Implantation of Bone Marrow Stromal Cell Sheets Derived from Old Donors Supports Bone Tissue Formation

Manabu Akahane¹ · Takamasa Shimizu² · Yusuke Inagaki³ · Tsutomu Kira² · Takuya Egawa² · Akinori Okuda² · Tadanobu Onishi² · Tomoaki Imamura¹ · Yasuhito Tanaka²

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Abstract The purpose of this study was to evaluate the osteogenesis ability of osteogenic matrix cell sheets (OMCS) derived from old donor cells. Bone marrow stromal cells (BMSC) were obtained from young (7-week-old) and old (1-year-old) Fischer344 rats donors and cultured with modified Eagle's medium (MEM group) alone or containing dexamethasone (Dex; 10 nM) and ascorbic acid phosphate (AscP; 0.28 mM) (Dex/AscP group). We prepared four *in vitro* experimental groups: (1) young MEM, (2) young Dex/AscP, (3) old MEM and (4) old Dex/AscP. Cell proliferation and osteogenic marker mRNA expression levels were evaluated *in vitro*. To assess bone formation *in vivo*, the cells of each group were combined with beta tricalcium phosphate (TCP) disks followed by implantation in recipient rats. The *in vitro* study showed significant differences in the mRNA expression of osteocalcin, ALP, and BMP2 between MEM and Dex/AscP groups. Bone formation following implantation was observed upon histological analyses of all groups. TCP combined with OMCS (OMCS/TCP group) resulted in enhanced bone formation compared to that following combination with BMSC (BMSC/TCP). The osteocalcin content of the OMCS/TCP group 4 weeks after implantation was significantly higher than that in the BMSC/TCP construct for both young and old donors. The present study clearly indicated that OMCS could be generated from BMSCs of old as well as young donors using a mechanical retrieval method. Thus, through its usage of OMCS, this method may represent a potentially effective therapeutic option for cell-based therapy in elderly patients.

Keywords Old donor · Cell sheet · Osteogenesis · Beta tricalcium phosphate

1 Introduction

Bone marrow stromal cells (BMSCs) have been commonly used for clinical application in regenerative medicine [1, 2] because they can be obtained from the patient's pelvis by

needle aspiration; thus, the invasiveness is much less compared with that in harvesting the patient's bone. As BMSCs contain mesenchymal stem cells, which are adult stem cells with great potential for differentiation to osteoblasts [1, 3], chondrocytes [2], and neurons [4], they have been utilized for regenerative medicine strategies to treat various diseases or tissue injury [5, 6]. In turn, scaffolds constitute an essential component of tissue engineering, playing important roles in the support of seeded cells, cell growth, and promotion of tissue repair. Notably, the combination of BMSCs with scaffolds such as beta tricalcium phosphate (TCP) and hydroxyapatite (HA) can promote the formation of new bone tissue after subcutaneous transplantation [3, 7–9]. Recently, cell sheet technology has received considerable attention as a favorable cell transplantation method because the use of cell sheets

✉ Manabu Akahane
makahane@naramed-u.ac.jp

¹ Department of Public Health, Health Management and Policy, Faculty of Medicine, Nara Medical University, Shijo 840, Kashihara, Nara 634-8521, Japan

² Department of Orthopedic Surgery, Nara Medical University, Shijo 840, Kashihara, Nara 634-8522, Japan

³ Department of Arthroplasty and Regenerative Medicine, Nara Medical University, Shijo 840, Kashihara, Nara 634-8522, Japan

allows the transplantation of cultured cells together with extracellular matrices.

Several methods have been reported to generate cell sheets, such as using thermo-responsive polymer-grafted culture dishes [10, 11] and mechanical retrieval [12–14]. We previously reported a method of cell sheet derivation using a mechanical retrieval technique with a scraper, in which a cell sheet, designated as an osteogenic matrix cell sheet (OMCS), could be transplanted free of scaffold. This method resulted in abundant bone formation following transplantation compared with conventional techniques using cell suspensions [13–15]. Using this strategy of scaffold-free OMCS transplantation, we have proposed solutions for hard tissue regeneration, such as fracture nonunion [15] and ligament reconstruction [16]. However, our previous experimental studies were conducted using only young donor rats and rabbits. For clinical application of the OMCS in the case of elderly patients, further experiments using BMSCs from old donors are necessary.

The number of elderly people is increasing in well-developed countries such as Japan [17], thus characterizing the country as an “aging society.” Accordingly, fall-related injuries are becoming an important concern especially among the elderly population, as falling often results in health problems such as femoral neck fracture or compression fractures of the vertebrae, leading to disability in daily life [18, 19]. Furthermore, severe cases sometimes result in large bone defects or nonunion requiring subsequent bone reconstructive surgery, implying that the requirement for treatment of these issues will increase concurrently. In particular, regenerative medicine will likely represent a potent treatment option in such cases.

However, as previous studies have reported that aging is associated with a decline in the osteogenic potential of bone marrow cells [7, 18, 20–22], a concern exists regarding whether regenerative medicine would be effective for the elderly population. It also remains unclear whether OMCS may be generated using BMSCs from old donors or whether OMCS derived from old donor cells may be applied to bone regeneration or exhibit osteogenesis potential similar to that of young donor OMCS. To address these issues, in the present study we conducted *in vitro* and *in vivo* experiments to evaluate the osteogenesis of OMCS created from BMSCs of old donor rats.

2 Materials and methods

2.1 Experimental materials

Minimal essential medium (MEM) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) were purchased from Nacalai Tesque (Kyoto, Japan). Culture flasks,

dishes, and plates were from Corning (Armonk, NY, USA) and fetal bovine serum was from Gibco, Life Technologies (Carlsbad, CA, USA). Trypsin/ethylenediaminetetraacetic acid (EDTA) and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA) and Ascorbic acid phosphate (AscP) and buffered formalin were from Wako Pure Chemical Industries (Kyoto, Japan). Dexamethasone (Dex) was from Sigma (St. Louis, MO, USA). Primer and probe sets and reagents for real-time quantitative polymerase chain reaction (PCR) were purchased from Applied Biosystems (Foster City, CA, USA). Sterile, porous TCP ceramic scaffolds (SuperPore; 5 mm diameter, 2 mm thickness, 75% porosity) were purchased from Hoya (Tokyo, Japan). CellTiter 96 Aqueous One Solution Reagent was from Promega (Madison, WI, USA). The RNeasy Mini Kit was purchased from Qiagen Inc. (Venlo, The Netherlands). Fischer 344 rats were purchased from Japan SLC Inc. (Shizuoka, Japan). The Rat Osteocalcin ELISA Kit was purchased from DS Pharma Biomedical (Osaka, Japan). Sephadex G-25 columns were from Amersham Pharmacia Biotech AB (Uppsala, Sweden).

The study protocol was approved by the Animal Experimental Review Board of Nara Medical University (IRB No. 11345).

2.2 BMSC preparation

We have previously reported the preparation of BMSCs [13, 14, 23]. Briefly, bone marrow was obtained from the femur shafts of donor seven-week-old Fischer 344 male rats. Both femur ends were cut and the bone marrow was flushed out using 10 ml standard culture medium (MEM with 15% fetal bovine serum and antibiotics) expelled from a syringe through a 21-gauge needle. Released cells were collected into two T-75 flasks containing 15 ml standard culture medium. Cells were cultured in an incubator under 5% CO₂ at 37 °C. Non-adherent cells were removed during media changes, which were performed three times per week. After reaching confluence, primary cultured cells were trypsinized from T-75 flasks using trypsin/EDTA and collected as a cell suspension of BMSCs. In the present study, BMSCs were separately prepared from young donor (7-week-old) and old donor (1-year-old) rats. BMSCs were prepared from three different donor rats because the characteristics of BMSCs from primary culture may vary between each individual animal.

2.3 Experimental groups for *in vitro* and *in vivo* studies

We prepared four *in vitro* experimental groups in the present study: (1) young MEM, (2) young Dex/AscP, (3) old

MEM, and (4) old Dex/AscP. These groups were subjected to cell proliferation and real time quantitative PCR assays as described below. Another four experimental groups were prepared for *in vivo* study: (1) young BMSC/TCP construct, (2) old BMSC/TCP construct, (3) young OMCS/TCP construct, and (4) old OMCS/TCP construct. These groups were subjected to histological observations and measurements of alkaline phosphatase (ALP) activity and osteocalcin content. A summary of the *in vivo* experiments is illustrated in Fig. 1.

2.4 Cell proliferation assay

The cell proliferation of young and old donor samples was measured using MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) [9]. Briefly, the released BMSCs from young or old donors were seeded into 96-well culture plates (1×10^4 cells/cm²) and cultured with 100 μ l medium for 5 days ($n = 5$). Then, the cell proliferation was determined using CellTiter 96 Aqueous One Solution Reagent. After removal of the medium and washing with PBS, 100 μ l medium and 20 μ l MTS solution was added per well and

Fig. 1 Summary of the *in vivo* experimental method

incubated for 1 h at 37 °C. Finally, formazan absorbance at 490 nm was measured using a microplate reader. Experiments were performed in triplicate.

2.5 RNA isolation and real-time quantitative PCR

We measured the gene expression levels of osteocalcin, ALP, bone morphogenetic protein 2 (BMP2), and collagen type I mRNA in each group. RNA for *in vitro* experiments was isolated from each group using RNeasy Mini RNA extraction kits [24]. Briefly, cells of each group cultured in 6-well plates were rinsed twice with PBS and treated with 600 µl kit reagent. The cells were scraped off into the reagent using a pipette tip, and converted to cDNA according to the manufacturer's protocol ($n = 3$). To measure mRNA expression levels, we conducted real-time quantitative PCR (ABI StepOne Plus Real Time PCR System; Applied Biosystems, Norwalk, CT, USA) using primers for rat cDNAs as previously described [9]. Target mRNA levels were compared after correcting to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA levels as an internal standard, to adjust for differences in the efficiency of reverse transcription between samples. The primers used to amplify the target mRNAs were as follows; *osteocalcin* (Hs01587814 g1), *Alp* (Hs01029144 m1), *Bmp2* (Hs00154192 m1), *collagen type I* (Rn00801649 g1), and *Gapdh* (Rn99999916 s1; all from Applied Biosystems). The thermal cycling conditions were 20 s at 95 °C for activation of the TaqMan Fast Universal PCR Master Mix, followed by 40 cycles of 1 s at 95 °C for denaturation and 20 s at 60 °C for annealing and extension. Experiments were performed in triplicate.

2.6 Preparation of the OMCS and β -TCP ceramics construct

The released BMSCs were seeded onto 100-mm culture dishes at a density of 1×10^4 cells/cm² to generate OMCS [13, 14, 25]. Briefly, the cells were cultured in standard medium with 10 nM Dex and 0.28 mM AsCP (cell sheet medium) for 14 days. Cell culture was maintained in a humidified atmosphere of 95% air with 5% CO₂ at 37 °C. The culture medium was renewed every 2 days for 2 weeks in the experimental period. After 2 weeks, the cells were rinsed twice with PBS, scraped off using a cell scraper, and harvested as an OMCS. The TCP ceramic scaffolds were wrapped with the cell sheet to create the OMCS and TCP ceramics construct (OMCS/TCP construct), for subsequent subcutaneous transplantation into the syngeneic recipient rats (Fig. 1).

2.7 Preparation of BMSC and TCP ceramics construct

We have previously reported the preparation of TCP constructs combined with BMSCs (BMSC/TCP construct) [25, 26]. Briefly, after release from the substratum, the BMSCs were centrifuged and suspended at a density of 1×10^6 cells/ml in standard medium. Then, the TCP ceramics were soaked in MEM containing 1×10^6 - BMSCs/ml until the air within the scaffolds was replaced with MEM (no more air bubbles were formed; approximately 2 h) in a CO₂ incubator at 37 °C to create the BMSC/TCP constructs. Notably, the solid and porous components of the microstructure in TCP ceramics are interconnected. After 2 h, the construct was transferred into a 12-well culture plate with 1 ml osteogenic-induction medium, consisting of standard medium, 0.28 mM AsCP, and 10 nM Dex for further subculture. The culture medium was renewed every 2 days for 2 weeks in the experimental period. After 14 days of subculture, the cultured BMSC/TCP constructs were transplanted into a subcutaneous site of syngeneic rats (Fig. 1).

2.8 Subcutaneous implantation of constructs

To assess the pattern of *in vivo* bone formation, the OMCS/TCP and BMSC/TCP constructs from young and old rats were implanted subcutaneously onto the backs of syngeneic 7-wk-old and 1-year-old male rats using a previously described procedure [9, 25]. Each group included seven ceramics for a single recipient rat. Each ceramic was transplanted subcutaneously via the separated incision. The ceramics were harvested after 4 weeks to evaluate the bone formation histologically and biochemically.

2.9 Histological analysis

For histological evaluations ($n = 2$), the ceramics harvested at 4 weeks after implantation were fixed in 10% neutral buffered formalin for 2 days, decalcified, and embedded in paraffin. The middle of the ceramic was cut into 8-µm sections parallel to the base and then stained with hematoxylin and eosin, or Masson trichrome staining for histological evaluation. The experiments were performed in triplicate.

2.10 Biochemical analysis

The ALP activities and osteocalcin contents of harvested constructs were measured as described previously [13]. Briefly, each construct ($n = 5$) was immediately crushed and homogenized in 1 ml 0.2% Nonidet P-40 (NP-40) containing 50 mM Tris-HCl and 1 mM MgCl₂ at 4 °C

Fig. 2 Cell proliferation assay between MEM and Dex/AscP medium in the **A** Young and **B** Old donor groups. MEM, standard culture medium; Dex/AscP, standard culture medium with Dex and AscP (cell sheet medium). Values are shown as the mean $\pm$ standard deviation. * $p < 0.05$

Fig. 3 **A** Osteocalcin mRNA expression between MEM and Dex/AscP medium in the young donor group. **B** *Alp* mRNA expression, **C** *Bmp2* mRNA expression, **D** Collagen type I mRNA expression.

MEM, standard culture medium; Dex/AscP, standard culture medium with Dex and AscP (cell sheet medium). Values are shown as the mean $\pm$ standard deviation. * $p < 0.05$

using a microhomogenizer, and then centrifuged at 12 000 g for 10 min at 4 °C. An aliquot (10 μ l) of the supernatant was combined with 56 mM 2-amino-2-methylpropanediol buffer containing 10 mM p-nitrophenylphosphate and 1 mM MgCl₂. After incubation for

30 min at 37 °C, 2 ml 0.2 N NaOH was added to stop the reaction and the ALP activity was calculated by measuring the absorbance of p-nitrophenol released at 410 nm using a spectrophotometer. ALP activity was represented as μ mol

Fig. 4 **A** Osteocalcin mRNA expression between MEM and Dex/AscP medium in the old donor group. **B** *Alp* mRNA expression, **C** *Bmp2* mRNA expression, **D** Collagen type I mRNA expression.

MEM, standard culture medium; Dex/AscP, standard culture medium with Dex and AscP (cell sheet medium). Values are shown as the mean $\pm$ standard deviation. * $p < 0.05$

of *p*-nitrophenol per construct released during 30 min incubation at 37 °C.

Subsequently, the osteocalcin content of the harvested construct was measured [13]. Osteocalcin was extracted from the sediments following extraction with 0.2% NP-40 in 4 ml 20% formic acid for 2 weeks at 4 °C. An aliquot (0.5 ml) of the formic acid extract was applied to a pre-packed Sephadex G-25 column (NAP-5 column) and eluted with 10% formic acid. The protein fractions were pooled and dried. After dissolution in 0.5 ml ELISA sample buffer, the osteocalcin content was assayed using a Rat Osteocalcin ELISA Kit [13] according to manufacturer instruction.

2.11 Statistics

Statistical analysis was performed using SPSS (Version 22, Chicago, IL, USA). All data are expressed as the mean $\pm$ standard deviation (SD). Overall differences between group means were analyzed by a *t* test. A *p* value of 0.05 was considered statistically significant.

3 Results

3.1 Cell proliferation assay

Figure 2 shows the results of cell proliferation using the MTS assay. There was a significant difference in cell proliferation between MEM and Dex/AscP culture in both young and old rat groups. Both groups tended to exhibit similar results, indicating that the cell sheet medium (Dex/AscP) stimulated cell proliferation.

3.2 *In vitro* mRNA expression levels

Figures 3 and 4 show the *in vitro* mRNA expression levels as determined by real time PCR of the young and old rat groups, respectively. There were significant differences in the mRNA expression of osteocalcin, ALP, BMP2, and collagen I between MEM and Dex/AscP culture in the young rat group (Fig. 3). Similar results were observed in the old rat group, except for collagen type I (Fig. 4).

Young BMSC/TCP group

Fig. 5 Representative figures of the harvested samples 4 weeks after transplantation. **A–D** Young BMSC/TCP group, **E–H** Young OMCS/TCP group, **I–L** Old BMSC/TCP group, **M–P** Old OMCS/TCP group. **A, B, E, F, I, J, M, N** Hematoxylin and eosin stain, and **C, D, G, H, K, L, O, P** Masson trichrome stain. The bone formation in OMCS/TCP appeared to be higher compared to that in BMSC/TCP

for both donor age groups. The right panel of each group represents higher magnified images of the rectangular area in the left panel. Bars indicate 100 and 20 μm in the lower and higher magnification images, respectively. BMSC/TCP, TCP combined with BMSC suspension; OMCS/TCP, TCP combined with OMCS. *bone tissue

3.3 Histology of the harvested samples

Figure 5 shows the representative histology of the harvested samples 4 weeks after implantation. Bone formation could be observed in the histology of both young (Fig. 5A–H) and old (Fig. 5I–P) groups. Masson trichrome staining (Fig. 5C, D, G, H, K, L, O, P) showed positive staining in all groups. The bone formation in the TCP/OMCS construct appeared to be greater than that in the TCP/BMSC construct for both age groups.

3.4 ALP activities and osteocalcin content

Figure 6 shows the ALP activities and osteocalcin content in the TCP constructs 4 weeks after implantation. There were significant differences between BMSC/TCP and OMCS/TCP constructs in ALP activity and osteocalcin

content in young donors (Fig. 6A, B). The ALP activities and osteocalcin content of OMCS/TCP constructs were approximately three- and five-fold higher than that of BMSC/TCP, respectively. However, in the old donors (Fig. 6C, D), a significant difference was not observed between BMSC/TCP and OMCS/TCP in ALP activity. The osteocalcin content of OMCS/TCP in old donors was decreased compared to that of young donors, indicating a decreased capacity for *in vivo* osteogenesis of old donors.

4 Discussion

Bone is continuously remodeled throughout life through the activities of osteoblasts and osteoclasts. However, the amount of bone resorbed by osteoclasts is not completely restored by the osteogenesis of osteoblasts in individuals of

Young OMCS/TCP group

Fig. 5 continued

advanced age. The imbalance between bone resorption and osteogenesis results in decreasing bone volume and strength in the elderly. Therefore, aging is considered a major risk factor for fractures such as of the femoral neck and spinal bone [27]. Changes in the activities of BMSCs with advancing age have been reported in humans and rodents, such as decreased proliferation, differentiation potential, and osteogenic potential, as well as increased senescence [18, 19, 28]. In particular, the loss of mesenchymal progenitor functionality with age might be due to the accumulation of oxidative damage [18]. Additionally, the number of adipocytes in bone marrow increases with advanced age in humans and levels of growth factors such as insulin-like growth factor 1 (IGF-1) decrease. Notably, IGF-1 constitutes the most abundant growth factor deposited in the bone matrix [29] as well as the primary osteogenic factor released from the bone matrix and promotes the differentiation of osteoprogenitor cells [30]. Therefore, reconstructive therapy using autologous cultured BMSCs

may not represent a satisfactory treatment option for elderly patients.

Previously, Inoue et al. [7] reported the effects of aging on bone formation in porous HA, comparing the activities of bone marrow cells from young and old rats. They implanted constructs of HA with BMSCs from young or old donors subcutaneously and concluded that the decreased bone formation associated with old BMSCs was due to the smaller population of BMSCs and/or their decreased capacity of differentiation to osteogenic cells. In addition, Lu et al. [20] reported that age affects vascularization during fracture repair. Tissue vascularization is essential for successful bone healing [31]. In particular, angiogenesis, which comprises the sprouting of new vessels from existing vessels, and vasculogenesis, which refers to the formation of new vessels from endothelial progenitor cells, can contribute to fracture repair through vascularization of the fracture callus [32]. Furthermore, Chen et al. [33] reported that aging and multiple passaging affected the

Old BMSC/TCP group

Fig. 5 continued

proliferation and osteogenic differentiation of human BMSCs, and that less effect was observed in human adipose-derived mesenchymal stem cells, suggesting that these cells may represent a potentially effective therapeutic option for cell-based therapy in elderly patients. Additionally, Mendes et al. [22] reported that a significant age-related decrease was observed in the growth rate of human BMSCs and that an increase in donor age significantly decreased the frequency of cases in which bone formation was observed when human BMSCs were seeded on porous calcium phosphate scaffolds and subcutaneously implanted in nude mice.

In the present study, our results showed abundant bone formation when the OMCS derived from either young or old donors were combined with TCP and transplanted subcutaneously, and demonstrated that very similar levels of osteogenesis occurred with use of either young or old donor cells. The osteogenesis of OMCS was higher than that of the cell suspension even when cells from old donors were used. We generated OMCS through culture in Dex/

AscP medium; therefore, the OMCS may have included differentiated cells such as osteoblasts and extracellular matrices such as collagen type I prior to implantation [9]. This may represent an advantage of our method toward facilitating osteogenesis following implantation as compared to other transplantation methods using cell suspensions. Our results also indicated that the culture conditions using standard culture dishes containing MEM with Dex and AscP allowed for the mechanical retrieval (scraping) of the confluent cell layer as a single cell sheet structure for both old as well as young donors. Therefore, we consider that the OMCS transplantation method may represent a preferred technique for the reconstruction of bone tissue such as during fracture and bone defect repair even for elderly patients.

Notably, the cells used in the present study were not of a single cell type as we harvested bone marrow cells and expanded them without cell sorting. The higher cell population obtained following culture in MEM included mesenchymal stem cells as well as vascular endothelial cells

Old OMCS/TCP group

Fig. 5 continued

and fibroblasts, among others. In contrast, the cells cultured in MEM with Dex and AscP mostly consisted of osteoblasts as culturing with Dex and AscP stimulated differentiation into the osteoblastic lineage. In particular, previous studies have reported that Dex prevents apoptosis of BMSCs [34] and promotes MSC proliferation [35], whereas another study reported that AscP stimulated extracellular matrix (ECM) secretion and cell proliferation [36]. In the present study, BMSCs cultured in MEM containing Dex and AscP exhibited stimulated cell proliferation compared to those cultured in MEM alone, consistent with the previous reports. These effects including stimulated differentiation, promoted proliferation, and ECM secretion resulted in enhanced osteogenesis of OMCS/TCP constructs following transplantation in both young and old rats. This may represent an advantage of cell sheet transplantation as prepared by our method compared to cell suspension techniques.

There are a few of limitations in the present study. First, we did not compare the characteristics of cell sheets created by our method to those created by other techniques

such as with a thermosensitive polymer. Therefore, other technique of generating cell sheets from BMSCs should be tested. However, our method of culturing in cell sheet medium (standard MEM with Dex and AscP) enhanced the osteogenic differentiation of the cell sheet, which is advantageous for bone regeneration. Second, we transplanted OMCS/TCP constructs subcutaneously. However, we have not assessed the osteogenic potential of BMSCs from young and old donors in other conditions such as fracture, nonunion, and bone defect sites. Further study is necessary to determine the efficacy of our method under these conditions. Third, loading cell number was not identical between OMCS/TCP and BMSC/TCP constructs. However, the ability to load more cells on TCP as allowed by cell sheet transplantation such as OMCS/TCP compared to the numbers obtained using cell suspension techniques may be considered advantageous as physicians require a greater degree of osteogenesis in clinical cases. Fourth, the present study was performed using rat BMSCs. As human BMSCs would be used in the clinical application of OMCS generated using our technique, the osteogenesis of human

Fig. 6 ALP activities **A** and **B** Osteocalcin content 4 weeks after implantation in the young donor group. **C** ALP activity and **D** Osteocalcin content 4 weeks after implantation in the old donor

BMSCs from young and old donors should be evaluated. Future studies will address these issues.

In conclusion, the present study clearly demonstrated that OMCS could be derived from BMSCs of old as well as young donors using a mechanical retrieval method. OMCS derived from both young and old donors induced abundant bone formation *in vivo* when combined with TCP and transplanted subcutaneously. Thus, through its usage of OMCS, this method may represent a potentially effective cell-based therapeutic option for bone regeneration, especially in elderly patients.

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Author Contributions MA designed this study and wrote the first draft of the manuscript. TS and TK conducted animal experiments. TE, AO, and TO conducted biological analyses. YI, TI, and YT jointly interpreted the results.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to disclose.

Ethical statement All experimental protocols using animals were approved by the Animal Experimental Review Board of Nara Medical University (IRB No. 11345) prior to initiating the experiments. The

group. BMSC/TCP, TCP combined with BMSC suspension; OMCS/TCP, TCP combined with OMCS. Values are shown as the mean $\pm$ standard deviation. * $p < 0.05$

animals were housed in a temperature-controlled environment at approximately 21 °C under a 12 h light/12 h dark cycle with free access to food and water.

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