Characterization of the Enhanced Bone Regenerative Capacity of Human Periodontal Ligament Stem Cells Engineered to Express the Gene Encoding Bone Morphogenetic Protein 2

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Human periodontal ligament stem cells (hPDLSCs) are considered an appropriate cell source for therapeutic strategies. The aims of this study were to investigate the sustainability of bone morphogenetic protein 2 (BMP2) secretion and the bone regenerative capacity of hPDLSCs that had been genetically modified to express the gene encoding BMP2 (*BMP2*). hPDLSCs isolated from healthy third molars were transduced using replication-deficient recombinant adenovirus (rAd) encoding *BMP2* (hPDLSCs/rAd-BMP2), and the cellular characteristics and osteogenic potentials of hPDLSCs/rAd-BMP2 were analyzed both *in vitro* and *in vivo*. hPDLSCs/rAd-BMP2 successfully secreted BMP2, formed colonies, and expressed immunophenotypes similar to their non-transduced counterparts. As to their osteogenic potential, hPDLSCs/rAd-BMP2 formed greater mineralized nodules and exhibited significantly higher levels of expression of *BMP2* and the gene encoding alkaline phosphatase, and formed more and better quality bone than other hPDLSC-containing or recombinant human BMP2-treated groups, being localized at the initial site until 8 weeks. The findings of the present study demonstrate that hPDLSCs/rAd-BMP2 effectively promote osteogenesis not only *in vitro* but also *in vivo*. The findings also suggest that hPDLSCs can efficiently carry and deliver BMP2, and that hPDLSCs/rAd-BMP2 could be used in an attractive novel therapeutic approach for the regeneration of deteriorated bony defects.

Introduction

THE CLINICAL LIMITATIONS of conventional bone reconstruction systems continue to drive the development of more attractive substitutes and strategies, and have led to the introduction of a new technology using additional signaling molecules and cells—bone tissue engineering.

As a strong ossification inducer, bone morphogenetic protein 2 (BMP2) has demonstrated significant osteoinductive properties in many experimental and clinical studies.^{1–7} Since being approved for clinical use by the U.S. Food and Drug Administration, BMP2 has become available commercially in the fields of orthopedics and dentistry.^{8,9} However, despite favorable effects on bone regeneration, problems associated with the direct application of BMP2 have been reported. Since BMP2 is rapidly dispersed and cannot be maintained sufficiently at the wound site, it lasts only a short time *in vivo*,¹⁰ which means that considerable amounts of BMP2 are required to ensure acceptable bone tissue formation. The resulting direct application of BMP2

in high doses has been reported to cause complications clinically, including ectopic bone formation,¹¹ cyst-like bone void emergence,¹² and significant soft-tissue swelling.¹³ Thus, a new modality for BMP2 treatment is required to overcome these shortcomings.

Gene delivery using various vectors has been suggested as a novel approach, in which target gene materials are injected into and around the wound to infect the host cells, enabling them to produce the therapeutic proteins such as BMP2 themselves.^{14,15} However, the main problems associated with direct gene transfer are that lots of the available vectors are degraded by serum proteases and specific cell targeting is difficult to achieve.¹⁶

The current approach involves *ex vivo* gene delivery, in which cellular carriers are used to provide a sustained release of the therapeutic proteins for more efficient tissue regeneration.^{17–21} Stem cells are the currently preferred cell source for *ex vivo* gene delivery, because they offer several advantages.²² The paracrine effects of stem cells can enhance tissue genesis through the cellular differentiation of host pluripotent

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progenitor cells,²³ and they can differentiate into multiple lineage cells to regenerate tissues.²⁴ More specifically, stem cells can modulate the inflammatory reactions of host immune cells against allogenic and xenogenic foreign bodies, such as scaffolds.²⁵ Thus, stem cell-based gene delivery systems were tested for osseous tissue engineering, and BMP2-releasing stem cells, including bone marrow stem cells and adipose tissue-derived stem cells, were found to induce favorable osseous tissue formation.^{26,27}

Stem cells used as a source for cell-based gene delivery would ideally be nonimmunogenic, highly proliferative, thus available in large quantities, easy to harvest with minimal invasiveness to the donor, and susceptible to genetic modification by gene-delivering vectors.¹⁶ Human periodontal ligament stem cells (hPDLSCs) represent a promising source thanks to their less injurious and highly proliferative characteristics. hPDLSCs have proven stem cell characteristics, including osteogenic-differentiating and immune-modulating properties.^{28–30} More importantly, hPDLSCs can be harvested from medical waste materials such as discarded extracted teeth without additional surgery that may cause patients to experience physical deformity, pain, and considerable expense. They have been shown to be superior to bone marrow stromal cells, adipose tissue-derived stromal stem cells,³¹ and periosteal cells,³² which have resulted in hPDLSCs being considered a promising cell type for cell-based gene delivery and tissue engineering applications.

Despite the great advantages of hPDLSCs, to the best of our knowledge, there has been very little research into bone tissue-engineering approaches using hPDLSCs and delivery of the gene encoding BMP2 (*BMP2*) for bone defect reconstruction. Therefore, the aims of the present study were to determine (1) whether hPDLSCs genetically modified with recombinant adenovirus (rAd) vectors containing *BMP2* (hPDLSCs/rAd-BMP2) can effectively produce BMP2, (2) whether hPDLSCs/rAd-BMP2 retain the typical properties of stem cells, and (3) whether hPDLSCs/rAd-BMP2 can improve the osteoinductive potential both *in vitro* and *in vivo*.

Materials and Methods

Isolation of hPDLSCs from teeth

hPDLSCs were isolated from third molar teeth that had been extracted from a healthy, nonsmoking adult. The isolation protocols were approved by the Ethics Committee of Inha Hospital (Approval No. IUH IRB 12-150), and informed consent was obtained from the subject before enrollment in this study. The isolation of hPDLSCs was performed as described previously.³³ In brief, PDL tissues were scraped from the extracted tooth and digested with 2 mg/mL collagenase (Wako Pure Chemical Industries, Tokyo, Japan) and 1 mg/mL dispase (Gibco, Grand Island, NY). The collected cells were seeded into a T75 cell culture flask (BD Falcon Labware, Franklin Lakes, NJ) and cultured in a growth medium (refreshed every 3 or 4 days) at 37°C in a humidified atmosphere of 5% CO₂. The growth medium contained the alpha minimum essential medium (Gibco), 15% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 µM ascorbic-acid-2-phosphate (Sigma-Aldrich, St. Louis, MO), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). Cells at passages three or four were used in the following experiments (Fig. 1).

Preparation and transduction of rAd vectors *in vitro*

The rAd expressing enhanced green fluorescent protein (EGFP) and human BMP2 was kindly provided by Prof. Young-chul Sung from the Pohang University of Science and Technology in South Korea. The hPDLSCs were transduced with rAd expressing either EGFP (rAd-EGFP) or BMP2 (rAd-BMP2) and designated hPDLSCs/rAd-EGFP and hPDLSCs/rAd-BMP2, respectively. In brief, rAd particles (20 multiplicity of infection, rAd-EGFP or rAd-BMP2) and FeCl₃ (final concentration 50 µM) were each separately diluted in the same volume of serum-free medium. After mixing the two solutions, they were incubated for 30 min at room temperature, and then added to hPDLSCs that had been washed with phosphate-buffered saline (PBS). After incubation for 1 h, the cells were provided with a fresh serum-containing growth medium.

Efficiency of the rAd-BMP2-transduced hPDLSCs

Flow cytometry for EGFP. After a 24-h culture, hPDLSCs or hPDLSCs/rAd-EGFP were harvested with 0.5 mM EDTA (Gibco) and washed twice with a staining buffer (BD Biosciences, San Diego, CA). The fluorescent expression of the EGFP was then detected using a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Analysis of BMP2 secretion and cellular activity. The culture supernatants of the hPDLSCs or hPDLSCs/rAd-BMP2 were collected on culture days 1, 3, and 7. To ensure accurate analysis at each time period, the supernatant was collected during 1-day periods as follows: days 0–1, 2–3, and 6–7. The quantity of secreted BMP2 protein was analyzed with a BMP2 enzyme-linked immunosorbent assay kit (Quantikine; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. On the same days (i.e., 1, 3, and 7), the cellular activity was simultaneously evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (0.5 mg/mL; Amresco, Solon, OH). Dimethyl sulfoxide (Amresco) was treated after a 3-h incubation, and the optical densities were read at 540 nm with the aid of a microplate reader (Molecular Devices, Sunnyvale, CA).

Cellular characterization of transduced hPDLSCs/rAd-BMP2

Colony forming. hPDLSCs or hPDLSCs/rAd-BMP2 were seeded at a density of 1×10^2 cells/plate on 100-mm culture dishes and cultured for 14 days. The cells were then fixed with 4% paraformaldehyde and stained with crystal violet (Sigma-Aldrich). The number of colony-forming units (CFUs) was counted for analysis.

Cell proliferation. As the instructions provided with the CellTrace CFSE (carboxyfluorescein diacetate succinimide ester) Cell Proliferation Kit (Molecular Probes, Eugene, OR), hPDLSCs or hPDLSCs/rAd-BMP2 (1×10^6 cells) were incubated in cell probes at 2 µM for 15 min at 37°C. The growth medium was refreshed and they were cultured for 1, 3, or 7 days. The fluorescence intensities of the cells were measured with a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Immunophenotyping for stem cell surface markers. hPDLSCs or hPDLSCs/rAd-BMP2 were harvested with 0.5 mM EDTA, washed once with a washing solution (BD Biosciences, San Diego, CA), and then incubated with primary antibodies raised against CD44, CD105, CD146, STRO-1, and CD19 (Abcam, Cambridge, MA) for 60 min at 4°C in the dark. The hematopoietic marker CD19 was used as a negative control. The cells were washed twice and then incubated with secondary antibodies (fluorescein isothiocyanate [FITC]-conjugated antibody for CD19, CD146, and CD105, and phycoerythrin-conjugated antibody for CD44 and STRO-1) for 30 min at 4°C in the dark. The cells were then analyzed with a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Osteogenic induction of hPDLSCs/rAd-BMP2 in vitro

Mineralized nodule formation. hPDLSCs or hPDLSCs/rAd-BMP2 (1×10^4 cells/well) were cultured in 24-well plates with an osteogenic induction medium for 28 days. The osteogenic induction medium comprised a growth medium containing 10^{-8} M dexamethasone (Sigma-Aldrich) and 1.8 mM KH_2PO_4 (Sigma-Aldrich), and was refreshed at 3-day intervals. After fixing with 4% paraformaldehyde for 10 min, mineralized nodules were stained with 2% Alizarin Red S (pH 7.2; Sigma-Aldrich) and then observed under a light microscope (Olympus CK41; Olympus Optical, Tokyo, Japan). The calcified nodules were stained red in Alizarin Red S, so the areas of red matrixes were measured using an automated image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD).

Real-time polymerase chain reaction for osteogenic or cementogenic marker genes. hPDLSCs or hPDLSCs/rAd-BMP2 were cultured with the osteogenic induction medium for 0, 1, 3, 7, or 14 days. The total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsberg, CA) and was used as a template for the synthesis of cDNA with an oligo(dT) primer (Maxime RT Premix; iNtRon Biotechnology, Daejeon, Korea). The subsequent real-time polymerase chain reaction (PCR) was performed with the SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA) and the StepOnePlus real-time PCR system (Applied Biosystems). The PCR amplification was conducted using the following osteogenic gene markers (Table 1): *BMP2*, and the genes encoding alkaline phosphatase (*ALP* [*ALP*]), osteocalcin (*OCN* [*OCN*]), and cementum protein 1 (*CEMP1* [*CEMP1*]). The relative mRNA expressions were quantified by comparison with the internal standard (*GAPDH*).

Subcutaneous ectopic transplantation of hPDLSCs/rAd-BMP2 in vivo

Transplantation. The ethical procedures and scientific care of the animal experiments were approved by the Institutional Animal Care and Use Committee of Inha University (Approval No. 111122-118). In total, 14 immunocompromised mice (6-week-old Balb/c nude mice; Orient Bio, Sungnam, Korea) were used. Three or four ectopic dorsal sites of each mouse were randomly assigned to the following experimental groups ($n = 5$ per group): scaffold alone (gr1), recombinant human BMP2 (rhBMP2)-soaked scaffold (gr2), hPDLSC-loaded scaffold

TABLE 1. PRIMER SEQUENCES FOR REAL-TIME POLYMERASE CHAIN REACTION

Gene (GenBank No.)	Primer sequence
<i>BMP2</i> (NM_001200)	ACTACCAGAAACGAGTGGG AA (forward) CATCTGTTCTCGGAAAACC TGAA (reverse)
<i>ALP</i> (NM_001127501)	AACATCAGGGACATTGA CGTG (forward) GTATCTCGGTTTGAAGCT CTTCC (reverse)
<i>OCN</i> (NM_000711)	GGACTGTGACGAGTTGG CTG (forward) CCGTAGAAGCGCCGATA GG (reverse)
<i>CEMP1</i> (NM_001048212.3)	GGGCACATCAAGCACTGA CAG (forward) CCCTTAGGA AGTGGCTGT CCAG (reverse)
<i>GAPDH</i> (NM_002046)	CCATGAGAAGTATGACAA CAGCC (forward) GGGTGCTAAGCAGTTGGTG (reverse)

BMP2, bone morphogenetic protein 2; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; *CEMP1*, cementum protein 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

(gr3), rhBMP2- and hPDLSC-loaded scaffold (gr4), and hPDLSC/rAd-BMP2-loaded scaffold (gr5). Particulate ceramic scaffold containing hydroxyapatite and tricalcium phosphate at a ratio of 20:80 (MBCP Plus; Biomatlante, Bretagne, France) was used as a cellular carrier (80 mg), and incubated in PBS without or with 1 μg of rhBMP2 (Cowellmedi, Pusan, Korea). After 24 h of incubation, the cells (1×10^6 cells/scaffold) were either loaded onto scaffolds or not loaded onto scaffolds. After a further incubation for 2 h at 37°C in 5% CO_2 , the scaffolds/cells (depending on the experimental group) were transplanted subcutaneously into the dorsal area of the mice. The animals were killed after 2 or 8 weeks, and the transplanted area was analyzed.

Tracing of transplanted hPDLSCs/rAd-BMP2 (*in vivo* imaging). hPDLSCs and hPDLSCs/rAd-BMP2 were labeled with fluorescent nanoparticles (NEO-LIVE Magnoxide-675 cell labeling kit; Biterials, Seoul, Korea). In accordance with the manufacturer's recommendations, each cell was treated with a solution containing 0.4 mg/mL nanoparticles for 36 h at 37°C in 5% CO_2 , and then transplanted using the same aforementioned method of subcutaneous ectopic transplantation. Four new mice were assigned to the two groups ($n = 2$ per groups) of hPDLSCs and hPDLSCs/rAd-BMP2. To avoid skin and food autofluorescence, the experimental mice were fed with an alfalfa-free diet (AIN-76A Purified Rodent Diet; Dyets, Bethlehem, PA). The entire body of each mouse was scanned through the MaestroEX imaging device (Cambridge Research & Instrumentation, Woburn, MA), and the total luminescent signals of the transplanted cells were analyzed using the Maestro In-Vivo Imaging System (version 2.1; Cambridge Research & Instrumentation).

FIG. 1. Schematic diagram of the study. hPDLSCs were isolated from healthy third molars and then transduced so as to induce BMP2 release using rAd-BMP2. The cellular characteristics and osteogenic potential of the resulting hPDLSCs/rAd-BMP2 were analyzed both *in vitro* and *in vivo*. hPDLSCs, human periodontal ligament stem cells; rAd-BMP2, recombinant adenovirus–bone morphogenetic protein 2; GFP, green fluorescent protein; CFU, colony-forming units; PCR, polymerase chain reaction. Color images available online at www.liebertpub.com/tea

Histomorphometric and immunohistochemical analyses

The explanted specimens were fixed in 4% paraformaldehyde, decalcified with 10% EDTA solution containing 1% paraformaldehyde, embedded in paraffin, and then sectioned at 4 μ m. The tissue sections were deparaffinized before staining.

Histomorphometry. The sectioned specimens were stained with hematoxylin–eosin, and visualized with the aid of a light microscope (Axio Scope; Carl Zeiss, Oberkochen, Germany). The histomorphometric analysis of mineralized tissue formations was performed using an automated histometric analysis system (Image-Pro Plus; Media Cybernetics). The percentage of newly formed mineralized tissues was calculated as the area of calcified tissues (excluding bone marrow and fibrovascular tissues) within the total augmented area.

Immunohistochemistry. The sections were treated with 10 mM sodium citrate (pH 6) at 90–95°C for 10 min for antigen retrieval, blocked with a serum-containing diluent solution (Invitrogen) for 1 h at room temperature, and then incubated in 0.2% Tween 20 (Amresco) in PBS for 20 min at room temperature. After rinsing twice with PBS, the sections were incubated with the respective rabbit anti-mouse antibodies raised against human mitochondrial ribosomal protein L11 (hMito), BMP2, ALP, OCN, or CEMP1 overnight at 4°C. On the next day, they were washed three times and incubated with FITC-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After washing three times, the sections were mounted onto glass slides with a solution containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). The immunohistochemically stained sections were observed with the aid of a confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany).

FIG. 2. Evaluation of transduction efficiency of rAd-BMP2 into hPDLSCs. (A) hPDLSCs/rAd-EGFP expression, showing that the success rate of transduction was $62.5 \pm 3.2\%$. (B) Although the proliferation rates were lower in hPDLSCs/rAd-BMP2 than in hPDLSCs, significantly greater amounts of BMP2 were released from hPDLSCs/rAd-BMP2 at all culture time points, and increased gradually until day 7. EGFP, enhanced green fluorescent protein; OD, optical density. Color images available online at www.liebertpub.com/tea

Statistical analysis

The data in this study were analyzed statistically using the Wilcoxon rank-sum test and the Kruskal–Wallis test with SPSS (version 19.0; SPSS Predictive Analytics, Chicago, IL). Tukey's *post hoc* multiple comparison tests were carried out to analyze the differences between groups. The statistical significance level was set at $p < 0.05$.

Results

Transduction efficiency of rAd-BMP2 for hPDLSCs (EGFP expression and BMP2 secretion)

In the flow cytometry test of transduction efficiency, $62.5\% \pm 3.2\%$ (mean $\pm$ SD) of the cells expressed the green EGFP signal (Fig. 2A). In the analysis of BMP2 secretion, the transduced hPDLSCs/rAd-BMP2 produced significantly higher levels of BMP2 at all culture times than their non-transduced counterparts, and the amount of BMP2 secreted increased gradually up to day 7: 0.79, 5.84, and 14.07 ng/mL at days 1, 3, and 7, respectively (Fig. 2B). However, the release of

BMP2 protein by hPDLSCs rarely increased, although their proliferation increased monotonically throughout the culture period.

Stem cell characterization of hPDLSCs/rAd-BMP2 (CFU assay, CFSE staining, and surface marker detection)

hPDLSCs/rAd-BMP2 produced significantly smaller colonies than hPDLSCs (Fig. 3A), and the rate of cellular doubling was smaller at the 7th day, although it was similar on the 1st and 3rd days (Fig. 3B). On the other hand, the CD44, CD105, CD146, STRO-1, and CD19 immunophenotypes of the hPDLSCs/rAd-BMP2 were similar to those of hPDLSCs (Fig. 3C).

Osteogenic characteristics of hPDLSCs/rAd-BMP2 in vitro (Alizarin Red S staining and real-time PCR)

The total amount of the mineralized nodules after 28 days was significantly greater in hPDLSCs/rAd-BMP2

FIG. 3. Characterization of transduced hPDLSCs/rAd-BMP2. (A) At 14 days after seeding, hPDLSCs/rAd-BMP2 produced significantly smaller colonies than hPDLSCs. (B) In the CFSE assay, hPDLSCs/rAd-BMP2 exhibited less replication than hPDLSCs after day 7. (C) The overall immunophenotypes of hPDLSCs and hPDLSCs/rAd-BMP2 were similar for CD44, CD105, CD146, and STRO-1 and for CD19, as the negative control marker. CFSE, carboxy-fluorescein diacetate succinimidyl ester. Color images available online at www.liebertpub.com/tea

FIG. 4. Osteogenic differentiation of hPDLSCs/rAd-BMP2 *in vitro*. (A) Many mineralized nodules were formed in all areas for both the hPDLSCs and hPDLSCs/rAd-BMP2, but the total amount of nodules after 28 days was significantly greater for hPDLSCs/rAd-BMP2 than for hPDLSCs. (B) Real-time polymerase chain reaction revealed that hPDLSCs/rAd-BMP2 were associated with significantly higher expressions of *BMP2* at all time points studied, with the expression being maximal at day 3. The expression of *ALP* was also significantly higher in hPDLSCs/rAd-BMP2 after day 7, although *OCN* and *CEMP1* were barely expressed in either cell type. *BMP2*, bone morphogenetic protein 2; *ALP*, alkaline phosphatase; *OCN*, osteocalcin; *CEMP1*, cementum protein 1. Color images available online at www.liebertpub.com/tea

than in hPDLSCs (Fig. 4A). A high level of gene expression was observed for *BMP2* in hPDLSCs/rAd-BMP2 at all of the studied times, and was maximal at day 3, whereas the *BMP2* expression of hPDLSCs was very low (Fig. 4B). Although low levels of *ALP* and *OCN* expres-

sion were observed in both cell types, those of hPDLSCs/rAd-BMP2 were higher than those of hPDLSCs, and they increased from day 7 to 14. On the other hand, the expressions of *CEMP1* could barely be detected in either cell type.

FIG. 5. Cell survival of transplanted hPDLSCs/rAd-BMP2 (*in vivo* imaging). The intensities of signals from the transplanted hPDLSCs and hPDLSCs/rAd-BMP2 with fluorescent nanoparticles decreased gradually over time. However, the signals were sustained at least until 4 weeks and were present only within the transplanted area. Color images available online at www.liebertpub.com/tea

Mineralized tissue formation by transplanted hPDLSCs/rAd-BMP2 *in vivo*

In vivo imaging. The fluorescence signals of the cells were constrained only to the initial transplanted area and persisted until at least 28 days, although they gradually decreased over the recorded time period (Fig. 5). The signal intensities of hPDLSCs and hPDLSCs/rAd-BMP2 were almost the same throughout the analysis period.

Histology. In specimens harvested following 2 weeks of healing, abundant connective tissues and woven mineralized tissues were observed in some of the groups (Fig. 6A): in gr1 (scaffold only control), only loose connective tissues accompanied by some blood vessels were observed; in gr2

(+rhBMP2), there were primary osseous constructions that consisted of cartilage tissues, including hypertrophic chondrocytes and nonmineralized osteoids; in gr3 (+hPDLSCs), highly dense connective tissues that contained well-arranged ligament-like fibers occupied the spaces between the grafted materials, and some osteoid-like nonmineralized matrixes were observed; in gr4 (+rhBMP2 + hPDLSCs), randomly oriented collagen fibers were interwoven adjacent to the scaffolds; and gr5 (+hPDLSCs/rAd-BMP2), specimens exhibited woven bone that was associated with bone cartilage and bead-like chondrocytes. At 8 weeks of healing, the histology revealed more well-developed and mature mineralized tissue formations (Fig. 6A): in gr1, dense connective tissues and many blood vessels were revealed, but the calcified tissue augmentation was minimal; in gr2, many

FIG. 6. Mineralized tissue formation by hPDLSCs/rAd-BMP2 *in vivo*. (A) All except gr1 specimens exhibited new mineralized tissue formation by 8 weeks. The inserted black boxes show the areas of interest at higher magnification. (B) Histo-morphometry revealed that the amount of newly formed mineralized tissues was significantly greater in gr2 and gr5 specimens at 2 weeks, and greatest in gr2, whereas gr4 and gr5 specimens exhibited a significantly greater new calcified tissue formation at 8 weeks, with it being greatest in gr5 (* $p < 0.05$; $\times 100$ and $\times 400$). Color images available online at www.liebertpub.com/tea

lamellae were observed in the mineralized tissues, but a considerable amount of fatty marrows and a large number of polymorphic nucleated cells were also found around the bone tissues; gr3 and gr4 exhibited similar forms of new mineralized tissues, in which the mineralized structures included large infiltrations of Sharpey's fiber-like collagen bundles; and gr5 exhibited excellent new bone formation, containing reversal lines and osteoblast-like cells entrapped within them, little fat tissue, very few polymorphic immune cells, and more importantly, bony bridges linking the grafted particles.

Histomorphometry. In specimens harvested following 2 weeks of healing, the amount of mineralized tissue regeneration was significantly greater in gr2 and gr5 than in the other groups, and was markedly greater in gr2 (Fig. 6B, $p < 0.05$). In contrast, the amount of newly formed mineralized tissues at 8 weeks was significantly greater in gr4 and gr5, and gr5 exhibited the greatest bone augmentation. However, these tissues were only rarely found in gr1, and their levels barely increased between the 2- and 8-week healing periods in gr2.

Immunohistochemistry. In the explanted specimens of 2- and 8-week healing, the amounts of hMito, BMP2, ALP, OCN, and CEMP1 were evaluated through immunohistochemistry (Fig. 7). In gr1, none of these proteins were detected, while in gr2, only ALP was detected in both the 2- and 8-week specimens. In gr3, hMito, ALP, and CEMP1 were highly expressed at both time points, whereas OCN in the 2- and 8-week specimens and BMP2 in the 8-week specimen were more weakly expressed, and no BMP2 was detected in the 2-week specimen. In gr4, all five proteins (hMito, BMP2, ALP, OCN, and CEMP1) were expressed at both time points, although the BMP2 signal in the 8-week specimen was slightly decreased compared to that at 2 weeks. The specimens in gr5 were also positive for all proteins, except for CEMP1 in the 8-week specimen.

Discussion

The present study produced hPDLSCs/rAd-BMP2 that released significantly large amounts of BMP2 proteins and promoted osteogenesis for new bone formation. Any differences in cellular characteristics between hPDLSCs and hPDLSCs/rAd-BMP2 were first determined. Although hPDLSCs/rAd-BMP2 were less proliferated, these findings are consistent with previous stem cell study findings that the number of rAd-infected rat bone marrow stem cells and the total quantity of DNA of dental pulp stem cells decreased with time, respectively.^{34,35} More importantly, the percentages of stem cell markers were similar in the two cell types and comparable to those found in previous studies.³³ This means that the hPDLSCs/rAd-BMP2 retained the stem cell characteristics of hPDLSCs even after the transduction process, and as a result, they were able to act as a cellular carrier that could release BMP2 continuously.

In our *in vitro* experiments, the hPDLSCs/rAd-BMP2 produced markedly more BMP2 than did hPDLSCs, and they formed significantly more mineralized nodules under osteoinductive conditions. The hPDLSCs/rAd-BMP2 successfully expressed the inserted BMP2 gene, and the secreted proteins improved the mineralized matrix formations through either an autocrine or a paracrine process. Given that the gene

expressions of ALP (an early-phase osteogenic marker) and OCN (a late-phase osteogenic marker gene) increased from the 7th day until the experiment endpoint, it is likely that hPDLSCs/rAd-BMP2 would become more osteogenic over time. Further studies are necessary to carry out experiments over longer periods of time to confirm the later stage bone regeneration capacity of these cells. On the other hand, the expression of CEMP1—which is a specific marker of cementoblasts and their progenitors³⁶—was scarce, and it is thought that the osteoinductive condition induced the differentiation toward an osteogenic phenotype rather than cementogenic, as already found in a previous study.³⁷

The present study was the first attempt to monitor the survival and migration patterns of hPDLSCs and hPDLSCs/rAd-BMP2 with an *in vivo* imaging system over a long period. Although fluorescent signals were rarely detected at 6 and 8 weeks on *in vivo* imaging, the 8-week hMito immunohistochemistry showed that the transplanted cells were alive. The results demonstrate that the transplanted hPDLSCs and hPDLSCs/rAd-BMP2 can survive in the recipient body for at least 8 weeks.

At 8 weeks post-transplantation, the *in vivo* histology indicated that the amount of mineralized tissue formation was significantly greater in gr5 specimens than in any of the other groups, and the regenerated tissues had typical features of BMP2-induced bone formation: hypertrophic chondrocytes at the initial phase, cells entrapped in lacunae, and lamellations as seen in mature bone tissue.^{38–40} Although the amount of bone tissue was less in gr5 than in gr2 after 2 weeks of healing, it was greater than in gr2 at 8 weeks. Moreover, abundant fatty marrow formation was observed in gr2, whereas very little fat tissues were detected in gr5. Therefore, the newly regenerated bone tissues in gr5 are thought to be clinically superior to those of gr2, since they have a higher mineral density, and the presence of a large amount of fatty marrow can cause poor bone quality. On the other hand, the rapid but transient osteogenesis observed in gr2 specimens may be attributable to the exhaustion of the rhBMP2 within the initial 2-week period. Many previous investigations found that BMP2 diffused rapidly, with its level decreasing to less than 10% within 7 days in several scaffold materials; its direct application thus has only short-term actions.¹⁰

In gr3 and gr4, cementum-like tissue formation was observed, including the mineralized structures infiltrated by Sharpey's fiber-like collagen bundles, as shown in previous studies,^{28,41} and the immunohistochemistry experiments revealed the presence of CEMP1 until 8 weeks, whereas in gr5, it did not last until 8 weeks and in gr2, it was never observed. In addition, the features of newly formed mineralized tissues of the rhBMP2-treated gr2 and gr4 were found to be very different: gr2 exhibited typical bone regeneration, whereas gr4 exhibited cementum-like tissue formation. From this it can be assumed that the rhBMP2 in gr4 was only effective over a short period of time or the hPDLSCs in gr4 might inhibit rhBMP2-induced bone formation. The transient effect of direct application of rhBMP2 was shown in many previous reports. In addition, the BMP2 suppression capability of PDL-derived cells had already been shown, whereby the osteogenic induction of rhBMP2 was inhibited by treatment with a hPDL cell-conditioned medium. These results might be due to the chordin that was expressed in and

FIG. 7. Immunohistochemistry of the mineralized tissues formed by hPDLSCs/rAd-BMP2. The presence of osteogenic-related proteins was evaluated using immunohistochemistry of the explanted specimens retrieved following 2 and 8 weeks of healing. **(A)** At 2 weeks, the gr4 and gr5 specimens were positive for all five targeted proteins, whereas gr2 specimens were only positive for ALP, and gr3 was positive for hMito, ALP, OCN, and CEMP1. **(B)** At 8 weeks, gr3 and gr4 specimens were positive for all five proteins, whereas gr5 specimens were positive for all except for CEMP1 (arrow, positively stained cells; $\times 800$). Color images available online at www.liebertpub.com/tea

secreted from hPDL cells acting as a signal to inhibit the reactions of BMP.⁴² Therefore, the sparse bone formation despite the application of rhBMP2 at 2 weeks post-transplantation in gr4 may have been attributed to the complicated causes of very little osteogenic induction by the short-period effect of rhBMP2 in gr4 and the inhibitory mechanism of hPDLSCs against rhBMP2.

At both 2- and 8-week post-transplantation, the histology revealed that gr2 specimens exhibited many inflammatory cells, whereas the other groups (gr1, gr3, gr4, and gr5) exhibited either none or fewer such cells. Therefore, the infiltrations of polymorphonuclear cells in gr2 are likely to have been the result of foreign body reactions against the direct application of BMP2. Considering the immunosuppression capabilities of stem cells shown in many other studies,^{29,30,43} the very small immune reactions in the other BMP2-containing groups (gr4 and gr5) may have been due to the presence of stem cells, hPDLSCs, or hPDLSCs/rAd-BMP2.

In summary, the present findings demonstrate that hPDLSCs, which are genetically modified to express *BMP2*, are able to produce a sustained release of the BMP2 and effectively promote osteogenesis, while retaining the typical characteristics of human mesenchymal stem cells. Moreover, the hPDLSCs/rAd-BMP2 exhibited great osteogenic potential both *in vitro* and *in vivo*; in particular, they produced remarkable mineralized tissue formation with typical bone-regenerating characteristics. The amount and quality of the bone formed were both greater than in the nontransduced hPDLSCs or BMP2-treated groups. More importantly, these results show that the regenerative characteristics of cells can be effectively manipulated through genetic modification. This is the first study to use hPDLSCs as cellular carriers in gene delivery for the continuous release of target proteins, such as BMP2. Although further animal experiments of bone defect models are needed to confirm the bone regenerative effects of hPDLSCs/rAd-BMP2, the present findings provide an attractive and efficient novel therapeutic approach for the regeneration of deteriorated bone defects caused by accidental injuries and osteolytic diseases.

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Disclosure Statement

No competing financial interests exist.

References

- Cheng, H., Jiang, W., Phillips, F.M., Haydon, R.C., Peng, Y., Zhou, L., Luu, H.H., An, N., Breyer, B., Vanichakarn, P., Szatkowski, J.P., Park, J.Y., and He, T.C. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). *J Bone Joint Surg Am* **85-A**, 1544, 2003.
- Knippenberg, M., Helder, M.N., Zandieh Doulabi, B., Wuisman, P.I., and Klein-Nulend, J. Osteogenesis versus chondrogenesis by BMP-2 and BMP-7 in adipose stem cells. *Biochem Biophys Res Commun* **342**, 902, 2006.
- Mussano, F., Ciccone, G., Ceccarelli, M., Baldi, I., and Bassi, F. Bone morphogenetic proteins and bone defects: a systematic review. *Spine (Phila Pa 1976)* **32**, 824, 2007.
- Jang, J.W., Yun, J.H., Lee, K.I., Jung, U.W., Kim, C.S., Choi, S.H., and Cho, K.S. Osteoinductive activity of biphasic calcium phosphate with different rhBMP-2 doses in rats. *Oral Surg Oral Med Oral Pathol Oral Radiol* **113**, 480, 2012.
- Okubo, Y., Bessho, K., Fujimura, K., Kusumoto, K., Ogawa, Y., Tani, Y., and Iizuka, T. Comparative study of intramuscular and intraskeletal osteogenesis by recombinant human bone morphogenetic protein-2. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* **87**, 34, 1999.
- Yang, H.S., La, W.G., Bhang, S.H., Kim, H.J., Im, G.I., Lee, H., Park, J.H., and Kim, B.S. Hyaline cartilage regeneration by combined therapy of microfracture and long-term bone morphogenetic protein-2 delivery. *Tissue Eng Part A* **17**, 1809, 2011.
- Kim, J.W., Jung, I.H., Lee, K.I., Jung, U.W., Kim, C.S., Choi, S.H., Cho, K.S., and Yun, J.H. Volumetric bone regenerative efficacy of biphasic calcium phosphate-collagen composite block loaded with rhBMP-2 in vertical bone augmentation model of a rabbit calvarium. *J Biomed Mater Res A* **100**, 3304, 2012.
- Jung, R.E., Glauser, R., Scharer, P., Hammerle, C.H., Sailer, H.F., and Weber, F.E. Effect of rhBMP-2 on guided bone regeneration in humans. *Clin Oral Implants Res* **14**, 556, 2003.
- Boyne, P.J., Lilly, L.C., Marx, R.E., Moy, P.K., Nevins, M., Spagnoli, D.B., and Triplett, R.G. *De novo* bone induction by recombinant human bone morphogenetic protein-2 (rhBMP-2) in maxillary sinus floor augmentation. *J Oral Maxillofac Surg* **63**, 1693, 2005.
- Uludag, H., D'Augusta, D., Palmer, R., Timony, G., and Wozney, J. Characterization of rhBMP-2 pharmacokinetics implanted with biomaterial carriers in the rat ectopic model. *J Biomed Mater Res* **46**, 193, 1999.
- Wong, D.A., Kumar, A., Jatana, S., Ghiselli, G., and Wong, K. Neurologic impairment from ectopic bone in the lumbar canal: a potential complication of off-label PLIF/TLIF use of bone morphogenetic protein-2 (BMP-2). *Spine J* **8**, 1011, 2008.
- Zara, J.N., Siu, R.K., Zhang, X., Shen, J., Ngo, R., Lee, M., Li, W., Chiang, M., Chung, J., Kwak, J., Wu, B.M., Ting, K., and Soo, C. High doses of bone morphogenetic protein 2 induce structurally abnormal bone and inflammation *in vivo*. *Tissue Eng Part A* **17**, 1389, 2011.
- Smucker, J.D., Rhee, J.M., Singh, K., Yoon, S.T., and Heller, J.G. Increased swelling complications associated with off-label usage of rhBMP-2 in the anterior cervical spine. *Spine (Phila Pa 1976)* **31**, 2813, 2006.
- Asahara, T., Kalka, C., and Isner, J.M. Stem cell therapy and gene transfer for regeneration. *Gene Ther* **7**, 451, 2000.
- McMahon, J.M., Conroy, S., Lyons, M., Greiser, U., O'Shea, C., Strappe, P., Howard, L., Murphy, M., Barry, F., and O'Brien, T. Gene transfer into rat mesenchymal stem cells: a comparative study of viral and nonviral vectors. *Stem Cells Dev* **15**, 87, 2006.
- Bagnis, C., and Mannoni, P. Stem cell-based gene therapy. *Oncologist* **2**, 196, 1997.
- Shin, J.H., Kim, K.H., Kim, S.H., Koo, K.T., Kim, T.I., Seol, Y.J., Ku, Y., Rhyu, I.C., Chung, C.P., and Lee, Y.M.

- Ex vivo* bone morphogenetic protein-2 gene delivery using gingival fibroblasts promotes bone regeneration in rats. *J Clin Periodontol* **37**, 305, 2010.
18. Tsuda, H., Wada, T., Ito, Y., Uchida, H., Dehari, H., Nakamura, K., Sasaki, K., Kobune, M., Yamashita, T., and Hamada, H. Efficient BMP2 gene transfer and bone formation of mesenchymal stem cells by a fiber-mutant adenoviral vector. *Mol Ther* **7**, 354, 2003.
 19. Meinel, L., Hofmann, S., Betz, O., Fajardo, R., Merkle, H.P., Langer, R., Evans, C.H., Vunjak-Novakovic, G., and Kaplan, D.L. Osteogenesis by human mesenchymal stem cells cultured on silk biomaterials: comparison of adenovirus mediated gene transfer and protein delivery of BMP-2. *Biomaterials* **27**, 4993, 2006.
 20. Lou, J., Xu, F., Merkel, K., and Manske, P. Gene therapy: adenovirus-mediated human bone morphogenetic protein-2 gene transfer induces mesenchymal progenitor cell proliferation and differentiation *in vitro* and bone formation *in vivo*. *J Orthop Res* **17**, 43, 1999.
 21. Conget, P.A., and Minguell, J.J. Adenoviral-mediated gene transfer into *ex vivo* expanded human bone marrow mesenchymal progenitor cells. *Exp Hematol* **28**, 382, 2000.
 22. Rios, H.F., Lin, Z., Oh, B., Park, C.H., and Giannobile, W.V. Cell- and gene-based therapeutic strategies for periodontal regenerative medicine. *J Periodontol* **82**, 1223, 2011.
 23. Gneccchi, M., Zhang, Z., Ni, A., and Dzau, V.J. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* **103**, 1204, 2008.
 24. Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. Multilineage potential of adult human mesenchymal stem cells. *Science* **284**, 143, 1999.
 25. Le Blanc, K., and Ringden, O. Immunomodulation by mesenchymal stem cells and clinical experience. *J Intern Med* **262**, 509, 2007.
 26. Chang, S.C., Chuang, H.L., Chen, Y.R., Chen, J.K., Chung, H.Y., Lu, Y.L., Lin, H.Y., Tai, C.L., and Lou, J. *Ex vivo* gene therapy in autologous bone marrow stromal stem cells for tissue-engineered maxillofacial bone regeneration. *Gene Ther* **10**, 2013, 2003.
 27. Drago, J.L., Choi, J.Y., Lieberman, J.R., Huang, J., Zuk, P.A., Zhang, J., Hedrick, M.H., and Benhaim, P. Bone induction by BMP-2 transduced stem cells derived from human fat. *J Orthop Res* **21**, 622, 2003.
 28. Seo, B.M., Miura, M., Gronthos, S., Bartold, P.M., Batouli, S., Brahimi, J., Young, M., Robey, P.G., Wang, C.Y., and Shi, S. Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* **364**, 149, 2004.
 29. Kim, H.S., Kim, K.H., Kim, S.H., Kim, Y.S., Koo, K.T., Kim, T.I., Seol, Y.J., Ku, Y., Rhyu, I.C., Chung, C.P., and Lee, Y.M. Immunomodulatory effect of canine periodontal ligament stem cells on allogenic and xenogenic peripheral blood mononuclear cells. *J Periodontol Implant Sci* **40**, 265, 2010.
 30. Wada, N., Menicanin, D., Shi, S., Bartold, P.M., and Gronthos, S. Immunomodulatory properties of human periodontal ligament stem cells. *J Cell Physiol* **219**, 667, 2009.
 31. Jung, I.H., Park, J.C., Kim, J.C., Jeon, D.W., Choi, S.H., Cho, K.S., Im, G.I., Kim, B.S., and Kim, C.S. Novel application of human periodontal ligament stem cells and water-soluble chitin for collagen tissue regeneration: *in vitro* and *in vivo* investigations. *Tissue Eng Part A* **18**, 643, 2012.
 32. Tsumanuma, Y., Iwata, T., Washio, K., Yoshida, T., Yamada, A., Takagi, R., Ohno, T., Lin, K., Yamato, M., Ishikawa, I., Okano, T., and Izumi, Y. Comparison of different tissue-derived stem cell sheets for periodontal regeneration in a canine 1-wall defect model. *Biomaterials* **32**, 5819, 2011.
 33. Jung, I.H., Kwon, B.S., Kim, S.H., Shim, H.E., Jun, C.M., and Yun, J.H. Optimal medium dormulation for the long-term expansion and stemness maintenance of periodontal ligament stem cells. *J Periodontol* **84**, 1434, 2013.
 34. Deng, W., Bivalacqua, T.J., Chattergoon, N.N., Jeter, J.R., Jr., and Kadowitz, P.J. Engineering *ex vivo*-expanded marrow stromal cells to secrete calcitonin gene-related peptide using adenoviral vector. *Stem Cells* **22**, 1279, 2004.
 35. Yang, X., van der Kraan, P.M., Bian, Z., Fan, M., Walboomers, X.F., and Jansen, J.A. Mineralized tissue formation by BMP2-transfected pulp stem cells. *J Dent Res* **88**, 1020, 2009.
 36. Alvarez-Perez, M.A., Narayanan, S., Zeichner-David, M., Rodriguez Carmona, B., and Arzate, H. Molecular cloning, expression and immunolocalization of a novel human cementum-derived protein (CP-23). *Bone* **38**, 409, 2006.
 37. Komaki, M., Iwasaki, K., Arzate, H., Narayanan, A.S., Izumi, Y., and Morita, I. Cementum protein 1 (CEMP1) induces a cementoblastic phenotype and reduces osteoblastic differentiation in periodontal ligament cells. *J Cell Physiol* **227**, 649, 2012.
 38. Shapiro, F. Bone development and its relation to fracture repair. The role of mesenchymal osteoblasts and surface osteoblasts. *Eur Cell Mater* **15**, 53, 2008.
 39. Junqueira, L.C.J.C., and Mescher, A.L. Junqueira's Basic Histology: Text and Atlas, 12th ed. New York: McGraw-Hill, 2010.
 40. Roach, H.I. Trans-differentiation of hypertrophic chondrocytes into cells capable of producing a mineralized bone matrix. *Bone Miner* **19**, 1, 1992.
 41. Kim, Y.T., Park, J.C., Choi, S.H., Cho, K.S., Im, G.I., Kim, B.S., and Kim, C.S. The dynamic healing profile of human periodontal ligament stem cells: histological and immunohistochemical analysis using an ectopic transplantation model. *J Periodontol Res* **47**, 514, 2012.
 42. Mizuno, N., Ozeki, Y., Shiba, H., Kajiya, M., Nagahara, T., Takeda, K., Kawaguchi, H., Abiko, Y., and Kurihara, H. Humoral factors released from human periodontal ligament cells influence calcification and proliferation in human bone marrow mesenchymal stem cells. *J Periodontol* **79**, 2361, 2008.
 43. Le Blanc, K., Tammik, L., Sundberg, B., Haynesworth, S.E., and Ringden, O. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* **57**, 11, 2003.

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