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ORIGINAL RESEARCH

Human Dental Pulp Facilitates Bone Regeneration in a Rat Bone Defect Model

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Abstract: The objective of this work was to investigate the osteogenetic ability of the human dental pulp stem cells (hDPSCs) derived from human third molars. We induced alkaline phosphatase (ALP) activity and bone morphogenetic protein 2 (BMP-2) mRNA expression, the markers for bone formation, in hDPSCs by using osteoinductive factors. The implantation of hDPSCs with collagen sponge promoted osteogenesis and fracture healing in the femur of an immunocompromised rat, which was a bone defect model for pseudoarthrosis. Histological analyses revealed that after implantation of the hDPSCs, the size and number of osteoblasts and the rates of osteoid production and mineralization increased to an appreciable extent, whereas the rate of bone resorption decreased. We believe that hDPSC implantation is a simple and safe procedure that can be beneficial in bone regeneration therapy in clinical practice.

Keywords: regeneration, dental pulp, stem cell, bone defect, osteoblast

Introduction

Stem cells are cells that have the ability to self-renew and differentiate into various tissues, including nerves, adipose, cartilage, and bone. Human dental pulp stem cells (hDPSCs) were shown to self-renew and differentiate into multiple lineages in vivo by Gronthos et al in 2000.[1] These cells were found to form dentin-like tissue and to differentiate into osteoblast-like cells that formed bone in vitro.[2] In the presence of specific stimuli, these hDPSCs differentiated into several cell types, including neurons, adipocytes, and chondrocytes.[3] Interestingly, vascular endothelial cells and hDPSCs were found to synergistically differentiate into osteoblasts and endothelial cells, respectively.[4]

In the case of a bone injury or bone nonunion, stem cells can remodel the hard tissue by differentiating into bone cells. Bone marrow grafting is a technique that is commonly used for intractable bone healing. Another approach is the substitution of bony tissue by permanent implants made of titanium alloy, ceramics, polymers, and other composite materials to repair the defects and to restore both physical and physiological functions of the bone.

Thus, we hypothesized that hDPSCs exposed to the appropriate environmental signals would differentiate into functionally active osteoblasts. Additionally, we demonstrated that collagen sponge and fibrin glue are useful scaffolds that are adequate for filling any gaps. We used fibrin glue and collagen sponge as delivery vehicles and implanted thawed hDPSCs into the femur of an immunocompromised rat. We studied the ability of the hDPSCs to differentiate into osteoblasts in the presence of inducing agents. Our aim was to investigate hDPSCs, which are easy to obtain and can be stored for long periods for potential application in clinical regeneration therapy.

Materials and Methods
Culture and differentiation of human dental pulp stem cell series (DP series)

Dental pulp stem cell series were extracted from human third molars in accordance with the guidelines approved by the institutional review board of Gifu University Medical Hospital. All dental pulp series numbers (DP series) 1, 11, 27, 29, and 100 were isolated as previously described by Takeda et al.[5] We used the DP100 cell for transplantation, and DP series numbers 1, 11, 27, and 29 were used in vitro study. DP100 were originally isolated from a 14-year-old individual followed as previously described by our report.[5] Single-cell suspensions of 1×10^5 dental pulp per well were seeded into 6-well plates containing alpha modification of Eagle's medium (Gibco-BRL Life Technologies Co., Carlsbad, CA), followed by incubation at 37 °C in 5% CO_2. When DP100 reached subconfluence, cells were frozen in liquid nitrogen for use as first-passage cells. Enhanced green fluorescent protein (EGFP) was transduced using murine stem cell virus (MSCV)-typed retroviral vectors, which allow high transduction efficiency and continued expression of the transgene in DP100 in order to determine their viability in vitro (not for implantation in this study).[6] Frozen DP100 were thawed at room temperature, followed by culturing using a Mesenchymal Stem Cell Basal Medium (MSCBM) (Lonza Group Ltd., Basel, Switzerland) kit supplemented with 0.2 mg/mL gentamicin sulfate (Gibco-BRL Life Technologies Co., Carlsbad, CA) at 37 °C in 5% CO_2. To induce differentiation into osteoblasts/odontoblasts, DP100 were cultured in MSCBM medium supplemented with 0.1 μM dexamethasone (Wako Chemical Co., Osaka, Japan), 50 μg/mL ascorbic acid (Wako Chemical Co., Osaka, Japan) and 0.1% β-glycerophosphate (Sigma-Aldrich Co., St. Louis, MO) and were plated at 1.0×10^5 cells/well in 6-cm dishes (Nunc, Roskilde, Denmark) from passage number two.[7] Cells were embedded in Cellmatrix® (Nitta Gelatin Inc., Osaka, Japan) consisting of fibrin glue for three-dimensional cultures. After 7 days, cells were centrifuged and embedded in a type I collagen sponge (Nitta Gelatin Inc. Osaka, Japan) for transplantation as passage number three.[8]

Measurement of alkaline phosphatase activity

Quantitative alkaline phosphatase activity was determined by comparison between cells harvested in supplemented and nonsupplemented MSCBM medium (as control) culture. DP100 were plated at a density of 1.0×10^4 cells in 6-well plates containing MSCBM absorbed into collagen sponges. Cells were pulverized in an Astrason ultrasonic processor (Misonix Inc., Farmingdale, NY) containing 10 mM Tris-HCl buffer solution, and alkaline phosphatase activity was measured according to the instructions

for the LabAssay™ ALP kit (Wako Chemical Co., Osaka, Japan) using ρ-nitrophenylphosphate as the substrate. One unit of enzyme activity was defined as the release of 1 unit of ρ-nitrophenol per minute and was quantified by monitoring the absorbance at 405 nm using a micro-plate reader (Model 680; Bio-Rad Laboratories, Inc., Hercules, CA).[9,10]

Semiquantitative RT-PCR

Total RNA was isolated from DP1, DP11, DP27, and DP29 by using a Nano Drop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Aliquots of 5 µg of total RNA were reverse transcribed using the SuperScript™ First-Strand Synthesis system for RT-PCR (Gibco-BRL Life Technologies Co., Carlsbad, CA). Primers 5′-*CAGAGACCCACCCCCAGCA*-3′ (forward) and 5′-*CTGTTTGTGTTTGGCTTGAC*-3′ (reverse) were used to amplify the BMP-2 sequence, the primers 5′-*ACTGGCTCTTTTAGGCACTGACTC*-3′ (forward) and 5′-*TGTTCCCTTGTAGCTGCGTCCTT*-3′ (reverse) were used to amplify the hepatocellular growth factor (HGF) sequence, the primers 5′-*ACTCCCCCTGAAAACCAAAGCC*-3′ (forward) and 5′-*GGCTTACACTTCGGGCACTTAC*-3′ (reverse) were used to amplify the hepatocyte growth factor receptor (c-MET) sequence, and the primers 5′-*GAAGGTGAAGGTCGGAGTC*-3′ (forward) and 5′-*CAAAGTTGTCATGGATGACC*-3′ (reverse) were used to amplify the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence. Amplification consisted of enzyme activation at 96 °C for 3 minutes, followed by amplification for 32 cycles of 96 °C for 45 seconds, 57 °C for 45 seconds, and 72 °C for 60 seconds by using iCycler thermalcycler (Bio-Rad Laboratories, Inc., Hercules, CA). After resumption the samples, the reaction were analyzed by 1% agarose gel electrophoresis with Mupid® submarine electrophoresis system (Advance Co., Ltd., Tokyo, Japan) for 30 minutes.

Bone defect model and surgical implantation of scaffold

Immunocompromised male F344/NJcl-mu/mu rats, weighing 350 to 450 g, were purchased from CLEA Japan, Inc. (Tokyo, Japan), and were acclimated to laboratory conditions for one week prior to experiments. All procedures used in animal experiments were approved by the Institutional Animal Care and Use Committee of Gifu University School of Medicine. All animals underwent surgery under sterile conditions, with anesthesia induced by diethyl ether and intraperitoneal injection of pentobarbital (40–50 mg/kg body weight). An incision was made over the thigh from the lateral knee joint extending proximally through the greater trochanter, and the muscle septum between the quadriceps and hamstring muscles was gently divided to approach the shaft of the femur. The skin flap was pierced with two stainless steel K-wires, each 1.5 mm in diameter. The wires were crossed over the central axis near the condylar groove and greater trochanter of the bone and were extended until they reached the outer surface of the other side of the bone fragments. Then, cross bridge fixation was made between the pins with epoxy resin (Cemedine Co., Ltd., Tokyo, Japan). A bone defect model was applied using a modification of the method described by Matsumoto et al in 2006.[11] Segmental osteotomy was performed in the center of the pins using a hard metal drill bar 1.5 mm in diameter to cut the bone to 1.5 mm in width. The femoral periosteum was cauterized circumferentially for 2 mm on each side of bone defect by-end. Animals were divided into three groups of five animals: a control group with no implant in the gap; a group with a collagen sponge scaffold implanted in the gap; and a group with a collagen sponge scaffold containing DP100 implanted in the gap.

Radiographic examination

Femurs were scanned using an X-ray machine (43855D; Faxitron X-Ray LLC, Lincolnshire, IL) at 17 kV/14 seconds (3 mA) on days 7, 28, and 56 after surgery in five specimens from each of the three groups, and the healing process at the damaged site was monitored. Mineralization of the fracture site was defined as callus formation and was evaluated by two independent observers using three categories: (1) complete bridging (four cortices bridged); (2) incomplete bridging (one to three cortices bridged); or (3) no bridging (no cortex bridging). Interobserver variability was determined using the κ (kappa) statistic.

Induction of calcein

Calcein was given to the rats via interperitoneal injection in 20 mg/kg to label mineralizing bone. Calcein marker injections were given at 6 and 2 days before being sacrificed as previously described.[12]

Evaluation of bone histomorphology

After the observation period, specimen femurs were fixed in 70% ethanol. Sections were stained with Villanueva bone stain for 6 days, dehydrated in an increasing ethanol series, defatted in an acetone and methyl-methacrylate monomer mixture, and embedded in methyl-methacrylate without decalcification. Sclerotic cross sections (~500 μm) were cut with a bone saw, followed by grinding to a thickness of 15 to 20 μm. All procedures were performed at the Ito Bone Histomorphometry Institution (Niigata, Japan). Quantitative histological analysis of bone formation and resorption in the interflagmentary gap was determined based on total volume of tissue removed (TV), osteoblast surface (Ob.S), osteoid surface (OS), osteoclast surface (Oc.S), and the eroded surface (ES) to bone surface (BS) ratio. These individual parameters were used to evaluate the Ob.S/BS and OS/BS parameters, which are regarded as markers related to bone formation, while Oc.S/BS and ES/BS are related to bone resorption, as calculated using a semi-automatic image analyzing system with bone morphometric instrument software (System Supply Co., Ltd., Nagano, Japan). For each parameter, standard bone histomorphometrical nomenclature, symbols, and units were used as described by the American Society for Bone and Mineral Research (ASBMR) Histomorphometry Nomenclature Committee.[13]

Statistical analysis

Interobserver variability for radiological evaluation was assessed using the κ statistic. Strength of agreement was assessed based on the κ value as follows: 0.81 to 1.00, almost perfect; 0.61 to 0.80, substantial; 0.41 to 0.60, moderate; 0.21 to 0.40, fair; 0 to 0.20, slight, and less than 0, poor.[14] Comparison of bone morphometric data from the three groups was performed using one-way analysis of variance (ANOVA) for independent samples. Tukey's honestly significant difference (HSD) was used to investigate the relationships for multiple combinations in all groups. Significant differences were set at the 95% confidence level. All statistical analyses were performed using GraphPad PRISM® Version 5.01 (GraphPad Software, La Jolla, CA).

Results

At the third passage (P3), DP100 were initially plated at 1.0×10^4 cells/10 cm well in medium in order to induce osteoblastic/odontoblastic differentiation after EGFP transduction. We compared cell viability in the normal medium and the fibrin glue-containing medium. One week later, cell viability in the fibrin glue-containing medium was approximately 70% of that in the normal medium. The proliferative rate of cells in both media was found to be similar. Spindle cells appeared to overlap in fibrin glue at 7 days after EGFP transduction (Fig. 1). Levels of ALP activity increased gradually over time in both the control and supplemented group, but decreased significantly ($P < 0.05$) at 21 days (P6) after induction (Fig. 2). We found that representative sample DP1 expressed BMP-2 m-RNA the that confirmed the levels of BMP-2 that extracted from DP 1, 11, 27, and 34 were upregulated at early stages (at P4, P5), and that levels of BMP-2 decreased with increasing passage number (Fig. 3). X-ray images were taken at 28 days and 56 days after surgery in order to determine the extent of fracture healing in each group. Representative X-ray images from each group are shown in Figure 1.
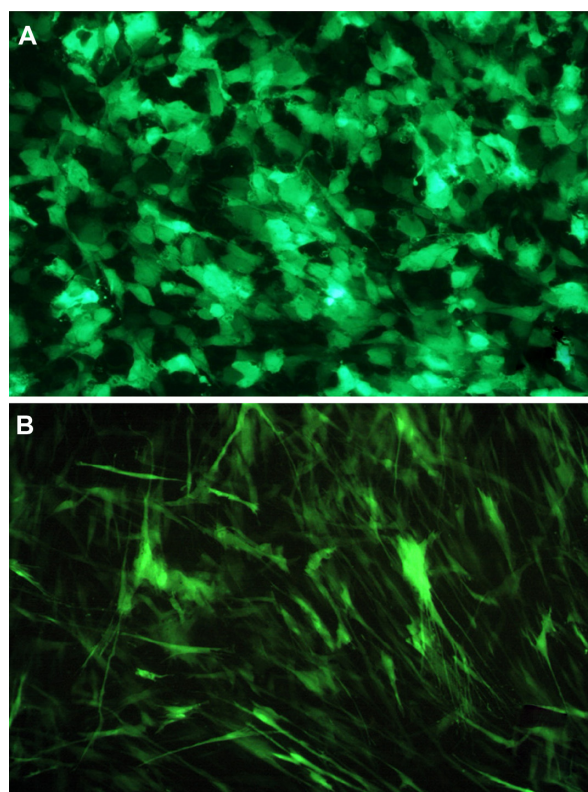

Figure 1 (**A**) Fluorescence image of DP100 at 7 days after transduction with EGFP. (P3), original magnification, ×400. (**B**) Fluorescence image of DP100 at 14 days after transduction with EGFP. (P4), original magnification, ×400.
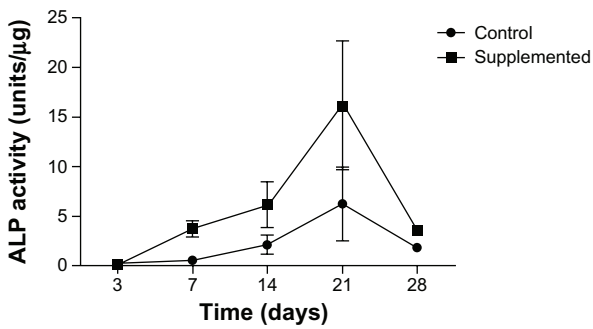**Abbreviation:** P, Passage number.

Figure 2 Quantitative determination of ALP activity in DP100 cultured in normal and supplemented medium (units per 1 µg of protein).
Notes: Cells were initially plated at passage number 3, and were counted on days 3, 7, 14, 21, and 28. The results differed significantly at day 21 ($P < 0.05$).

The DP100-implanted group showed better callus formation from the early stages when compared with the other groups. In the DP100-implanted group, three of five cases showed callus formation on day 28, and in one of these, complete bridging callus formation was observed (Figure 4). Only one case in each of the other two groups showed incomplete callus formation. On day 56, this trend became more marked. The DP100-implanted group showed complete bridging callus in four of five cases, whereas the collagen sponge-grafted group showed complete bridging callus in only one case and incomplete callus in one case. In the control group, no callus was observed. Interobserver agreement regarding radiological evaluation was moderate, with a κvalue of 0.571 (Table 1). The histological appearance of rat femurs was evaluated after 56 days. We observed osteoid, calcified tissue, and other cartilage matrix between the defect sites on

Villanueva-stained sections. Initially, we confirmed the appearance of osteoblasts, along with osteoid bands and filling with mineralization tissue, at defect sites in DP100-implanted samples and compared these with normal osteogenesis sites in the same specimen by microscopy using natural light as well as polarization and fluorescence conditions. Under natural light conditions, we observed cell morphological characteristics, such as number, maturation, and size of osteoblasts; width of the osteoid band; and number of osteocytes. DP100-implanted samples were superior with regard to cell size, number, and the osteoid band, as compared to normal osteogenesis. Under polarization conditions, we observed bone conformation (lamellar and woven bone). Lamellar bone is regarded as having better intensity and maturity than woven bone. When we observed DP100-implanted samples, there were more with woven bone when compared with normal osteogenesis samples. On fluorescence microscopy, we observed the double green lines of calcein labeling at the ossification front. In DP100-implanted samples, the spaces between the first and second calcein lines were wider than in normal osteogenesis samples. These findings suggest more vigorous new bone formation and calcium deposition in DP100-implanted samples (Fig. 5). We also performed histological analysis in the control and collagen-implanted groups. Control and collagen-implanted groups showed little calcification tissue and substantial erosion was observed (Fig. 6). Statistical analyses of each parameter in the three groups are given in Figures 7–9. In the DP100-implanted group,
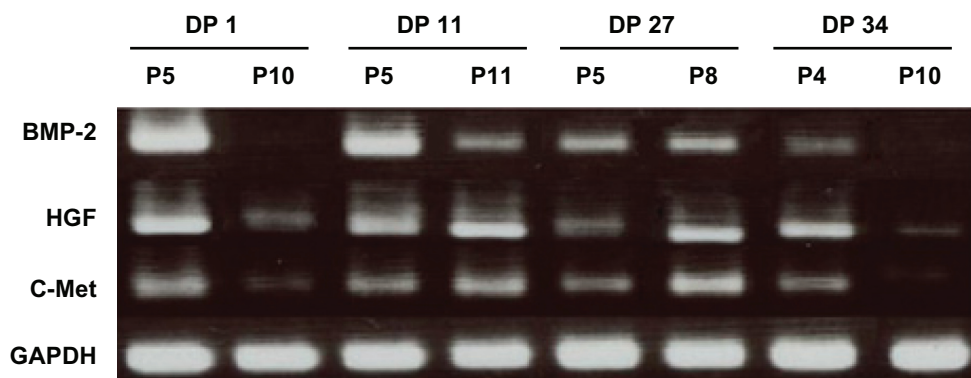

Figure 3 (**A**) Results of quantitative analysis of mRNA expression by real-time reverse-transcription polymerase chain reaction (RT-PCR). RNA was extracted from the cell line DP1. Decreased expression of BMP-2/GAPDH mRNA was seen with increasing passage number. Levels of BMP-2/GAPDH mRNA expression were obtained subsequent to normalization against constitutively expressed GAPDH mRNA. (**B**) The dental pulp stem cell lines DP1, 11 and 34, and hBMSCs showed upregulated protein levels of BMP-2 on Western blot analysis. GAPDH was used as a loading control.
Abbreviations: DP, dental pulp; BMP, bone morphogenetic protein; HGF, hepatocellular growth factor; c-Met, HGF receptor; P, passage number.

Table 1. Evaluation of radiological score.

	28 days			56 days		
	(A)	(B)	(C)	(A)	(B)	(C)
Control	0	1	4	0	1	4
Collagen sponge-implanted group	0	1	4	1	1	3
hDPSC-implanted group	1	2	2	4	0	1

Notes: (A) Complete bridging; (B) Incomplete bridging; (C) No brudging.

the bone formation parameters and number of osteoblast cells were significantly higher than those in the control and collagen sponge-implanted groups. In contrast, the control and collagen sponge-implanted groups showed significant lower in ES/BS ratio when compared with the hDPSC-implanted group.

Discussion

hDPSCs have been used in various studies on bone formation, which occurs via a complex process. The results of one such study suggested that hDPSCs differentiate into odontoblastic, adipogenic, and neural cell types.[1–3]

In the present study, we confirmed that the secreted ALP activity that peaked 21 days after induction of bone differentiation was significantly higher than in the nonsupplemented culture. Similar results were observed following induction by an osteogenesis/odontogenesis agent, which induced cell differentiation within the same time period,[7,10] This may provide a basis for the optimal timing of cell transplantation in future studies.

Histomorphologic evaluation of interfragmental bone formation parameters indicated that the number of osteocytes and osteoblasts and the amount of osteoid were higher in the DP100-implanted group when compared with the control and collagen sponge-implanted groups. This suggests that maintaining the balance in osteoblast and osteoclast counts plays a very important role in the process of bone metabolism and the mechanism of pseudarthrosis. We thought that DP100 differentiating into osteoblasts adhere to the collagen scaffold; significantly fewer osteoblasts and lower bone formation parameters were seen in the nonunion control. Furthermore, our findings are in agreement with previous results indicating that a series of endochondral ossification stages occur in the presence of DP100.[15]

Nevertheless, we could not determine whether transplanted DP100 are involved up to the final stages of the bone formation process from regenerated bone segment. The present study suggests that osteoblasts are derived from DP100 cultured in collagen sponge scaffolds following induction of osteogenesis.
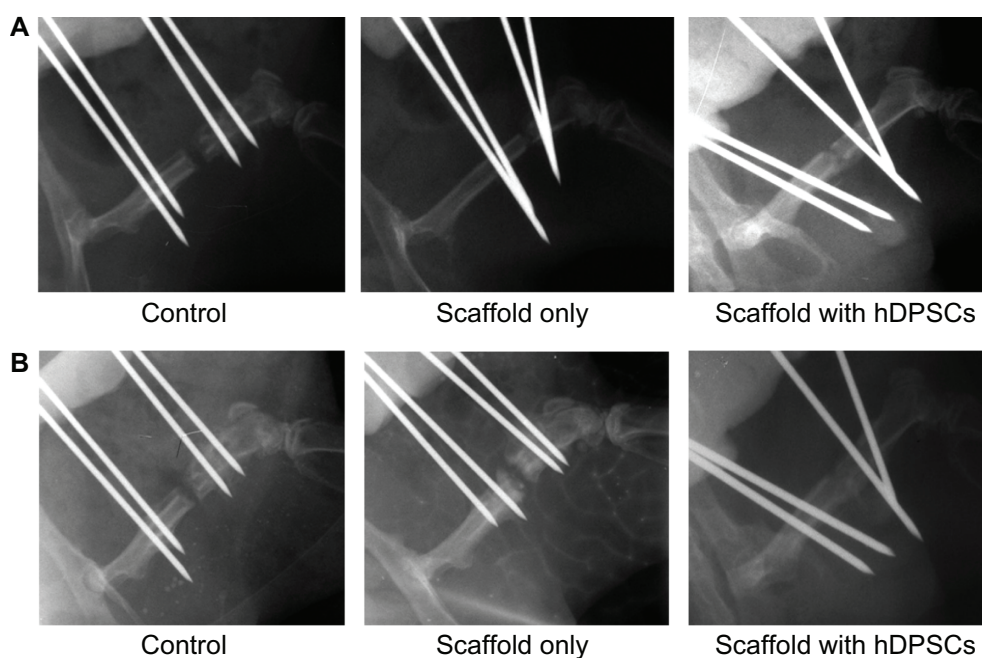

Figure 4. Representative X-ray photographs of rat femurs at 28 days and 56 days after surgery; comparison of control, scaffold only and scaffold with DP100. (**A**) At 28 days after surgery, there were no bridging calluses in the control and scaffold-implanted group, while the DP100-implanted group showed incomplete bridging (right panel). (**B**) At 56 days after surgery, the control and scaffold-implanted groups showed no apparent bridging callus, and only the DP100-implanted group showed bone union.
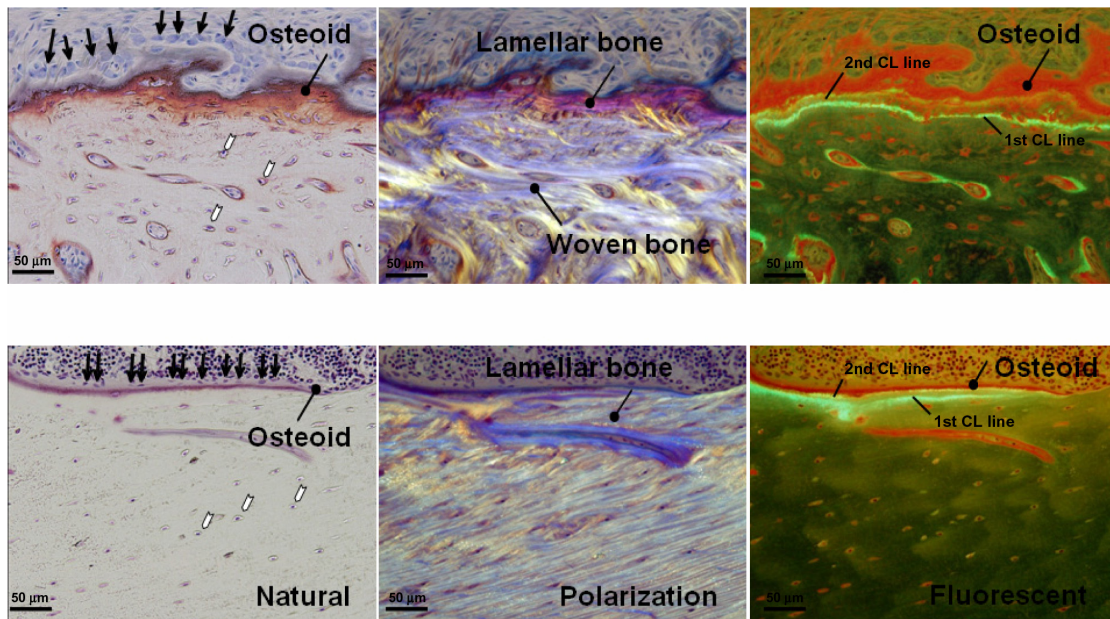
Figure 5. Villanueva bone staining of calcein-induced section at 56 days after DP100 implantation (upper panels) and normal cortical layer in endosteum from the same specimen (lower panels).
Notes: Black arrows indicate the synchronization of Ob with osteoid (upper left panel). White arrowheads indicate osteocytes. The core area of ossification is indicated by the double green line (right panel).
Abbreviations: Ob, osteoblast; Ot, osteocyte.

Alternatively, invasion by host mesenchymal cells may be promoted by DP100 expressing ossification transcription factors at the initial stages of ossification.[16] The time course of BMP-2 mRNA expression supports the hypothesis that ALP activity is partly regulated by BMP-2. Thus, several regulators may affect osteogenesis, including transforming growth factor-β (TGF-β), basic fibroblast growth factor (FGF), insulin-like growth factor I (IGF-I), tumor necrosis factor-α (TNF-α), IL-β1, and BMP. Moreover, BMP may play a very important role in the early stages of cell proliferation and differentiation, thus regulating bone formation.[17]
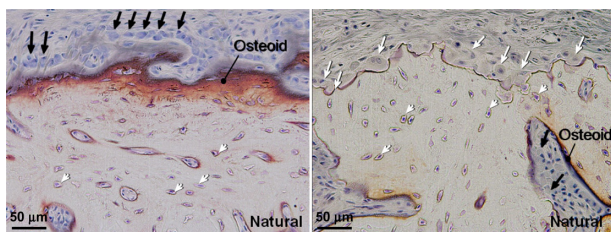

Figure 6. Villanueva bone staining at 56 days after DP100 implantation (left panel).
Notes: Cubical osteoblasts (black arrowheads) are seen adjacent to osteoid (brown area), which indicates active osteogenesis. Right panel shows aggressive bone resorption by osteoclasts in the cavity (large white arrowhead), which indicates active bone resorption. Small white arrowhead indicates osteocyte (natural light).

Our findings suggest that hDPSCs may not always form dental pulp, andtheir dynamics may be affected by surrounding cells and niches.[4,18,19] hDPSCs are easily collected from third molars extracted for orthodontics and this can performed with local anesthesia on the same day, as these teeth are usually disposed of as medical waste and no complex procedures are necessary for their culture, proliferation, and storage. There is also no risk of infection or need for general or lumbar spinal anesthesia, which would require hospitalization. These previous reports support the use of hDPSCs as diversified cellar resources for bone regeneration. Use of our experimental system increases the possibility that hDPSCs may be useful as a grafting material to treat bone defects instead of bone marrow stromal cells;[20,21] however, collection of cells of quantity are lower than bone marrow stromal cells. Moreover, we demonstrated a newly designed experimental protocol for regenerative bone formation, scaffold development, and hDPSCs maintenance. We also examined human leukocyte antigen (HLA) types of hDPSCs. Others have previously estimated that hDPSCs with 50 specific HLA homozygous haplotypes would match around 73% of Japanese of population at the three HLA loci (HLA-A, B, and DRB1) that are important for tissue and cell transplantation.[22]
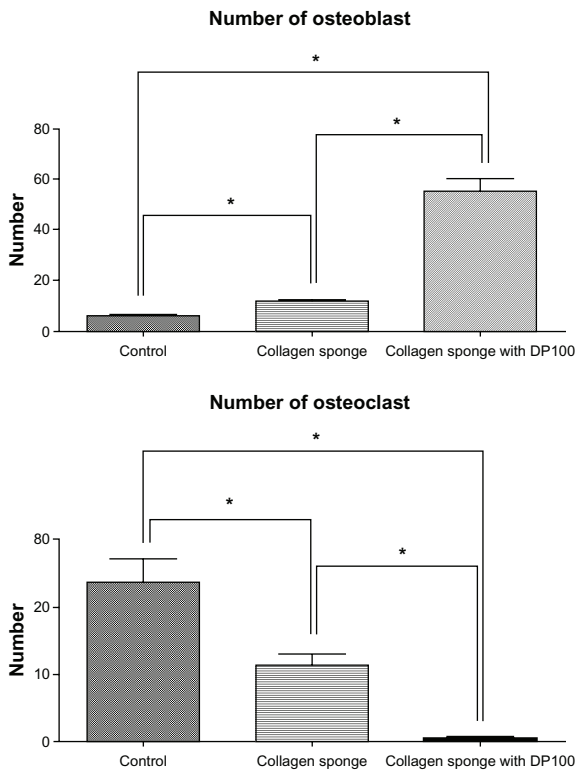
Number of osteoblast

Number of osteoclast

Figure 7. Counts of the number of osteoblast and osteoclast from osteotomy gap, (n = 14 from each of the groups).
Notes: *$P < 0.05$. Statistical analysis were evaluated by one-way analysis of variance (ANOVA) at a level of significance of $P < 0.05$. Tukey's HSD post hoc procedure was performed to determine which groups of cell numbers were significantly different from each other.
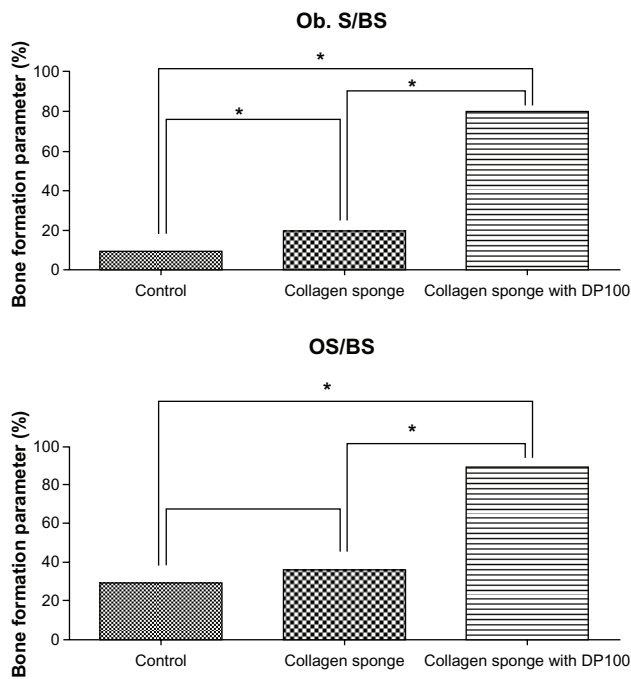
Ob. S/BS

OS/BS

Figure 8. Quantitative histological analysis of percentage bone formation within the osteotomy gap in each of the groups.
Note: Asterisks mark indicate that is significantly different from Tukey's honest significant difference ($P < 0.05$).
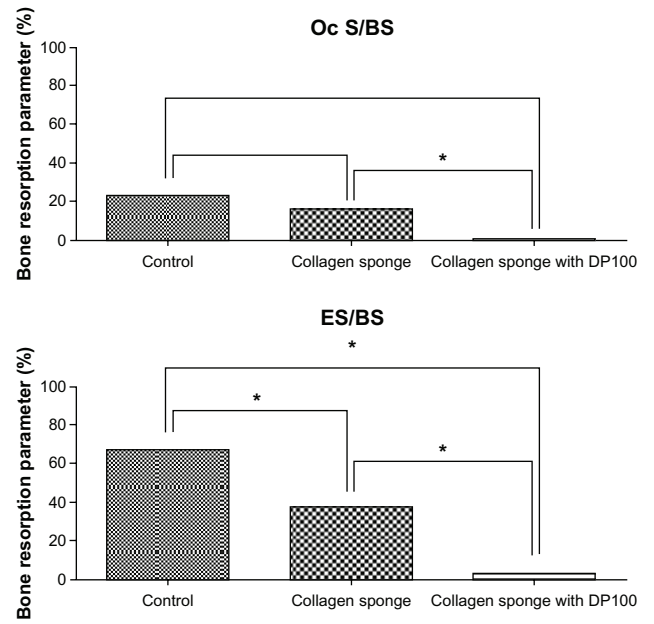
Oc S/BS

ES/BS

Figure 9. Quantitative histological analysis of percentage bone resorption. **Notes:** Control and collagen sponge-implanted groups showed a significantly higher ES/BS ratio than the DP100-implanted group. Asterisks mark sharing that is significantly different from Tukey's HSD ($P < 0.05$).

Our institution has already structured an hDPSCs banking system consisting of more than 200 patients we had been detected two donors homozygous for different set of three HLA loci. These two lines, DP74 and DP94, were expected to match around 20% of the Japanese population without serious immunological rejection. This approach, therefore, provides an innovative and novel biologically based clinical treatment using hDPSCs as well as pluripotent stem cells derived from these cells.

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Author Contributions

Conceived and designed the experiments: G.K. and T.O. Analyzed the data: G.K. Wrote the first draft of the manuscript: G.K. Contributed to the writing of the manuscript: G.K. Agree with manuscript results and conclusions: T.O., K.T, and K.S. Jointly developed the structure and arguments for the paper:

T.K., A.N., M.S., I.T., A.M., K.Y., K.H., T.K., A.H., and K.S. Made critical revisions and approved final version: T.O. All authors reviewed and approved of the final manuscript.

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Competing Interests

Author(s) disclose no potential conflicts of interest.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

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Supplementary Data

None.