



## Original article

# Dynamic cultivation with radial flow bioreactor enhances proliferation or differentiation of rat bone marrow cells by fibroblast growth factor or osteogenic differentiation factor



Yuuhei Kanda <sup>a</sup>, Itsuro Nishimura <sup>a</sup>, Toru Sato <sup>b</sup>, Aiko Katayama <sup>a</sup>, Taichi Arano <sup>a</sup>, Yoshito Ikada <sup>c</sup>, Masao Yoshinari <sup>d,\*</sup>

<sup>a</sup> Department of Fixed Prosthodontics, Oral Health Science Center, Tokyo Dental College, Tokyo, Japan

<sup>b</sup> Department of Fixed Prosthodontics, Tokyo Dental College, Tokyo, Japan

<sup>c</sup> Division of Life Science, Nara Medical University, Kashihara, Japan

<sup>d</sup> Oral Health Science Center, Tokyo Dental College, Tokyo, Japan

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## ABSTRACT

Dynamic cultivation using a radial flow bioreactor (RFB) has gained increasing interest as a method of achieving bone regeneration. In order to enhance bone generation in large bone defects, it is necessary to use an RFB to expand the primary cells such as bone marrow cells derived from biotissue. The present study aimed to evaluate the cell expansion and osteogenic differentiation of rat bone marrow cells (rBMC) when added to basic fibroblast growth factor containing medium (bFGFM) or osteogenic differentiation factor containing medium (ODM) under dynamic cultivation using an RFB. Cell proliferation was evaluated with a DNA-based cell count method and histological analysis. An alkaline phosphatase (ALP) activity assay and immunohistochemistry staining of osteogenic markers including BMP-2 and osteopontin were used to assess osteogenic differentiation ability. After culture for one week, rBMC cell numbers increased significantly under dynamic cultivation compared with that under static cultivation in all culture media. For different culture media in dynamic cultivation, bFGFM had the highest increase in cell numbers. ALP activity was facilitated by dynamic cultivation with ODM. Furthermore, both BMP-2 and osteopontin were detected in the dynamic cultivation with ODM. These results suggested that bFGFM promotes cell proliferation and ODM promotes osteogenic differentiation of rBMC under dynamic cultivation using an RFB.

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## 1. Introduction

Large bone defects caused by trauma, inflammation, tumors, or congenital abnormalities are often treated with bone grafts. Current treatments of large bone defects are based on autologous or allogeneic bone grafts that have several limitations. Autologous bone grafts are preferred but the treatment suffers from a limited supply and donor site morbidity [1]. Allogeneic bone grafts, although in more abundant supply, have been implicated in disease transmission [2].

Bone tissue engineering is a rapidly developing field aimed at satisfying the need for replacing diseased or damaged bone. Furthermore, it is well accepted that ideal bone transplants involve cell-scaffold constructs in which target cells are able to expand and develop according to their type. Transplantation of rat bone marrow cell-scaffold constructs in cranial defect models leads to a substantial increase in bone formation compared to that in scaffolds containing no cells at all [3]. However, the scaffolds intended to bridge such defects would be sufficiently large to complicate in vitro culture, because bone defects that require such surgical correction are large. Diffusion of nutrients into the scaffold and excretion of metabolites may not satisfy the metabolic requirements of the seeded cells and might result in the suppression of cell growth. Accordingly, a solution to this problem is to culture seeded scaffolds in a system that can enhance nutrient delivery, and various culturing systems have been developed as a result.

\* Corresponding author.

E-mail address: [yosinari@tdc.ac.jp](mailto:yosinari@tdc.ac.jp) (M. Yoshinari).

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Bioreactors, of which spinner flasks [4] and rotating wall vessels [5] are two examples, provide the necessary conditions for promoting and maintaining tissue culture. In these types of bioreactors, scaffolds are fixed or allowed to float and the culture medium is changed at specific intervals. However, cell growth and mineralization for spinner flasks and rotating wall vessels is limited to the outside of the scaffolds because the limitations on internal nutrient transport are not eliminated. The perfusion bioreactor is another example. In this device, the scaffolds are fixed and the medium is continuously circulated throughout the chambers [6–10].

The radial flow bioreactor (RFB) is one type of perfusion bioreactor [11–14]. This type has the ability to maintain an ideal cell culture environment by radial provision of the medium, enabling the construction of comparatively larger tissues. Furthermore, the RFB offers highly functional 3D cultivation. To allow an even distribution of oxygen, the medium is pumped to the center of the chamber from the periphery under low shear stress.

In a previous study, it was reported that osteoblast-like cells and human mesenchymal stem cells (hMSCs) were expanded uniformly over a 3D scaffold under dynamic cultivation using an RFB, and the cellular characteristics of the hMSCs were not changed when compared to static cultivation in DMEM without bone differentiation medium [11,12]. In addition, it was reported that both the proliferation and bone differentiation of hMSCs were accelerated in 3D culture with dynamic cultivation by osteogenic differentiation factor [13].

Instead, in large bone defects, it is necessary to expand the primary cells derived from biotissue using an RFB in order to enhance bone generation. Bone marrow-derived cells can be induced to follow one of many lineages by the addition of various induction factors to their growth medium. Specifically, directed differentiation of rat bone marrow cells toward osteogenic lineage *in vitro* is facilitated in the presence of dexamethasone,  $\beta$ -glycerol phosphate, and ascorbic acid [15].

However, there have been no reports primary cells such as bone marrow cells being cultured using an RFB and evaluating their biological characteristics. Therefore, the present study aimed to evaluate the cell expansion and osteogenic differentiation of rat bone marrow cells when added to growth factor or osteogenic differentiation factor under dynamic cultivation using an RFB.

## 2. Materials and methods

### 2.1. Isolation and culture of rBMC (bone marrow cells)

The animal experiments in this study were conducted in accordance with the Guidelines for the Treatment of Experimental Animals in Tokyo Dental College (Approval number: 272701).

Rat bone marrow cells were isolated and cultured using the method modified by method of Maniopoulos et al. [15]. Briefly, 6-week-old male SD rats (Sankyo Labo Service, Tokyo, Japan) were sacrificed by deep anesthesia with sodium pentobarbital (Kyoritsuiseiyaku, Tokyo, Japan) and the humeri, femora, and tibiae removed. The soft tissue was removed from the humeri, femora, and tibiae. The humeri, femora, and tibiae were washed twice in DMEM (Gibco Massachusetts USA) with 0.5 mg/mL gentamycin (Wako Osaka Japan) and 3 mg/mL Fungizone (Gibco, Massachusetts, USA). The epiphyses were cut off and the diaphyses flushed out with 10 mL osteoblast induction medium (Table 1). The released cells were collected in a 75-cm<sup>2</sup> plastic culture flask containing 10 ml of Osteoblast induction medium. The medium was changed after 3 days to remove the non-adherent cell population. The cells were subcultured every 7 days.

Osteoblast induction medium was used for primary culture and GM was used for culture of passage 1 and 2 (Table 1). Cells were removed from the culture flask by trypsinization, then counted and seeded in new 75-cm<sup>2</sup> plastic tissue flasks at a density of  $5 \times 10^3$  cells/cm<sup>2</sup>.

### 2.2. Preculture

The method used for cell seeding was based on studies by Arano and Katayama et al. [12,13] in order to optimize the initial cell attachment with high rate of cell density into the collagen sheets that have high porosity ratio and 3-mm thickness, and a preculture assay was performed that involve turning over the sheets. After passaged 2 times, the cells were harvested by 0.25% trypsin-EDTA treatment and seeded onto type 1 collagen sheets (Gunze, Kyoto, Japan) (pore size, 70–110  $\mu$ m; porosity ratio, 80%–95%; diameter, 18 mm; thickness, 3 mm). Briefly, collagen sheets were placed in a 12-well plate and cell suspension (80  $\mu$ L) containing  $2.5 \times 10^5$  cells was seeded onto them. The sheets were then incubated in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub> for 6 h. Next, the sheets were turned over and a further cell suspension (80  $\mu$ L) was added before another incubation for 6 h (Finally, the total cell seeding density was  $5 \times 10^5$  cells per sheet).

### 2.3. Dynamic cultivation

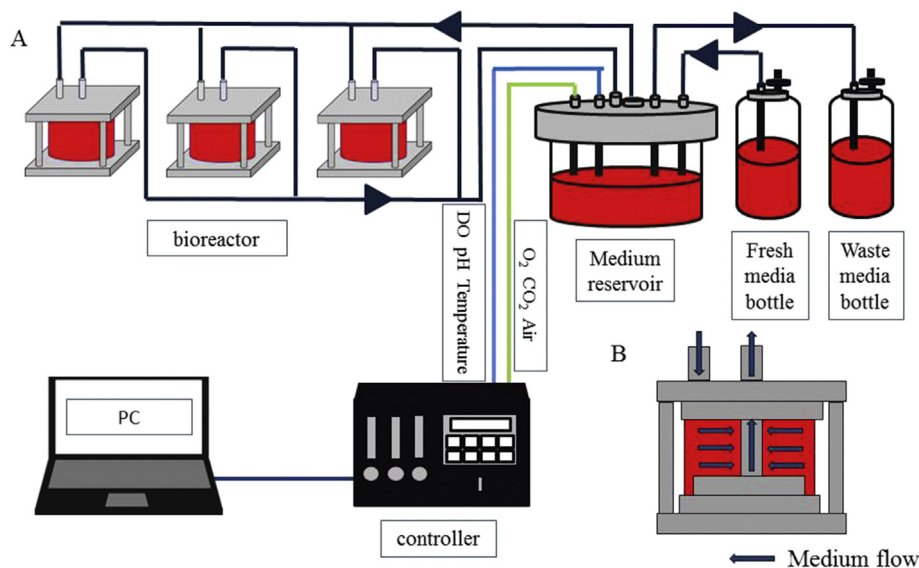
Dynamic cultivation was carried out according to previous studies [11–13]. Figs. 1 and 2 and Table 2 were based on those studies.

Fig. 1 shows the RFB (Able, Tokyo, Japan) and the RFB cell culture system used.

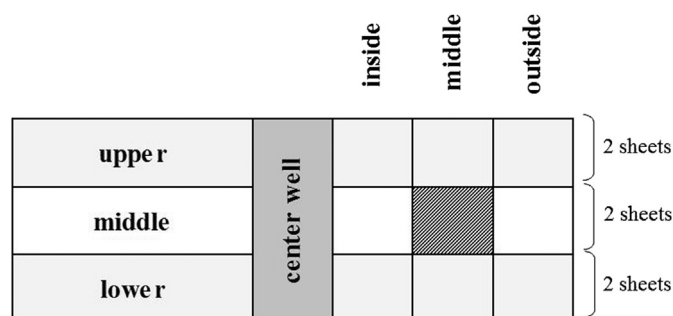
Cultivation condition for dynamic and static cultivation is shown in Table 2. GM, bFGFM or ODM was used for both dynamic and static cultivation.

**Table 1**  
Culture medium.

General medium(GM)	D-MEM (gibco Massachusetts USA) + 10% FBS (Sigma-Aldrich Missouri USA) + 30units/ml gentamycin (Wako Osaka Japan)
Osteoblast induction medium	General medium(GM) + 50 $\mu$ g/ml ascorbic acid–2-phosphate (Wako Osaka Japan) + 10mM Na $\beta$ -glycerophosphate (Sigma-Aldrich Missouri USA) + 10 <sup>-8</sup> M dexamethasone (Sigma-Aldrich Missouri USA)
Basic fibroblast growth factor containing medium (bFGFM)	General medium(GM) + 10ng/ml bFGF (R&Dsystems Minnesota USA)
Osteogenic differentiation factor containing medium (ODM)	General medium(GM) + 50 $\mu$ g/ml ascorbic acid–2-phosphate (Wako Osaka Japan) + 10mM Na $\beta$ -glycerophosphate (Sigma-Aldrich Missouri USA)



**Fig. 1.** Radial flow bioreactor (RFB) system used in this study. (A) Schematic of total system. Medium was circulated between RFB and medium reservoir using a circulation pump. During the experiment, dissolved oxygen (DO), pH, and temperature in the medium were monitored and controlled. The volume of the chamber medium was maintained at 100 mL, and fresh medium was added continuously. (B) Schematic of RFB. Medium in the RFB flows from the periphery to the center of the reactor chamber.



**Fig. 2.** Cross section of scaffolds (six layered collagen sheets cultured in the RFB) used for each analysis. Scaffolds in the RFB were divided horizontally and perpendicularly into nine areas consisting of six sheets (from top to bottom: upper, middle, and lower)  $\times$  three areas (inside, middle, and outside). DNA-based cell count and ALP activity were evaluated using three sheets (upper, middle, and lower). Histological analysis and immunocytochemical analysis were performed using the middle area of the middle sheet (shaded area).

**Table 2**  
Cultivation conditions.

	Dynamic	Static
Number of cells	$5.0 \times 10^5$ cells/sheet	
Temperature	37 °C	
CO <sub>2</sub>	–	5%
pH	7.4	–
DO	6.86	–
Medium flow rate	3 ml/min	–
Medium volume	100 ml/day	2 ml/well
Medium change	Changed daily after 3 days	Every 3 days
Scaffold	6 sheets/reactor	1 sheet/well

To form a scaffold, six precultured sheets were placed in the RFB in layers. The temperature (37 °C), pH (7.4), and dissolved oxygen (DO, 6.86 ppm) in the medium reservoir were controlled and monitored. The medium volume was maintained at 100 mL. After initiation of culture, the medium was changed every day beginning on the third day. The medium flow rate was set at 3 mL/min. Culture was continued for a total of 7 days.

Cross sections of scaffolds (six layered collagen sheets cultured in the RFB) were used for each analysis (Fig. 2). The scaffolds in the RFB were divided horizontally and perpendicularly into nine areas each consisting of six sheets (from top to bottom: upper, middle, and lower)  $\times$  these areas (inside, middle, and outside). DNA-based cell counts and ALP activity were evaluated using three sheets (upper, middle, and lower). Histological analyses and immunocytochemical analyses were performed using the middle area of the middle sheet (shaded area).

#### 2.4. Static cultivation

An individual precultured sheet was placed in each well of a 12-well plate. The culture medium was maintained at 2 mL. Culture was carried out in a humidified atmosphere at 37 °C and 5% CO<sub>2</sub> without control of DO or pH values. The culture medium was changed every 3 days. Culture was carried out for a total of 7 days.

Individual sheets cultured in the well were used for the DNA-based cell counts, histological analyses, ALP activity, and immunocytochemical analyses in static cultivation.

#### 2.5. DNA-based cell count

DNA-based cell counts were performed according to previous studies [11–13] for dynamic cultivation.

Total DNA was quantified with the NanoDrop 1000 Spectrophotometer (ND-1000, Thermo Fisher Scientific, Massachusetts, USA). Finally, cell numbers were calculated using a previously constructed working curve based on cell numbers determined with the Z1 Coulter Counter (Beckman Coulter, California, USA) and total DNA.

The mean DNA-based cell count of the three areas under dynamic cultivation was compared with that of a single collagen sheet under static cultivation.

#### 2.6. Histologic examinations

Scaffolds that were harvested after culture were fixed with 10% neutral-buffered formalin and dehydrated through a series of

ethanol washes. After being embedded in paraffin, the specimens were sliced into 3- $\mu\text{m}$  thick sections and stained with hematoxylin and eosin (H-E staining) according to standard protocols. The samples were then morphologically observed using a universal photomicroscope (Axiophot 2, Carl Zeiss, Oberkochen, Germany).

### 2.7. Alkaline phosphatase (ALP) activity

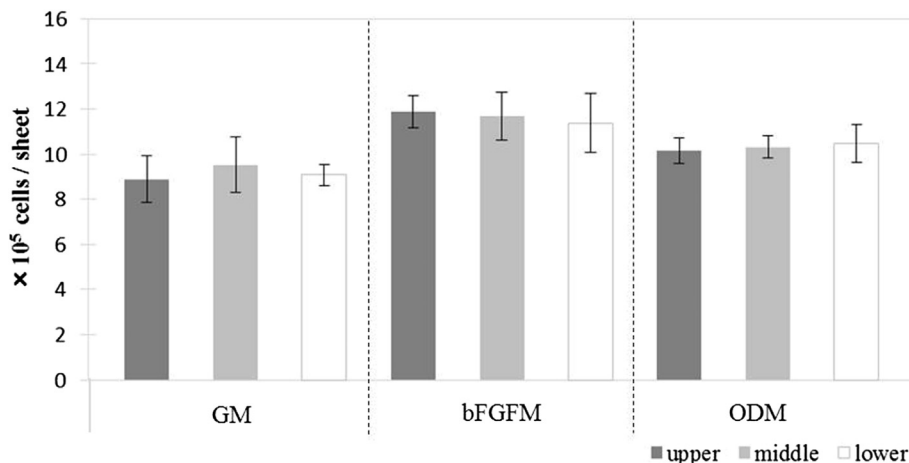
Scaffolds were harvested from the RFB and well plate, and placed in another 12-well plate. The scaffolds were rinsed with cold phosphate-buffered saline (PBS), cut into small fragments, and sonicated for 30 s after application of 200  $\mu\text{L}$  Triton-X/PBS. The lysates obtained were centrifuged at 15,000 rpm for 15 min, and the supernatant was used as sample. ALP activity was assayed using LabAssay ALP (Wako, Osaka, Japan). Sample absorbance was measured in a 96-well plate at 405 nm. The amount of total protein in the sample was then examined with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). Finally, ALP activity was expressed as units/ $\mu\text{g}$  protein.

The mean ALP activity of the three areas under dynamic cultivation was compared with that of a single collagen sheet under static cultivation.

### 2.8. Immunohistochemical staining

Proteins in the collagen sheet were visualized with antibodies of BMP-2 and osteopontin. The sections were washed in 10 nmol/L with pH 7.4 phosphate-buffered saline (PBS) and endogenous peroxidase activity was blocked by incubating sections with 0.3%  $\text{H}_2\text{O}_2$  in methanol for 30 min.

The sections were then reacted with the primary antibodies, BMP-2 polyclonal antibody diluted 1:50 (Proteintech Group, Chicago, USA) and Anti-Osteopontin (rabbit) polyclonal antibody (R&D Systems, Minnesota, USA) diluted 1:100, by incubating at 37 °C for 60 min. The sections were washed in PBS and then incubated with the secondary antibody, peroxidase-labeled anti-mouse IgG polyclonal antibody (Histofine Simple Stain Rat MAX-PO [MULTI]; Nichirei, Tokyo, Japan) for 30 min and washed with PBS. Subsequently, the sections were stained with 3,3'-diaminobenzidine (DAB substrate kit, Nichirei, Tokyo, Japan), washed in sterilized water, and counterstained with hematoxylin. The sections were then dehydrated according to established protocol and the sections were examined and photographed using a universal photomicroscope (Axiophot 2).



**Fig. 3.** Comparison of cell numbers (DNA-based cell count) in each area under dynamic cultivation. No significant difference was observed among the three areas ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD.

### 2.9. Statistical analysis

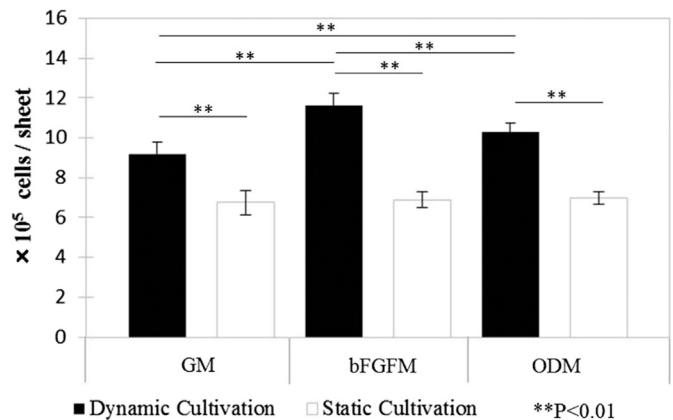
The DNA-based cell count and ALP activity were statistically analyzed using a two-way analysis of variance (ANOVA), followed by Tukey's multiple comparison test.

## 3. Results

### 3.1. DNA-based cell count

**Fig. 3** shows a comparison of the number of cells (DNA-based cell count) in each area under dynamic cultivation. The number of cells increased over that at the initial seeding ( $5.0 \times 10^5$  cells) in all areas and all media. Among each area of all culture media, no significant differences were observed in the number of cells.

Comparison of cell numbers among GM, bFGFM, or ODM under dynamic and static cultivation at 7 days is shown in **Fig. 4**. In each culture medium, a significant increase was noted in the number of cells under dynamic cultivation over that under static cultivation ( $**p < 0.01$ ), despite the small difference in number of cells that was observed between dynamic cultivation and static cultivation in GM.



**Fig. 4.** Comparison of cell numbers among GM, bFGFM, and ODM under dynamic and static cultivation. Under dynamic cultivation, the mean of three areas was chosen for comparison with that under static cultivation. No significant differences were observed in the number of cells among each area of all culture media ( $p > 0.05$ ). Data are expressed as mean  $\pm$  SD.

Under dynamic cultivation, the numbers of cells cultured in the different media were significantly greater in the order of bFGFM > ODM > GM (\*\*p < 0.01). In contrast to dynamic cultivation, no significant differences were observed in the number of cells among the culture media under static cultivation.

3.2. Histological analysis

Fig. 5 shows optical micrographs of hematoxylin-eosin staining.

A greater number of cells were observed in the collagen sheets under dynamic cultivation than under static cultivation in all culture media.

3.3. Alkaline phosphatase activity

Fig. 6 shows a comparison of ALP activity in each area under dynamic cultivation. No significant difference was observed in ALP activity among each area of all culture media.

A comparison of ALP activity among GM, bFGFM, or ODM under dynamic and static cultivation at 7 days is shown in Fig. 7. ALP activity cultured in ODM was significantly higher than that in GM and bFGFM under both dynamic and static cultivation (\*\*p < 0.01).

In ODM, ALP activity under dynamic cultivation was significantly higher than that under static cultivation (\*\*p < 0.01). No significant difference in ALP activity was found between dynamic cultivation and static cultivation in GM and bFGFM.

3.4. Immunocytochemical analysis

The results of immunocytochemical analysis stained with BMP-2 and osteopontin are shown in Figs. 8 and 9, respectively.

BMP-2 and osteopontin were confirmed by color development in both the dynamic and static cell cultures. The expression of BMP-2 was observed in only ODM under both dynamic cultivation and static cultivation (Fig. 8C, F). The expression of osteopontin was observed in ODM under dynamic cultivation, but not under static cultivation (Fig. 9C).

4. Discussion

In this study, dynamic and static cultivations of rat bone marrow cells (rBMC) loaded onto scaffolds were performed using rBMC

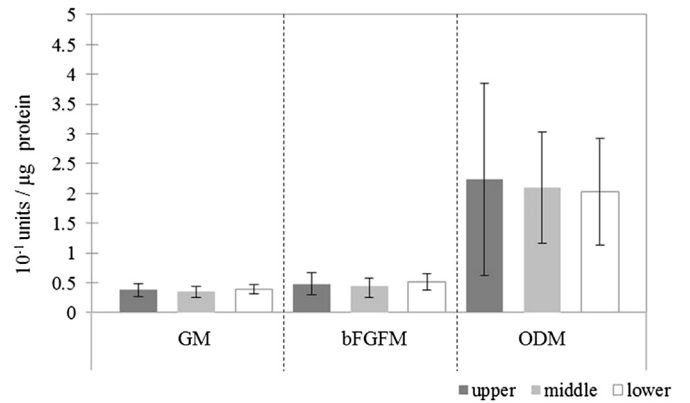


Fig. 6. Comparison of ALP activity (units/µg protein) in each area under dynamic cultivation. No significant difference was observed among the three areas (p > 0.05). Data are expressed as mean ± SD.

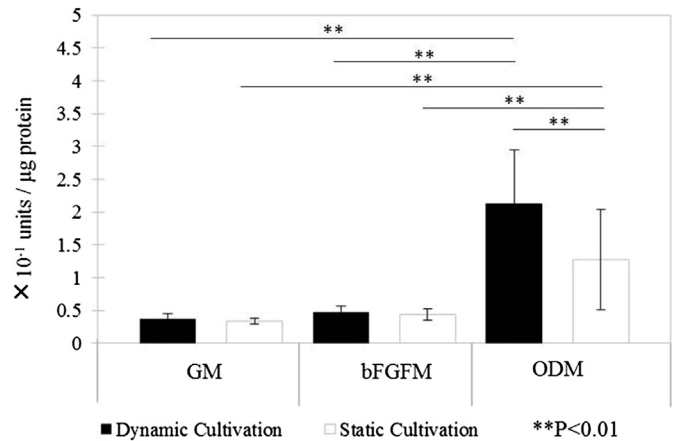


Fig. 7. Comparison of ALP activity among GM, bFGFM, and ODM under dynamic and static cultivation. Under dynamic cultivation, the mean of three areas was chosen for comparison with that under static cultivation. No significant difference was observed in ALP activity among each area of all culture media. (p > 0.05). Data are expressed as mean ± SD.

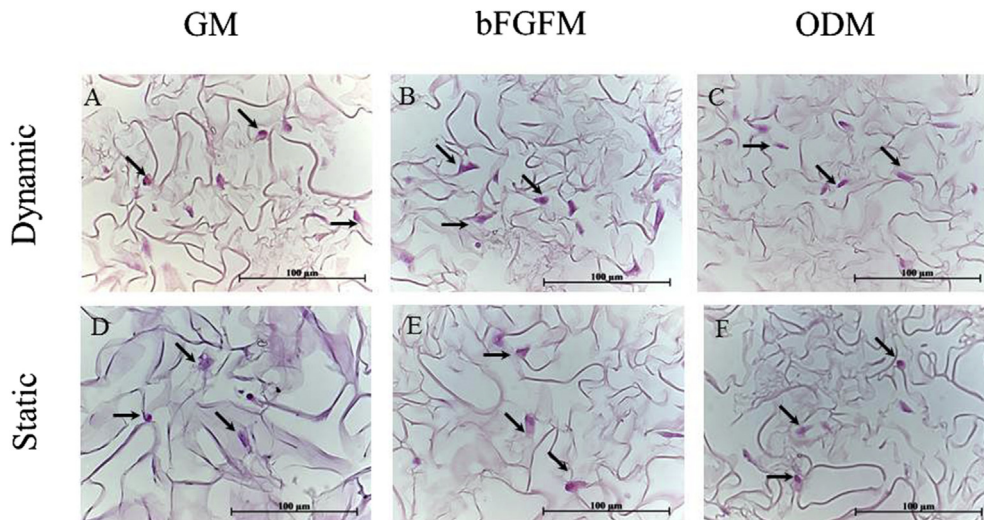
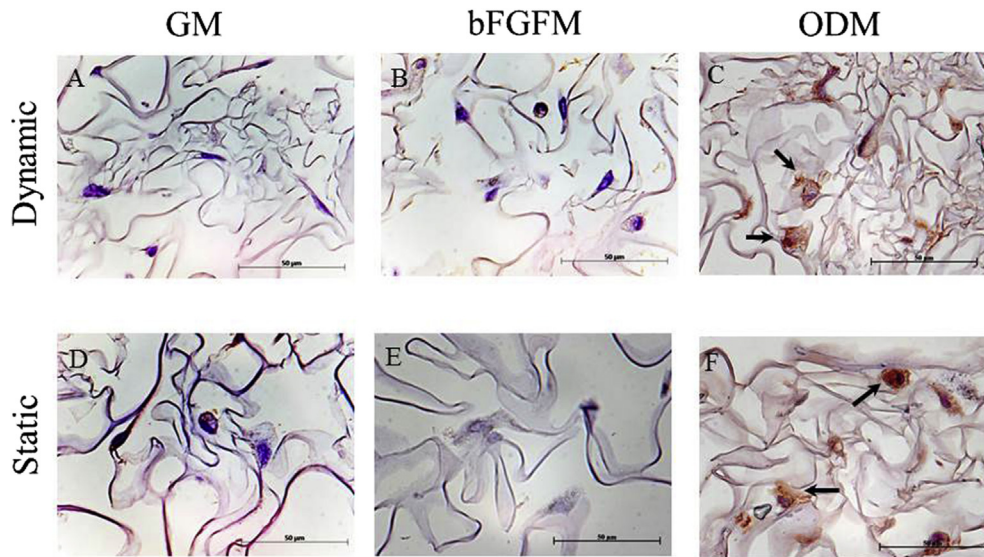
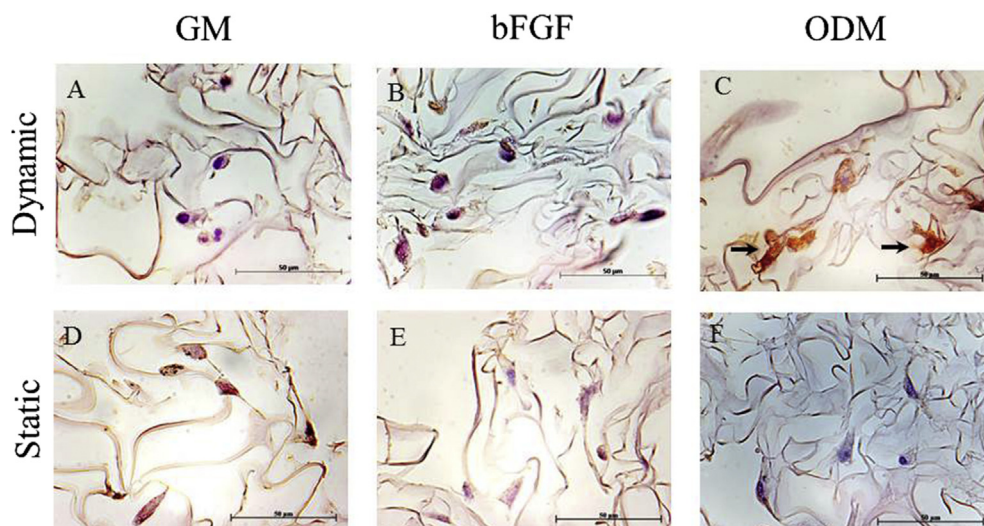


Fig. 5. Typical optical micrographs of specimens stained with hematoxylin–eosin (7 days). (A) GM dynamic cultivation, (B) bFGFM dynamic cultivation, (C) ODM dynamic cultivation, (D) GM static cultivation, (E) bFGFM static cultivation, (F) ODM static cultivation (Scale bar: 100 µm).



**Fig. 8.** Typical optical micrographs of specimens stained with BMP-2 antibodies. (A) GM dynamic cultivation, (B) bFGFM dynamic cultivation, (C) ODM dynamic cultivation, (D) GM static cultivation, (E) bFGFM static cultivation, (F) ODM static cultivation (Scale bar: 50 µm).



**Fig. 9.** Typical optical micrographs of specimens stained with osteopontin antibodies. (A) GM dynamic cultivation, (B) bFGFM dynamic cultivation, (C) ODM dynamic cultivation, (D) GM static cultivation, (E) bFGFM static cultivation, (F) ODM static cultivation (Scale bar: 50 µm).

when added the growth factor or osteogenic differentiation factor under dynamic cultivation.

bFGF was added to the culture medium for both dynamic and static cultivations. Noff et al. reported that bFGF promoted cell proliferation, while it had no effect on osteogenic differentiations [16]. Hori et al. reported that the combination of bFGF, ascorbic acid,  $\beta$ -glycerol phosphate, and dexamethasone promoted cell proliferation as well as osteogenic differentiation of rBMC [17]. In this study, we used bFGFM in order to verify that adding bFGF alone to a culture medium succeeds in the proliferation and osteogenic differentiation of rBMC in dynamic cultivation.

Only ascorbic acid and  $\beta$ -glycerol phosphate were added to the culture medium as osteogenic differentiation factors in this study. Holtorf et al. found that dynamic cultivation induced osteoblastic differentiation of marrow stromal cell-scaffold constructs in the absence of dexamethasone [10]. After three-dimensional culture for a long time (more than 1 week) in ODM including dexamethasone in an RFB culture system, mineralization was observed and

ODM could not be distributed evenly in the scaffold [18]. Accordingly, we used ODM without dexamethasone in order to prevent the mineralization that it caused.

In the present study, increased proliferation and high density of BMC was present in dynamic cultivation compared with static cultivation. In dynamic cultivation, essential nutrients, gas exchange, and removal of metabolites are all necessary for cell proliferation [14,19,20]. Moreover, 3D cell culture using a bioreactor prevents low-oxygen conditions and cell death [14,21]. This study is believed to have obtained the same rBMC' proliferation as the results of previous studies that used an RFB.

In this study, the number of cells cultured in different media were greater in the order of bFGFM > ODM > GM under dynamic cultivation. In the bioreactor culture system, osteogenic differentiation factors together with the mechanical stimuli produced by the bioreactor synergistically promoted rBMC' proliferation [10]. The interaction between bFGF and its receptor was reported to increase cell proliferation via activation of the MAPK/ERK pathway

[22]. Ascorbic acid also accelerated cell proliferation [23]. These actions were consistent with our results. After bFGF was added in the dynamic cultivation, the mechanical stimuli and bFGF synergistically promoted the greatest amount of cell proliferation.

In this study, ALP activity increased only in the ODM group, regardless of dynamic and static cultivations. Hanada et al. reported that Treatment with bFGF in the absence of dexamethasone results in no osteogenesis [24]. Audin et al. reported that the addition of dexamethasone in the primary culture of rBMC and the removal of dexamethasone at passage 2 did not compromise the expressions of ALP and osteopontin [25]. However, osteocalcin, the marker of mineralization was not detected.  $\beta$ -glycerol phosphate was reported to promote the differentiation of BMC into osteoblasts by up-regulating the expression both mRNA and protein of osteopontin [26]. In this study,  $\beta$ -glycerol phosphate in ODM is believed to promote the osteogenic differentiation of rBMC. On the other hand, only a basal medium or basal medium containing bFGF did not promote osteogenic differentiation of rBMC.

The present study showed that ALP activity was promoted in dynamic cultivation when rBMC were cultured in ODM. Expressions of BMP-2 and osteopontin were also detected in dynamic cultivation in ODM. Holtorf et al. reported that osteogenic differentiation factors (ascorbic acid,  $\beta$ -glycerol phosphate, and dexamethasone) combined with shear stress accelerated osteogenic differentiation and increased ALP activity [10]. Gomes et al. reported that when cells were cultured in ODM with dynamic cultivation, BMP-2 expression of rBMC was enhanced by the mechanical stimuli produced by the bioreactor [6]. Bancroft et al. also reported that osteopontin expression of rBMC was up-regulated by the shear stress when cells were cultured in ODM with dynamic cultivation [27]. These studies suggest that the addition of osteogenic differentiation factors and shear stress to the mechanical stimuli of an RFB promote the osteogenic differentiation of rBMC under dynamic cultivation.

Based on this study, cell proliferation and osteogenic differentiation are promoted in dynamic cultivation by adding bFGF and ODM, respectively. There is no consensus about which condition, cell proliferation or osteogenic differentiation, has a greater effect on bone regeneration. The results in this study suggested that the addition of bFGF is effective in the cases that focus on cell proliferation whereas ODM is effective in the cases that focus on osteogenic differentiation for in vivo transplantation scaffolds after dynamic cultivation with an RFB. Further studies are needed to confirm whether cell proliferation or differentiation of BMC is more significant for bone regeneration.

It is necessary to expand the primary cells in order to enhance the bone generation in large bone defects, because the primary cells harvested in bone marrow are extremely limited [28]. The results in this study showed that cell proliferation and osteogenic differentiation were promoted in dynamic cultivation by adding bFGF and ODM, respectively, indicating that the dynamic cultivation using RFB believe to be effective for bone generation in large bone defects.

## 5. Conclusion

The results of evaluating the cell expansion and osteogenic differentiation of rat bone marrow cells when added to growth factor or osteogenic differentiation factor under dynamic cultivation using an RFB support the following conclusions.

1. Dynamic cultivation promoted more cell proliferation than did static cultivation in all types of culture media. The addition of bFGF increased the cell proliferation of rBMC the most.

2. Dynamic cultivation accelerated the osteogenic differentiation of rBMC by adding osteogenic differentiation factors.

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