The Effect of Megakaryocytes and Platelets Derived from Human-Induced Pluripotent Stem Cells on Bone Formation

Masashi Sato[1)2)], Yasuhiro Shiga[1)], Naoya Takayama[2)], Masamitsu Sone[2)], Kentaro Kosaka[2)], Itsuro Motegi[3)], Norichika Mizuki[1)2)], Kazuhide Inage[1)], Yawara Eguchi[1)], Miyako Narita[1)], Sumihisa Orita[1)], Koji Eto[2)3)] and Seiji Ohtori[1)]

1) Department of Orthopaedic Surgery, Chiba University Graduate School of Medicine, Chiba, Japan
2) Department of Regenerative Medicine, Chiba University Graduate School of Medicine, Chiba, Japan
3) Department of Clinical Application, Center for iPS Cell Research and Application (CiRA), Kyoto University, Kyoto, Japan

Abstract:

Introduction: Platelet-rich plasma (PRP) is drawing attention as a substance that can promote bone formation. The growth factors present in PRP are stable for a long time after freeze-drying. However, the effects of PRP are inconsistent, and its effects on bone union in spinal surgery remain controversial. The immortalized megakaryocyte cell lines (imMKCLs) derived from human-induced pluripotent stem cells (hiPSCs) have been developed to produce numerous stable and clinically functional platelets. In this study, growth factors present in freeze-dried hiPSC-derived imMKCLs and platelets (iPS-MK/Plts) were evaluated, and their ability to promote bone formation was examined using a rat lumbar artificial bone grafting model.

Methods: We prepared freeze-dried iPS-MK/Plts and quantified their growth factors by enzyme-linked immunosorbent assays. Surgical grafting of artificial bone to the lumbar transverse processes was performed in 8-week-old female rats, which were divided into two groups: artificial bone graft (control) and artificial bone graft plus freeze-dried iPS-MK/Plts (iPS group). Transplantation was performed only on the left side. Eight weeks after the surgery, we captured computed tomography images and compared bilateral differences in the bone volume of the graft site in each rat. We also compared the left side/right side bone volume ratio between the two groups.

Results: The freeze-dried iPS-MK/Plts contained numerous growth factors. While there was no significant increase in bone volume on the transplanted side than that on the non-grafted side in the control group, bone volume significantly increased on the transplanted side in the iPS group, as evidenced by augmented mean left/right bone volume ratio of the iPS group compared with that of the control group. But the new bone observed in the iPS group was histologically normal.

Conclusions: Freeze-dried hiPSC-derived MKCLs and platelets contain several stable growth factors and have the potential for promoting new bone formation.

Keywords:
bone, platelet-rich plasma, freeze-dry, immortalized megakaryocyte cell lines, induced pluripotent stem cells, growth factors

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Introduction

Platelet-rich plasma (PRP), which is isolated by centrifuging human blood, contains numerous and varied growth factors; therefore, PRP has been used for tissue repair or regeneration in various clinical settings[1-4)]. A previous study reported that PRP combined with hydroxyapatite promoted bone formation in the spinal bone of rats[5)]. Another clinical study confirmed that the addition of PRP to bone grafts promoted bone formation during lumbar fusion[6)]. However, PRP cannot be stored for a long time due to the short shelf-life (only several days) of the growth factors present in it[7)]. As a solution to this issue, novel method, freeze-dried (FD)-PRP, has been developed[8,9)]. The major cytokines present in FD-

Corresponding author: Masashi Sato, a050022jp@gmail.com
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PRP were stable after at least 1 month at room temperature, and FD-PRP promoted osteogenesis in rats as much as fresh PRP[10,11].

Some studies have reported positive results after using PRP to promote bone union in spinal surgery[12,13], while others have reported negative results[14,15]. Thus, the effect of PRP on bone union remains controversial. One of the reasons why the effects of PRP are inconsistent is the variability and heterogeneity in the growth factor content. It has been reported that the concentration of growth factors present in PRP varies depending on the characteristics of an individual, particularly age[16]. PRP is autologously prepared from the patient's own blood to avoid transfusion reactions; thus, its effects may vary depending on the characteristics of the patient including age. To ensure a positive effect of PRP, platelets that contain consistent growth factors and can be used universally without any side effects are required. Therefore, we focused on the usefulness of artificial platelets derived from induced pluripotent stem cells (iPSCs).

The group of Kyoto University has recently demonstrated the feasibility of proliferative human megakaryocyte cell lines, immortalized megakaryocyte cell lines (imMKCLs)[17], and the derived platelets, iPS-platelets, for clinical application as an alternative transfusion product[18]. This newly developed technology enabled to prepare 100 billion order ($>10^{11}$) iPS-platelets consistently[19]. In addition, these platelets can express the required cytokines by genetic recombination of imMKCLs. If these platelets can promote bone formation, it would be possible to develop growth factor preparations with a stable effect on bone formation that do not depend on the characteristics of the recipient. This study examined the effect of human iPSC (hiPSC)-derived imMKCLs and platelets in promoting bone formation using a lumbar artificial bone grafting model in rats.

Materials and Methods

Experimental animals

Female Sprague-Dawley rats weighing 250-300 g were used in this study. This research was approved by the ethics committees of the authors' affiliated institution, and all animal protocols followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (1996 revision). The protocols for animal experiments were as per a previous study[20].

Cell culture and reagents

imMKCL (MKCL line 7)[17] was cultivated in Iscove's modified Dulbecco medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with a cocktail of 10 μg/mL human insulin, 5.5 μg/mL human transferrin, and 5 ng/mL sodium selenite (41400-045, Thermo Fisher Scientific, Waltham, MA, USA), a cocktail of 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (10378-016, Thermo Fisher Scientific), 0.45 mM 1-thioglycerol (M6145, Sigma-Aldrich), 50 μg/mL L-ascorbic acid (A4544, Sigma-Aldrich), and 15% highly filtered fetal bovine serum (10099-141, Thermo Fisher Scientific) in the presence of 50 ng/mL human stem cell factor (SCF; 193-15513; Wako, Tokyo, Japan), 400 ng/mL TA-316 (human thrombopoietin mimetic small compound, in-house synthesized)[19], and 100 μg/mL doxycycline (Dox) (Clontech, Mountain View, CA, USA) to maintain overexpression of c-MYC, BMI1, and BCL-XL[17].

FD imMKCLs and platelets prepared from hiPSCs

Dox-off culturing, to silence exogenous c-MYC, BMI1, and BCL-XL for platelet production from MKCLs, was performed using 1×10^5 cells/mL for 6 days in 125 mL Erlenmeyer cell culture flasks (#431143, Sigma-Aldrich) under shaking conditions at 37°C and 5% CO_2 in a Lab-Therm shaker (Kuhner, Basel, Switzerland) (Fig. 1a), which turbulent energy and shear stress promoted healthy platelet biogenesis[19]. Ten flasks, each containing 25 mL of medium, were used for culturing. Dox-off culture medium was prepared from Iscove's modified Dulbecco's medium supplemented with 5% human plasma (Japan Blood Products Organization, Tokyo, Japan), 1 IU/mL enoxaparin (Kaken Pharmaceutical, Tokyo, Japan), and 5 IU/mL urokinase (Mochida Pharmaceutical, Tokyo, Japan) as an anticoagulant. In addition, 50 ng/mL SCF, 400 ng/mL TA-316, 15 μM KP-457 (Kaken Pharmaceutical) for inhibitor of GPIbα shedding on platelets[21], 0.75 μM SR-1 (aryl hydrocarbon receptor antagonist, NARD Institute, Hyogo, Japan), and 10 μM Y27632(Rho-associated protein kinase inhibitor, Wako Pure Chemicals, Osaka, Japan) were added. SR-1 and Y27632 were used for imMKCL maturation and platelet biogenesis even in a liquid culture[19]. After 6 days of incubation, the culture media in all flasks were combined, and the mixed products of imMKCLs and platelets (iPS-MK/Plts) were collected by centrifugation at 170 G for 15 min. The cell pellet was suspended in Tyrode buffer (10% of the volume of the culture medium). Immunofluorescent staining of CD41 and CD42b was performed on a portion of the collected cells to confirm their maturation (Fig. 2a). FTIC anti-human CD41 antibody (BioLegend, San Diego, CA, USA), PE anti-human CD42b antibody (BioLegend), and DRAQ5 (BioStatus, Leicestershire, UK) were added in 100 μL of dox-off medium cell suspension in a 96-well plate. After standing at 37°C for 1 h, cell images were captured with an imaging cytometer (CQ1, Yokogawa Electric, Tokyo, Japan). Flow cytometry measurement was performed to determine the ratio of platelets to megakaryocytes. The cells labeled with APC anti-human CD41 antibody (#303709, BioLegend) and PE anti-human CD42b antibody (#55473, Becton, Dickinson & Co., Franklin Lakes, NJ, USA) were mixed with CountBright™ Absolute Counting Beads (C36950, Thermo Fisher Scientific) and counted with flow cytometer (FACSCanto II, Becton, Dickinson & Co.) (Fig. 2b, c). The iPS-MK/Plts were activated by adding 0.1% $CaCl_2$ and incubating at 37°C for 1 h. The flask was frozen at −60°C overnight and

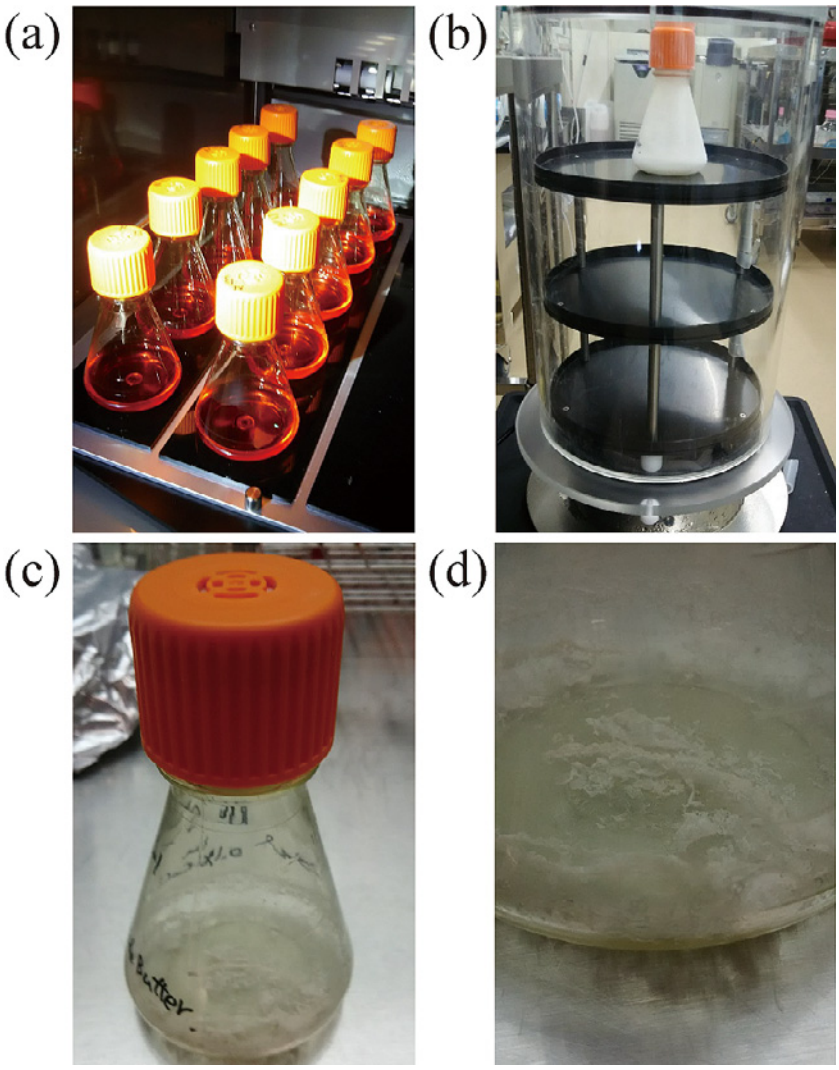

Figure 1. a. Cell culture in a shaker. b. Freeze-drying the flask in a lyophilizer after 6 days of doxycycline-off culturing and platelet activation. c. After freeze-drying. d. Closer view of c.

then lyophilized (FDU-1200, EYELA, Bohemia, NY, USA) overnight (Fig. 1b) to convert the contents of the flask to a powder (Fig. 1c, d).

Growth factor evaluation

The expression levels of growth factors, including transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF)-BB, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF), were quantified by enzyme-linked immunosorbent assay kits (Quantikine®, R&D Systems). These cytokines are tissue repair-associated growth factors present in PRP. FD-iPS-MK/Plts derived from 25 mL culture were resolved in 1 mL of distilled water and used in the cytokine assay. Three samples were measured for each cytokine. All assays were conducted according to the manufacturer's instruction. All growth factor levels were analyzed immediately after freeze-drying.

Lumbar posterolateral artificial bone grafting model

Lumbar fusion surgery was performed on 8-week-old female Sprague-Dawley rats. Two groups with six rats were created based on the graft: artificial bone graft (control group) and artificial bone graft plus FD iPS-MK/Plts (iPS group). A hydroxyapatite-collagen composite, Refit® (Hoya, Tokyo, Japan), was employed as the artificial bone graft. We used 0.5 mL of the artificial bone for each rat. In the iPS group, FD iPS-MK/Plts derived from 25 mL culture were dissolved in 1 mL sterile distilled water immediately before transplantation and mixed with 0.5 mL of the artificial bone. The surgical protocols employed in this study were adopted from a previous study[11]. Briefly, under general anesthesia by intraperitoneal injection of anesthetics [mixed with 0.15 mg/kg medetomidine hydrochloride (Nippon Zenyaku Kogyo, Fukushima, Japan), 2 mg/kg midazolam (Maruishi Pharmaceutical, Osaka, Japan), and 2.5 mg/kg butorphanol tartrate (Meiji Seika Pharma, Tokyo, Japan)], a midline skin incision of 3-4 cm was made on the back of the rat, and then an incision was made on the left paramedian fascia. This was followed by blunt dissection to expose the left lamina and the transverse processes of L4-L6. Corticotomy was not per-

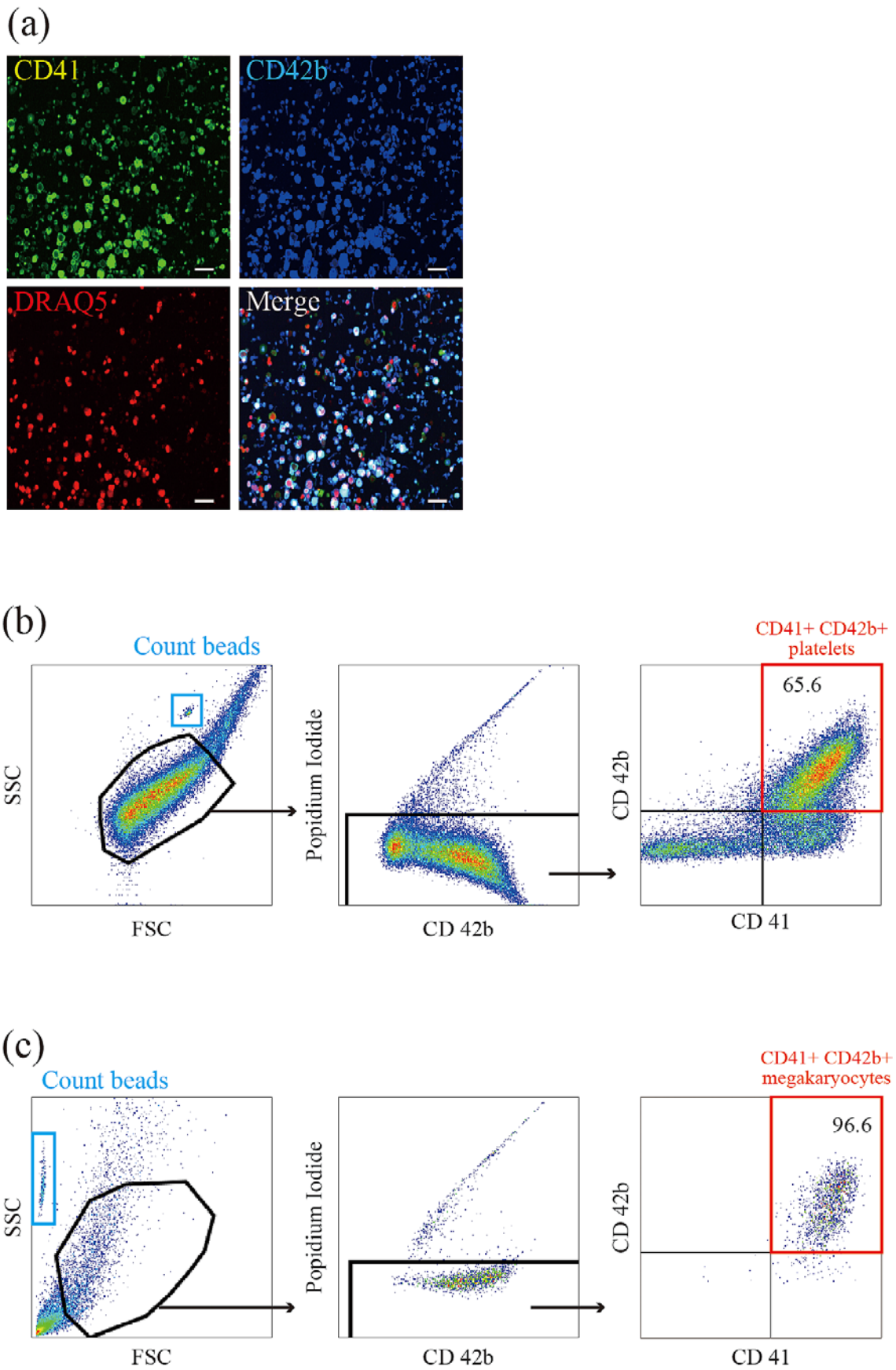

Figure 2. a. Immunofluorescence staining of megakaryocyte cell lines (MKCLs) after 6 days of doxycycline-off culturing. There are more CD41- and CD42b-positive cells than DRAQ5-positive cells. This demonstrates the maturation of MKCLs into platelets. Scale bars, 50 μm. b, c. Flow cytometry of iPS cell-derived platelets (b) and megakaryocytes (c) after 6 days of doxycycline-off culture.

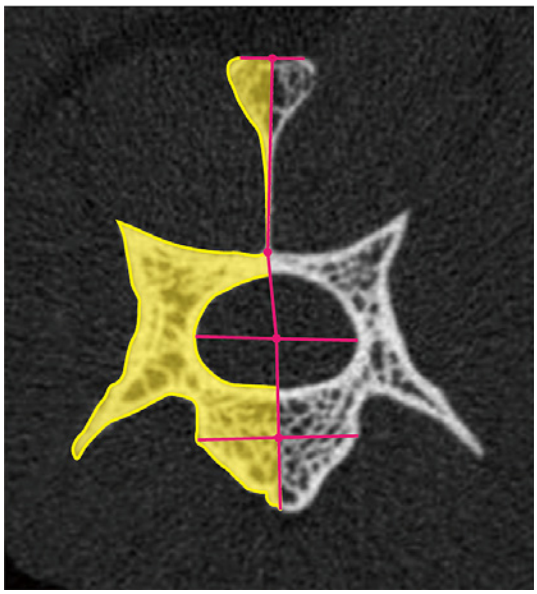

Figure 3. Range of bone volume measurement on CT images. Straight lines were connected between the midpoint of the upper edge and base of the spinous process, between the base of spinous process and the midpoint of the maximum lateral diameter of the spinal canal, and between the midpoint of the maximum lateral diameter and width of the vertebral body. By extending the straight line connecting the midpoint of the maximum diameter of the spinal canal and the midpoint of the vertebral body width, the vertebra was divided into left and right. The same contours were drawn on each side of all slices.

formed in any group. The artificial bone was filled on the dorsal side of the left transverse processes, and the fascia and skin were sutured.

Evaluation of bone augmentation

All rats were euthanized 8 weeks after the operation. The spine, including the transplanted site, was harvested together with the surrounding muscular tissue. After immersing them in a 10% neutral buffered formaldehyde solution for more than 24 h, computed tomography (CT) images of vertebral bodies of L4, L5, and L6 were obtained by horizontal cross-sectional scanning using a Latheta experimental animal X-ray system (LCT-200, Aloka, Tokyo, Japan). The scan range was set from the upper to the lower end plate, and the number of slices was set to 150 per vertebral body. Quantitative assessment was performed using Latheta software (version 3.2, Aloka). Straight lines were drawn on vertebrae on each slice and divided in half, and the left and right bone areas were set as separate regions of interest (Fig. 3). The bone volume was calculated by combining the regions of interest of each slice. There is no objective evidence in the literature that supports this drawing method because we devised it for this study. Thus, we confirmed beforehand that there was no significant difference in the bone volume between the left and right sides of the vertebral bodies that had not undergone artificial bone transplantation.

Table 1. Concentrations of Major Cytokines in Freeze-dried Human iPS Cell-derived Megakaryocytes and Platelets.

Cytokine	Concentration (pg/mL)
TGF-β1	31,093
PDGF-BB	1,982
VEGF	ND
EGF	686
BMP-2	974
BMP-4	831
BMP-7	ND

Each cytokine was measured by dissolving in distilled water.
TGF-β1: transforming growth factor-β1
PDGF-BB: platelet-derived growth factor-BB
VEGF: vascular endothelial growth factor
EGF: epidermal growth factor
BMP: bone morphogenetic protein
ND: not detected

Histological examination

After CT scanning, the spinal bone was decalcified. Then, paraffin sections were prepared and sliced to a 2 μm thickness. Hematoxylin and eosin staining (9130 and 9134, Sakura Finetek, Tokyo, Japan) was performed on each slice.

Statistical analyses

Student's t-test was used to compare volumes of the transverse processes of the left (the transplanted side) and right side (the non-transplanted side) in each group. The ratio of the bone volume on the left to right side was calculated for each vertebra. The t-test was also used to compare the mean volume ratios of the left to right sides between the two groups. The significance level was set at 5%. All statistical analyses were performed with EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria)[22]; it is a modified version of R Commander designed to add statistical functions frequently used in biostatistics.

Results

Growth factor analyses

Mixed products of iPSC-derived megakaryocyte (imMKCLs) and iPS-platelets (iPS-MK/Plts) were generated and quantified by flow cytometer. The average concentration of platelets was $2.83\times10^{6}\pm1.32\times10^{5}$ cells/mL, and that of megakaryocytes was $1.87\times10^{5}\pm1.34\times10^{4}$ cells/mL (n=4). In other words, iPS-MK/Plts were composed of 6.2% imMKCL and 93.8% iPS-platelet. Table 1 shows the quantitative analyses of the growth factors present in the FD iPS-MK/Plts. The expression of TGF-β1, PDGF-BB, EGF,

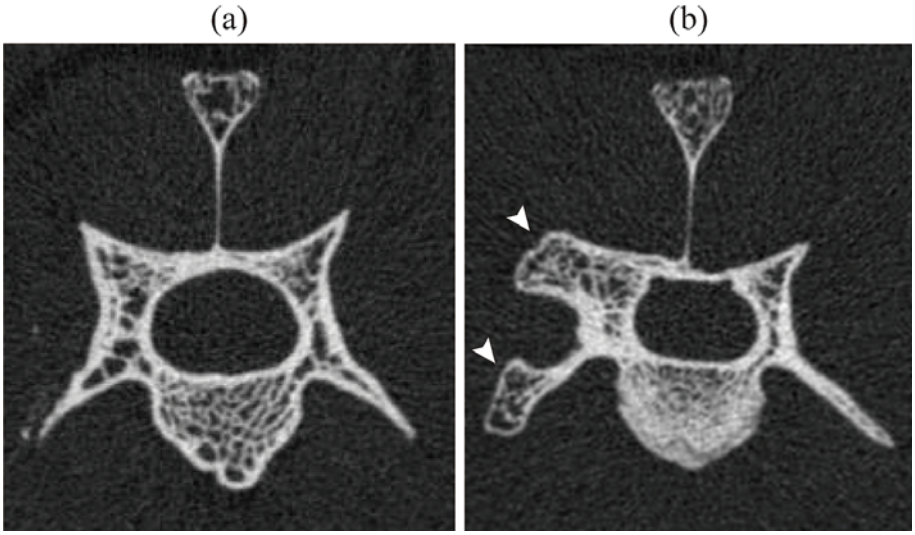

Figure 4. Bone images 8 weeks after surgery (L4). a. Control group. There is little bone formation on the grafting site. b. iPS group. Obvious bone formation (arrow head) is present on the lamina and transverse processes on the grafting site.

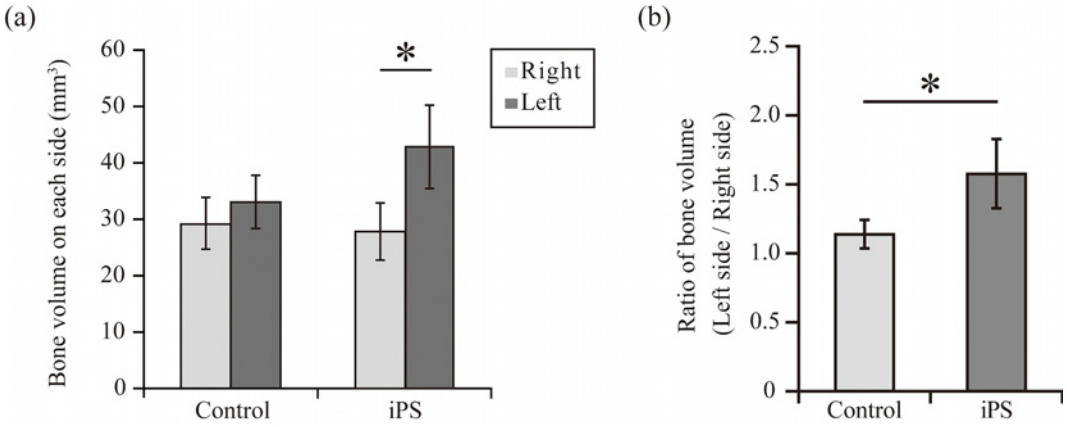

Figure 5. a. Bone volumes of lamina and transverse processes on the right and left sides. b. Bone volume left side/right side ratios. Values are presented as means±standard deviation. (*p<0.05, n=3 for both a and b).

BMP-2, and BMP-4 was observed in the FD iPS-MK/Plts, but VEGF and BMP-7 were not detected (below the lower limit).

CT images

Eight weeks after surgery, only a small amount of new bone formation was observed on the transverse processes of the left side in the control group than that on the right side (Fig. 4a). In the iPS group, new bone formation was observed in the transverse processes of the left side (Fig. 4b).

Comparison of bone formation

In the CT image analysis, there was no significant difference between the right and left mean bone volumes of the transverse processes in the control group, while the left mean bone volume was significantly larger than that of the right in the iPS group (Fig. 5a). The mean ratios of the left and right volumes in the iPS group were significantly larger than in the control group (Fig. 5b).

Histological evaluation

Hematoxylin and eosin staining was performed on the grafting site bone tissue obtained from each group (Fig. 6). In the control group, although slight ossification was observed around the transverse processes, there was little bone remodeling at the grafting site. On the other hand, obvious bone remodeling was observed in the transplanted site surrounding the artificial bone in the iPS group. The trabeculae of the new bone in the iPS group were dense and thick and were determined as normal bone tissue of good quality.

Discussion

In this study, iPS-MK/Plts were generated ex vivo, allow-

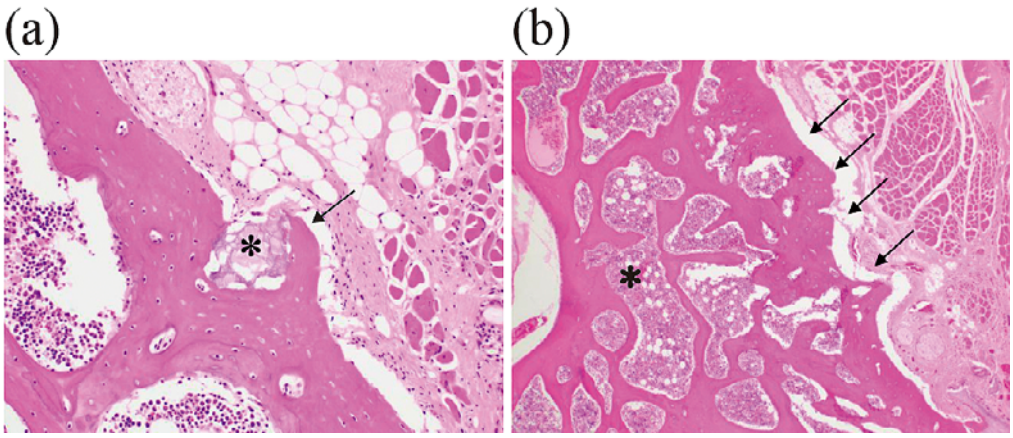

Figure 6. Histological images around the grafted artificial bone. a. Control group. Limited bone remodeling (arrow) is present around the artificial bone (*). b. iPS group. Active bone formation (arrows) is present around the artificial bone (*).

ing to the preparation of FD packaging as an alternative PRP source. We confirmed that various major cytokines known in native PRP were also present in this FD packaging powder derived from iPS-MK/Plts. These results suggest that hiPSC-derived new PRP alternative may exert comparable actions as donor-derived PRP in tissue repair and regeneration. It has been confirmed that the growth factors of FD-PRP are stable for a long period[10]. In this study, growth factors were evaluated immediately after freeze-drying; thus, it remains unknown whether growth factors of FD iPS-MK/Plts remain stable following long-term storage.

We then evaluated the effect and influence of the FD iPS-MK/Plts on bone formation using a lumbar artificial bone grafting model in rats. These FD iPS-MK/Plts, when combined with artificial bone, significantly accelerated new bone generation. In addition, the new trabeculae formed around the artificial bone were dense and thick and were histologically identified as normal ossific tissue. This bone remodeling was not facilitated in the control group. These results indicated that iPS-MK/Plts contribute to tissue regeneration effects. However, although bony bridging forming was observed between transverse processes in the animal model we adopted[11], in this study, we could confirm only new bone formation at the grafting site, but not bony bridging forming between transverse. One explanation of this is that the cross-reactivity between some human cytokines and rat tissues is low. The other reason might be the difference of cytokine concentration between the present study and our previous study. In this study, 1 mL of distilled water solution of FD iPS-MK/Plts derived from 25 mL culture was used for one rat. Based on the data in Table 1, we can speculate that it might include 31,093 pg/ml TGF-β, 1,982 pg/ml PDGF-BB, and 686 pg/ml EGF. On the other hand, in the previous PLF model, the amount of cytokines contained in the rat PRP used for one rat was 271,300 pg/mL of TGF-β and 23,100 pg/mL of PDGF-BB[11]. Therefore, the amount of FD preparation used in the present study is sufficient for the bone formation but not for inter-transverse processes bridging. At this point, we were not able to produce a large amount of FD formulation due to the limited capacity of the device to make FD. However, imMKCL can be easily expanded in vitro. We can increase the culture scale and prepare iPS-MK/Plts including optimal concentration of cytokines in the future.

Some of the regulatory factors of osteoblast differentiation such as BMP-2 and BMP-4 belong to the bone morphogenetic protein (BMP) family[23-26]. Recombinant human BMP-2 (rhBMP-2) is an osteoinductive growth factor approved by the US Food and Drug Administration for clinical use. However, many adverse effects have been reported after direct administration of rhBMP-2, such as ectopic bone formation, osteoclast-mediated bone resorption, and inappropriate adipogenesis[27]. The causes of these harmful effects and influences have not been elucidated to date. However, we hypothesize that these adverse effects may be caused by the hyperphysiological application of a single growth factor, whereas the interaction of various growth factors is important for promoting physiological osteogenesis. In our study, there were no notable adverse events observed during the 8-week observation period, and newly formed bone was considered to be normal tissue histologically. These results suggest that FD iPS-MK/Plts may be used safely to promote ossification physiologically. At present, there is a lack of knowledge about the optimal combination, concentration, and ratio of growth factors for both safety and efficacy even in PRP. However, the fact that imMKCL can be genetically engineered may allow us to validate optimal concentration and combination of growth factor in the future by forcibly expressing growth factors. Moreover, HLA class I-deficient imMKCLs and derived iPS-Plts will be applicable as universal FD device for avoiding immune rejection in the future[28].

On the other hand, this study has several limitations. First, the observation period was only 8 weeks; thus, the long-term safety of iPS-MK/Plts should be confirmed in the future. Second, the compatibility between human platelets and rats was not considered. Further studies are required to

confirm the safety and universality of iPS-MK/Plts.

In conclusion, this study demonstrated that the FD iPS-MK/Plts contain various growth factors. In addition, their ability to promote bone formation was observed in a rat lumbar artificial bone grafting model. The newly formed bone was histologically estimated as normal tissue. iPS-MK/Plts should contain the potential to act as a growth factor formulation, which promotes physiological new bone formation safely and stably.

Disclaimer: S. Orita is one of the Editors of Spine Surgery and Related Research and on the journal's Editorial Committee. He was not involved in the editorial evaluation or decision to accept this article for publication at all.

Conflicts of Interest: YS, KE, and S. Ohtori have applied for patents related to this manuscript. KE is a founder of Megakaryon and a member of its scientific advisory board without salary. This work was supported in part by grants from KYOTO SEISAKUSHO.

Ethical Approval: None. This study was not included any clinical trials.

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