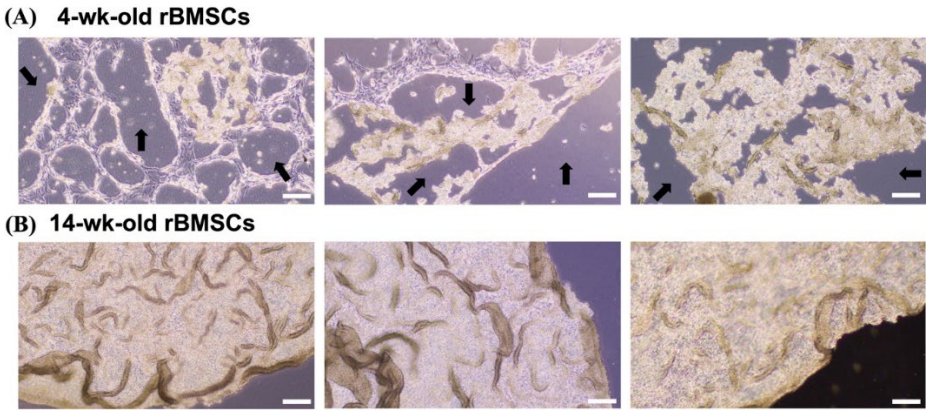

Figure 3 Morphology of rBMSC sheets by donor age. Representative images illustrated cell sheet formation and structure. (A) Sheets from 4-wk-old rBMSCs appeared fragmented, with a loosely connected cellular arrangement and gaps (arrows). (B) Sheets from 14-wk-old rBMSCs showed well-organized, dense cellular layers with a continuous structure. Scale bar: 100 μm. Images were representative of three independent experiments (n = 3).

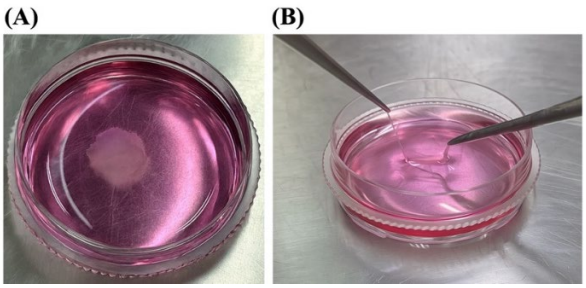

Figure 4 rBMSC sheets from 14-wk-old donors. Representative images of cell sheet. (A) The sheet remained continuous and structurally intact after detachment. (B) The detached sheet was handled with forceps.

DISCUSSION & CONCLUSION

rBMSCs were used in regenerative medicine for their osteogenic capacity. Donor age, however, can altered experimental outcomes. Motivated by preliminary observations of limited osteogenic differentiation in rBMSCs from 14-wk-old rats, we examined rBMSCs from 4-wk-old donors and compared osteogenic capacity and cell sheet formation between the two donor ages.

In this study, rBMSCs from 4-wk-old donors exhibited greater *in vitro* osteogenesis, showing high ALP activity and abundant mineralized nodules, whereas cells from 14-wk-old donors showed minimal ALP activity with no detectable mineralization. These results aligned with prior reports of age-associated declines in osteogenic differentiation (Fafián-Labora et al., 2015; Li et al., 2015; Zhang et al., 2008).

Unlike their osteogenic ability, sheet formation followed the opposite pattern. rBMSCs from 4-wk-old donors produced fragmented, discontinuous layers that failed to merge into an intact sheet. Previous studies that generated rBMSC sheets from donors younger than eight wks used temperature-responsive plates with ascorbic-acid-supplemented media, ranging from 0.1 to 25 mM (Ito et al., 2017; Maruyama et al., 2020; Mito et al., 2024; Wang et al., 2023). Because ascorbic acid enhanced collagen maturation and could modulate proliferation and matrix deposition in a dose-dependent manner (Choi et al., 2008), we excluded it to avoid confounding and to isolate the effect of donor age.

Under ascorbic-acid free conditions, longer culture periods were often required. A previous study reported successful sheet formation using rBMSCs from 8-11-wk-old male Sprague-Dawley rats after 14 days of culture without passage and without ascorbate (Li et al., 2012). We employed a 3-day culture period based on our previous experiments that 14-wk-old rBMSCs formed sheets within this interval, consistent with protocols in other studies (Maruyama et al., 2020; Mito et al., 2024) Across experiments, **rBMSC sheets were obtained from 14-wk-old donors but not from 4-wk-old donors.** For practical use in bone regeneration such as calvarial reconstruction, cell sheets should be continuous, structurally intact, and detachable for transfer to the target site. In our experiments, sheets derived from 4-wk-old rBMSCs did not meet these criteria and therefore required further optimization for cell sheet-forming structure.

This study had limitations. Analyses were restricted to *in vitro* endpoints using Wistar rats at two ages, and mechanical testing of the sheets was not performed. As such, the generalizability of these findings to other genetic backgrounds, age groups, and *in vivo* settings remains to be determined. Future studies should evaluate sheet formation across various donor ages, include mechanical characterization, and explore additional culture modifications to optimize rBMSC sheet protocols for regenerative applications.

In conclusion, donor age significantly influences rBMSC function, with younger donors exhibiting enhanced osteogenic differentiation while older donors demonstrate a greater

consistency in intact sheet formation. Future studies should focus on strategies to optimize sheet fabrication from juvenile rBMSCs without compromising their superior differentiation capacity.

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