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Ex vivo bone morphogenetic protein-2 gene delivery using gingival fibroblasts promotes bone regeneration in rats

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Abstract

Aim: The aim of the present study was to investigate bone regeneration following ex vivo bone morphogenetic protein-2 (BMP-2) gene delivery using human gingival fibroblasts (HGFs) in rat calvarial defects.

Materials and Methods: An 8 mm craniotomy defect was created in Sprague–Dawley rats. The animals were divided into four groups: (1) non-grafted group, the defect was left empty; (2) collagen matrix group, the defect was filled with collagen matrix only; (3) HGF group, the defect was filled with non-transduced HGFs on collagen matrix; (4) BMP-2/HGF group, the defect was filled with BMP-2 gene-transduced HGFs on collagen matrix. Animals were sacrificed at 2 and 4 weeks after surgery, and micro-computed tomographic and histologic observations were performed.

Results: The BMP-2/HGF group showed promoted osseous healing of calvarial defects, as compared with the other groups. At both 2 and 4 weeks, regenerated bone area was significantly greater in the BMP-2/HGF group than the other three groups. Quite a few number of transplanted HGFs were observed within the regenerated bone tissues.

Conclusions: The results of this study suggest that ex vivo BMP-2 gene delivery induces prominent bone regeneration in vivo and HGFs may be useful as target cells for ex vivo gene therapy.

Key words: bone morphogenetic protein-2 (BMP-2) gene; bone regeneration; ex vivo gene therapy; gingival fibroblasts; transplantation

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Bone loss is a commonly encountered problem in the field of dental surgery. There is an enormous need for bone regeneration to recover periodontal defects and develop the site for implant placement or other dental prostheses. Various materials have been used for bone regeneration including growth factors. Bone morphogenetic proteins (BMPs) are members of the transforming growth factor superfamily and induce primitive mesenchymal cell chemotaxis, proliferation, and differentiation into chondrocytes and osteoblasts (Alden et al. 2002). BMP-2 has osteoinductive potential and various delivery system scaffolds for BMP-2 such as collagen, gelatin, demineralized bone matrix, and hyaluronan have been suggested. Although topical delivery of BMP-2 with these scaffolds has been proven to have some promising effects for bone regeneration, there are some limitations. Because of the instability and quick dilution in vivo, therapeutic BMP-2 level at the defect site cannot be maintained sufficiently (Anusaksathien & Giannobile 2002). Therefore, gene therapy has been considered an alternative method for providing sufficient duration of action of the applied BMPs.

Tissue engineering of alveolar bone using gene therapy may offer potential

Conflict of interest and source of funding

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for optimal delivery of BMP molecules in the fields of periodontal and oral implant surgery. Jin et al. (2003) demonstrated the first successful evidence of periodontal tissue engineering using gene transfer of BMPs. In their study, the osseous lesions treated by BMP-7 gene delivery demonstrated rapid osteogenesis, cementogenesis, and predictable bridging of the periodontal bone defects. In a later study by Dunn et al. (2005), treatment of dental implant fixtures with BMP-7 gene delivery resulted in enhancement of alveolar bone defect fill and new bone-to-implant contact.

There are two basic strategies in BMP gene delivery. Recombinant vectors encoding BMP is directly delivered to in vivo sites (in vivo) or genetically transduced cells by vectors encoding BMP are implanted (ex vivo). In the case of the in vivo approach, it is difficult to control the specific cell population transduced genetically (Franceschi et al. 2004) and significant antibody response to the vector can be caused (Gelse et al. 2001). Ex vivo approach may somewhat overcome the problems stated above.

In the case of the ex vivo approach, the type of cells used for transplantation must be considered. Fibroblasts (Krebsbach et al. 2000, Jin et al. 2003), periosteum (Mason et al. 1998, Breitbart et al. 1999), bone marrow stromal cells (Lou et al. 1999, Musgrave et al. 2000), and muscles (Musgrave et al. 2000, Lee et al. 2001) have been used as the source of transplanted cells. Although these cells have been proven to be effective for ex vivo gene therapy, they are difficult to harvest and can cause morbidity in the donor site. As an alternative, gingival fibroblasts can be considered because they are relatively easy to harvest especially in the field of dental surgery and morbidity can be reduced. A previous study suggested that human gingival fibroblasts (HGFs) are capable of secreting transduced BMP-7 genes in vitro and in vivo (Krebsbach et al. 2000). However, research on ex vivo BMP-2 gene delivery using HGFs in vivo is scarce.

The aim of the present study was to investigate the effect of bone regeneration following ex vivo BMP-2 gene delivery in rat calvarial defects and to evaluate the feasibility of HGFs as a cell source for supplying BMP-2 in vivo.

Materials and Methods

Preparation of HGFs

Gingival connective tissue was obtained from the healthy gingiva of premolar teeth of one donor (18-year-old female) undergoing tooth extractions for orthodontic reasons. The tissues were cut into small pieces and placed in 35 mm dishes. The minced tissues were cultured in Dulbecco's modified eagles medium (DMEM) (Gibco, Carlsbad, CA, USA) containing 10% (v/v) foetal bovine serum (FBS) (Gibco) and 1% (v/v) penicillin–streptomycin solution (5000 U/ml penicillin and 50 μg/ml streptomycin) (Gibco), at 37°C in a humidified atmosphere of 95% air and 5% CO_2. When the HGFs that grew out from the tissue fragments reached confluence, the cells were released with 0.25% trypsin/0.1% EDTA solution (Gibco). The cells of passage three to five were harvested and used for this study. This study protocol was approved by the Seoul National University Dental Hospital Institutional Review Board (#CRI08023).

Construction of BMP-2 expressing adenovirus and transduction

A replication-defective human Ad5 containing a cDNA for human BMP-2 (ATCC, Manassas, VA, USA) in the E1 region of the virus (Ad5BMP2) was constructed by in vivo homologous recombination. Briefly, an expression cassette encoding BMP-2 gene was amplified in *Escherichia coli* and ligated to Adeno-X Viral DNA (BD Biosciences Clontech, San Jose, CA, USA). Recombinant adenoviruses were obtained from human embryonic kidney 293 cells transfected by the recombinant Adeno-X vectors. The HGFs were plated at a density of 1×10^6 cells/cm^2 and transduced with Ad5BMP2 in DMEM supplemented with 10% FBS and antibiotic–antimycotic solution. Virus was allowed to adsorb for 4 h and the media was then added.

In vitro expression of BMP-2

In vitro secretion of BMP-2 by Ad5BMP2-transduced HGFs was quantified by enzyme-linked immunosorbent assay (ELISA). HGFs were plated in a 24-well plate (5×10^4 cells/well) and viral transduction was performed as mentioned above. Control cells without viral transduction were also prepared in the same manner. The culture medium in each well was collected and replaced at 1, 4, 7, 10, and 14 days after transduction with adenovirus. The media collected at each examination point were frozen at -80°C for later analysis. The amount of BMP-2 in the collected media from each examination day was measured separately using a commercial enzyme immunoassay kit (Quantikine BMP-2 microplate, R&D systems, Minneapolis, MA, USA), a 96-well polystylene microplate coated with a mouse monoclonal antibody against BMP-2. All experiments were performed in triplicate.

Cell labelling for tracking

The Ad5BMP2-transduced HGFs were labelled with CM-Dil (Invitrogen, Eugene, OR, USA) according to the manufacturer's recommendation. Briefly, the cells were incubated with 5 μM CM-Dil for 5 min. at 37°C and additionally for 15 min. at 4°C. After incubation, the cells were washed with PBS.

Cell seeding into carrier

Sterilized collagen matrix (CollaCote™, Zimmer Dental Inc., Carlsbad, CA, USA) was trimmed to a circular disk with an 8 mm diameter. Cell suspensions (2×10^7 cells/ml) of HGFs and Ad5BMP2-transduced HGFs were prepared, respectively. In case of Ad5BMP2-transduced HGFs, the cell suspension was prepared 1 day after the gene transduction. Aliquots of 50 μl of each cell suspension were seeded onto the collagen matrix resulting in a seeding density of 10^6 cells/matrix.

Surgical procedure

Thirty-two male Sprague–Dawley rats (8 weeks old) were used in this study. Care and treatment of the animals were conducted in accordance with guidelines established by the Seoul National University Institutional Animal Care and Use Committee (Approval No SNU-081114-2). General anaesthesia was induced by an intramuscular injection of a combination of ketamine and xylazine. A 3 cm linear incision was made over the calvarium at midline and the full-thickness flaps were elevated. An 8 mm craniotomy defect was created using a trephine bur (3i Implant Innovation, Palm Beach Gardens, FL, USA) under sterile saline irrigation. The ani-

mals were divided into four groups as follows: (1) non-grafted group, the defect was left empty; (2) collagen matrix group, the defect was filled with collagen matrix; (3) HGF group, the defect was filled with non-transduced HGFs–collagen matrix construct; (4) BMP-2/HGF group, the defect was filled with Ad5BMP2-transduced HGFs–collagen matrix construct. The incisions were closed at layers with 5-0 chromic gut and 4-0 silk. All the animals received a single intramuscular injection of antibiotics directly after surgery. For 1 week, 20 mg cefazolin was injected. One milligram per kilogram body weight immunosuppressive (FK506, Cayman, Ann Arbor, MI, USA) was also injected during 2 weeks then twice a week until sacrifice.

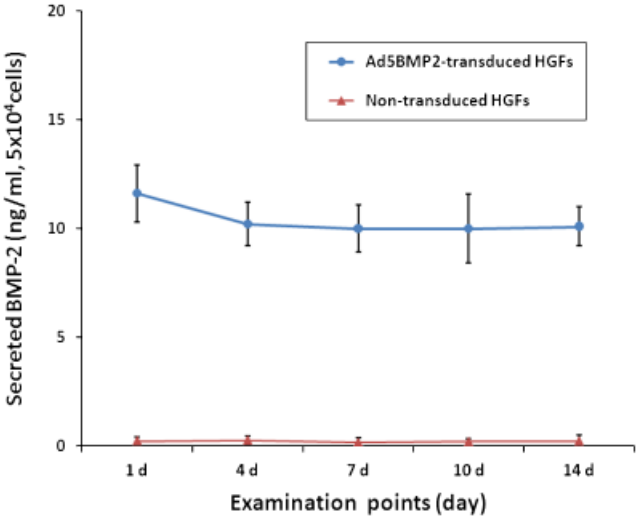

Fig. 1. An illustration of the amount of bone morphogenetic protein-2 (BMP-2) produced by human gingival fibroblasts (HGFs) transduced with Ad5BMP2, as determined using enzyme-linked immunosorbent assay. Non-transduced cells were used as the control. The value at each time point represents the amount of BMP remained in the media collected at the time point when the media was replaced. Error bars represent standard deviations.

Micro-computed tomography (micro-CT)

Four rats of each group were sacrificed at 2 and 4 weeks after surgery, respectively. The tissues including the surgical sites were harvested and fixed in 10% neutralized-buffered formalin solution and micro-CT was taken in each group using Skyscan 1172 (SkyScan, Kontich, Belgium).

Histologic procedure

The specimens were decalcified with 10% EDTA solution for 2 weeks and dehydrated through a series of ethanol solutions of increasing concentrations and embedded in paraffin. Five-μm-thick coronal sections through the centre of the circular defects were obtained and stained with Masson trichrome stain. The specimens prepared were examined by light microscopy.

After microscopic examination, a photograph of each slide was taken using a digital camera, and the resulting images were saved to a computer for histomorphometric analysis. A single masked, calibrated examiner (K. H. K.) examined all of the images. Intra-examiner error between pre- and post-examination and inter-examiner error compared with a standard (Y. M. L.) were $<5\%$. Measurement of newly formed bone was carried out using an automated image analysis system (Tomoro Scope Eye 3.5 Image Analyzer, Techsan Digital Imaging, Seoul, Korea). Regeneration of bone was quantified by measuring the percentage of the whole defect area filled by regenerated bone (area of bone regenerated in the defect divided by the whole defect area). The whole defect area was measured arbitrarily by the area surrounded by two imaginary lines along the inner and outer calvarial bone contour and both sides of defect border.

For cell tracking, the CM-DiI-labelled cells in the tissue sections were observed under a confocal laser scanning microscope (CLSM FV300, Olympus, Tokyo, Japan).

Statistical analysis

One-way analysis of variance with Fisher's LSD tests was used to evaluate the differences of bone regeneration (%) among the experiment groups. Statistical significance was set at $p<0.05$.

Results

BMP-2 expression by Ad5BMP2-transduced HGFs in vitro

The amount of BMP-2 expressed by HGFs was quantified by ELISA at various time points within a 14-day period in vitro. In each well, 5×10^4 cells were initially plated, transduced with virus, and cultured for up to 14 days. As shown in Fig. 1, Ad5BMP2-transduced HGFs consistently secreted BMP-2 during the entire period of analysis. For each examination point, 10–12 ng/ml of BMP-2 was detected in the culture media, whereas no detectable BMP-2 was produced by the non-transduced HGFs, control cells.

Micro-CT findings

At 2 weeks of healing, the radio-dense defect fill was prominent in the BMP-2/HGF group, whereas the defect fill was limited in the other three groups (Fig. 2a–d). At 4 weeks of healing, the defect fill advanced in all groups compared with 2 weeks (Fig. 2e–h). Particularly in the BMP-2/HGF group, the defect healing was so marked that the defect was almost closed with radio-dense tissue (Fig. 2h).

Histologic findings

In the non-grafted groups, the collagen matrix group, and the HGF group, only a limited amount of new bone formation along the periphery of the defect was observed at 2 weeks (Fig. 3a–c). The defect was mainly filled with fibrous tissues in the non-grafted group (Fig. 3a). In the collagen matrix group and the HGF group, the defect was almost filled with remnants of collagen matrix (Fig. 3b and c). On the other hand, in the BMP-2/HGF group, relatively less remnants of collagen matrix were observed and evidence of newly formed bone was observed to be mingled with degradation remnants of

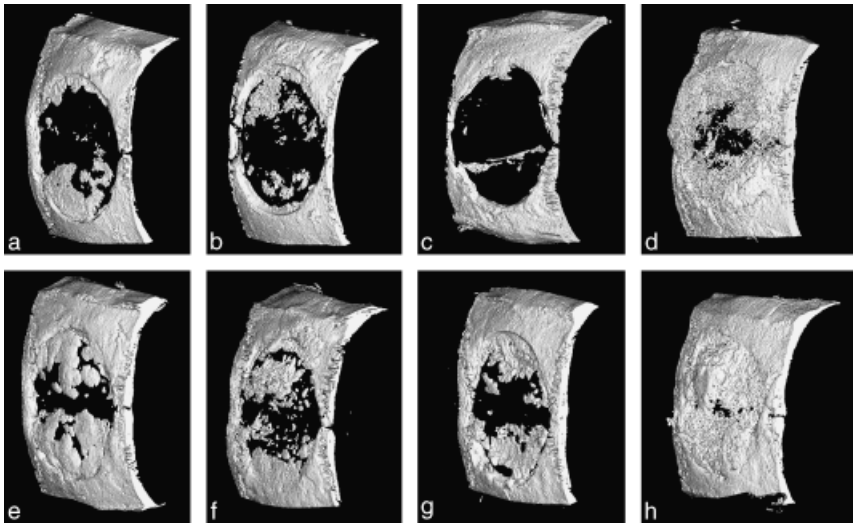

Fig. 2. Micro-CT images. At 2 weeks after surgery: (a) non-grafted group; (b) collagen matrix group; (c) human gingival fibroblast (HGF) group; (d) bone morphogenetic protein-2 (BMP-2)/HGF group. At 4 weeks after surgery: (e) non-grafted group; (f) collagen matrix group; (g) HGF group; (h) BMP-2/HGF group.

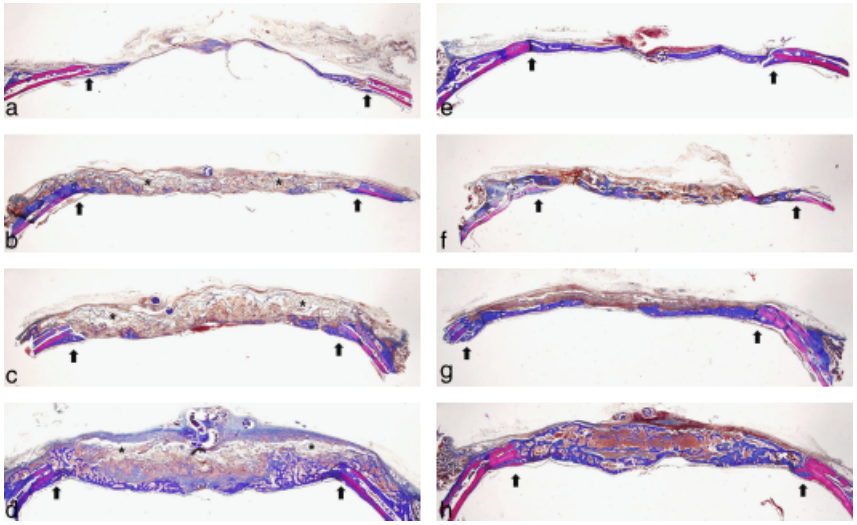

Fig. 3. Histological findings at 2 (left panels, a–d) and 4 weeks (right panels, e–h) after surgery (Masson trichrome stain, × 12.5). (a, e) non-grafted group; (b, f) collagen matrix group; (c, g) human gingival fibroblast (HGF) group; (d, h) bone morphogenetic protein-2 (BMP-2)/HGF group. Arrows indicate the defect margin. Remnants of collagen matrix (*) were observed at 2 weeks.

the collagen matrix. The BMP-2/HGF group showed marked primary spongework of newly formed woven bone, infiltrating into collagen matrix (Figs 3d and 4a).

At 4 weeks of healing, bone regeneration had advanced with fibrous tissues still occupying small central portions of the defects in the non-grafted group, the collagen matrix group, and the HGF group (Fig. 3e–g). However, the BMP-2/HGF group showed apparent bone regeneration and the defect almost coalesced with thick newly formed bone (Fig. 3h). The regenerated trabecular bone underwent remodelling, and the woven bone was gradually replaced by lamellar bone. New bone formation resulted in a trabecular pattern. The intertrabecular space contained fibrous connective tissue and bone marrow rich in blood vessels (Fig. 4b).

Histomorphometric measurements of the regenerated bone

Regenerated bone area (%) in the non-grafted group, the collagen matrix group, the HGF group, and the BMP-2/HGF group, was $22.24 \pm 4.79\%$, $25.75 \pm 19.61\%$, $19.78 \pm 16.8\%$, and $77.31 \pm 14.54\%$, respectively, at 2 weeks and $45.37 \pm 8.11\%$, $73.63 \pm 32.74\%$, $52.45 \pm 16.56\%$, and $122.87 \pm 17.17\%$, respectively, at 4 weeks. At both 2 and 4 weeks, regenerated bone area (%) was significantly greater ($p < 0.01$) in the BMP-2/HGF group than the other groups. But no significant differences (p-value range = 0.113–0.836) were observed among the three other groups at both 2 and 4 weeks (Fig. 5). (Corrections added on 5 January 2010, after first online publication: $p \leqslant 0.01$ was corrected to $p < 0.01$ in the previous sentence and in the legend of Fig. 5.)

Cell tracking in regenerated tissue

A large number of transplanted BMP-2-transduced HGFs were detected in the regenerated bone within the defect (Fig. 6). The labelled cells were distributed widely throughout the defect at 2 weeks after transplantation (Fig. 6a). At 4 weeks, transplanted BMP-2-transduced HGFs still remained although lower staining density was observed than at 2 weeks (Fig. 6b).

Discussion

Tissue engineering technology can be used to restore, maintain, or enhance tissues and organs (Giannobile 2002, Griffith & Naughton 2002). Recently, BMP gene delivery has offered new approaches for periodontal tissue engineering (Jin et al. 2003) and alveolar bone engineering at dental implant defects (Dunn et al. 2005). To date, critical-sized calvarial defects (Schmitz & Hollinger 1986, Schmitz et al. 1990) have been extensively used and tested as a pre-clinical experimental model for cranio-mandibulofacial reconstruction. Various approaches using BMP gene therapy for bone regeneration have been tested using this cranial defect model (Breitbart et al. 1999, Krebsbach et al. 2000, Lee et al. 2001, Blum et al. 2003, Nussenbaum et al. 2003, Franceschi et al. 2004). In the present study, it was demonstrated that genetically engineered HGFs can produce BMP-2 and can stimulate bone regeneration in

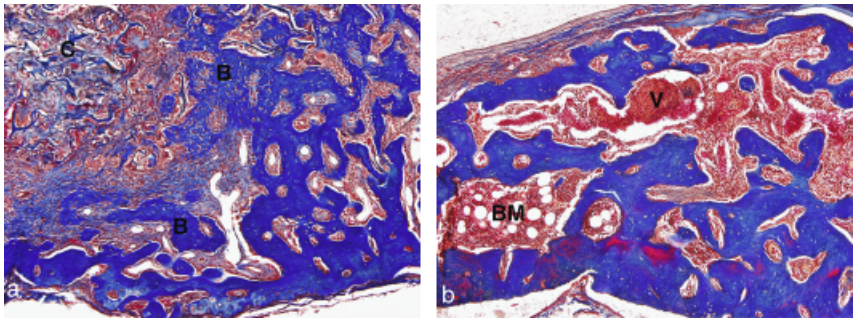

Fig. 4. Bone morphogenetic protein-2/human gingival fibroblast group at 2 (a) and 4 weeks (b) after surgery (Masson trichrome stain, × 100). At 2 weeks, newly formed bone (B) infiltrated into and mingled with degraded collagen matrix and degraded collagen matrix fragments (C) were observed. Note the advanced bone remodelling with an appearance of bone marrow (BM) and blood vessels (V) at 4 weeks.

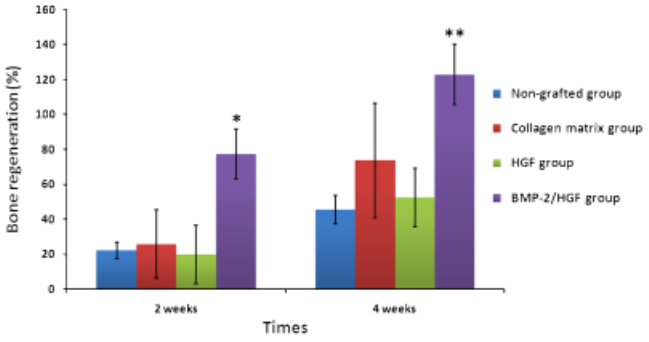

Fig. 5. Histomorphometric data of the percentage of regenerated bone. Statistically significant differences ($p < 0.01$) were shown between the bone morphogenetic protein-2 (BMP-2)/human gingival fibroblast (HGF) group and the other groups at 2(*) and 4 weeks (**), respectively. Error bars represent standard deviations.

rat calvarial defects. The finding that HGFs alone did not improve bone healing indicates that BMP-2 is a major osteogenic determinant in the healing process of rat calvarial defects.

The ELISA data in this study demonstrated that nanogram levels (10–12 ng/ml) of BMP-2 were produced by Ad5BMP2-transduced HGFs that were initially plated at a density of 5×10^4 cells in vitro. Although more cells (10^6 cells) were transplanted in the rat calvarial defects, the expected amount of BMP-2 produced by the transplanted cells in vivo was less than the quantity (0.5–115 μg) of exogenous BMP-2 required for generating new bone formation in vivo as reported previously (Wang et al. 1990). However, this amount of BMP-2 appears to be still effective for bone regeneration using an ex vivo approach. Considering that BMP-2 functions via a threshold paradigm (Reddi 1994), temporally distinct processes of chemotaxis, cell proliferation, and bone differentiation may be best supported with sustained, low-level BMP-2 delivery attainable only by the use of ex vivo gene therapy. In addition, sustained BMP-2 delivery during a period of 2–5 weeks would result in a cumulative amount of BMP-2 in the microgram quantities necessary to promote cell differentiation (Reddi 1994).

In the present study, the non-grafted group still showed considerable bone generation (45.37 ± 8.11%) at 4 weeks after surgery. Hence, other factors should be considered. In this study, immunosuppressive FK506 was used to diminish immune response to HGFs. Voggenreiter et al. (2005) stated that systemic application of FK506 had no biomechanical and histological effects of experimental fracture healing in the rat and resorption far in excess of formation led to a net bone loss in the trabecular bone of the tibia. However, a previous study reported that systemic application of FK506 enhanced bone formation in heterotopically implanted demineralized bone matrix in rats (Voggenreiter et al. 2000). It also has been shown that FK506 enhances osteoblastic differentiation in mesenchymal cells (Tang et al. 2002). Therefore, although it is still conflicting that FK506 increases bone healing, it may be an explanation for considerable bone regeneration in this present study.

Most studies testing the efficacy of ex vivo gene therapy utilizes an immunocompromised animal model like nude rat to prevent rejection of cell transplants. However, in this study, HGFs were transplanted into immunocompetent rats instead, providing a more challenging transplantation condition. The reason for this attributes to the intention of this study as attempts were made as a pre-clinical measure to first test the feasibility of HGFs regarding the efficacy of ex vivo BMP gene delivery under xenogenic transplantation condition, before advancing to human trial. For human clinical applications, the cell source of ex vivo gene therapy should be from human and an autogenous cell source should be optimal for transplantation.

In this study, the mean area of regenerated bone in the non-grafted group, the collagen matrix group, and the HGF group at 4 weeks was 45.37 ± 8.11%, 73.63 ± 32.74%, and 52.45 ± 16.56%, respectively. Although no statistically significant differences were observed among these three groups, the groups grafted with collagen matrix (collagen matrix group and HGF group) tended to show more bone regeneration than the non-grafted group. This might be explained by the space-maintaining property of collagen matrix.

Currently, a series of cell labelling techniques are applied in cell transplantation fields, which include the BradU, fluorescent dye (CM-Dil, CFSE, DAPI, etc.), reporter gene (GFP, Lac-Z, luciferase, etc.), Y chromosome marker, magnetic, and isotope labelling techniques (Yan et al. 2007). Among these, CM-Dil has a cellular membrane-staining property because it contains a thiol-reactive chloromethyl moiety that allows the dye to covalently bind to cellular thiols. Labelling cells with CM-Dil requires only a short staining step (30 min.), and standard histological procedures can be used. Therefore, the method is much simpler than labelling with a reporter gene (Ferrari et al. 2001). In addition, CM-Dil labelling provides a stable fluorescent tracking system not influenced by changes in surface markers or cell differ-

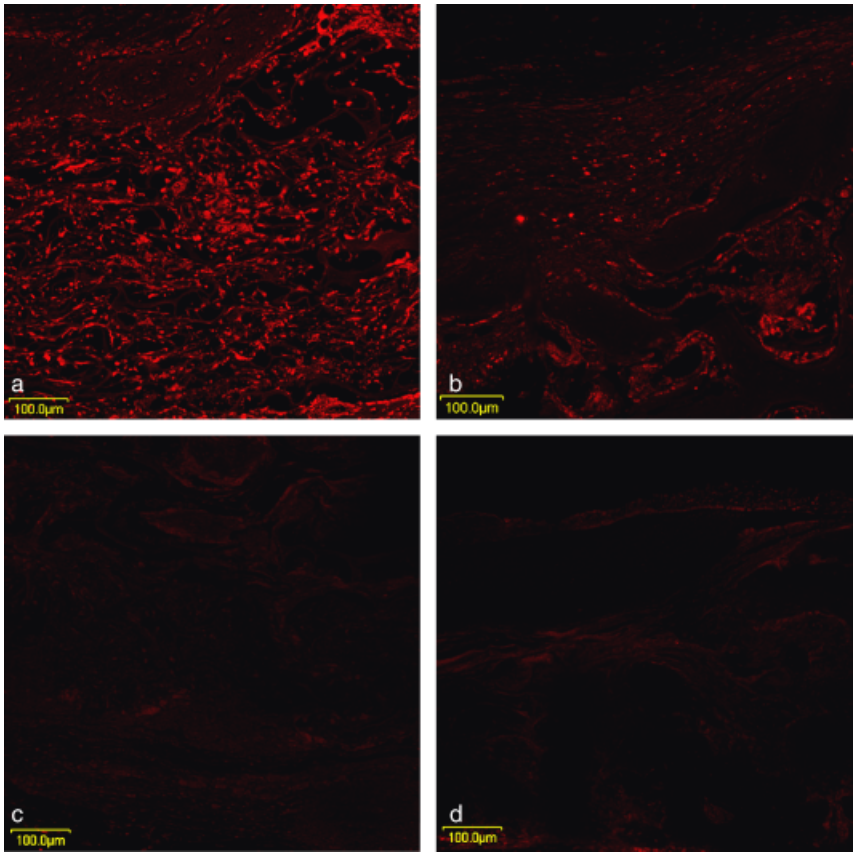

Fig. 6. Labelled Ad5BMP2-transduced human gingival fibroblasts (HGFs) in the regenerated bone tissue under a confocal laser-scanning microscope at 2 (a) and 4 weeks (b) after surgery (× 200). (c) and (d) are unlabelled controls at 2 and 4 weeks, respectively.

entiation, yet does not affect cell division (Weir et al. 2008). Because of these reasons, in the present study, CM-Dil was used for tracing transplanted HGFs transduced with Ad5BMP2 instead of a reporter gene. Well-distributed HGFs were observed under a confocal laser-scanning microscope at 2 and 4 weeks in this study. This indicates that HGFs were well seeded into the defect by means of the collagen matrix and had viability in vivo. This finding suggests that HGFs transduced with Ad5BMP2 produces BMP-2 protein during the healing phase for about 4 weeks after transplantation. BMP-2 expressed by Ad5BMP2-transduced HGFs affected cell differentiation in both an autocrine and paracrine manner. In addition, cells derived from recipient sites might also be stimulated by the BMP-2 during the healing phase of bone regeneration. Lower labelling density was shown at 4 weeks than at 2 weeks. It can be assumed that cell mitosis and apoptosis were the reason why the staining density of the cells was diminished over time after transplantation.

HGFs were used as target cells of viral transfection and considerable bone regeneration was observed in this study. Selecting the proper cell source for ex vivo gene delivery is an important therapeutic consideration. In a clinical setting, it would be advantageous if the cells can be obtained via routine dental outpatient biopsy of oral tissue such as gingival tissue with minimal morbidity (Krebsbach et al. 2000). The optimal cell type for ex vivo BMP gene delivery should not only demonstrate a high rate of transduction with the adenovirus vector but also be readily accessible or available in a sufficient number (Gugala et al. 2003). Previous studies (Franceschi et al. 2000, 2004, Krebsbach et al. 2000, Rutherford et al. 2002) have demonstrated that HGFs are easily cultivable and well-expanded cells that are capable of expressing transduced BMP gene with high transduction efficiency. Bone cell precursors have been used recently as target cells because such cells may directly participate in bone formation in addition to expressing BMP-2 (Gazit et al. 1999). However, Gugala et al. (2003) reported that osteoinduction by ex vivo adenovirus-mediated BMP-2 delivery was independent of cell type, which comprised of primary human bone marrow mesenchymal stem cells, primary human skin fibroblasts, and a human diploid foetal lung cell line. In addition, other studies have stated that HGFs-transduced BMP-7 gene were capable of secreting BMP-7 in vitro and in vivo (Krebsbach et al. 2000) and BMP-7-transduced HGFs were converted to osteoblasts and formed bone in vivo (Rutherford et al. 2002). In the present study, there was statistically significant difference in newly formed bone area between the HGF group and the BMP-2/HGF group. Therefore, it can be presumed that BMP-2 was effectively expressed by Ad5BMP2-transduced HGFs in vivo. HGFs are also readily available clinically compared with other cell types such as bone marrow cells.

In conclusion, this study confirms that ex vivo BMP-2 gene delivery induces prominent bone regeneration in vivo and HGFs may be useful as target cells for ex vivo gene therapy. Further studies are required for clinical application.

References

Alden, T. D., Varady, P., Kallmes, D. F., Jane, J. A. Jr. & Helm, G. A. (2002) Bone morphogenetic protein gene therapy. *Spine* **27**, S87–S93.

Anusaksathien, O. & Giannobile, W. V. (2002) Growth factor delivery to re-engineer periodontal tissues. *Current Pharmaceutical Biotechnology* **3**, 129–139.

Blum, J. S., Barry, M. A., Mikos, A. G. & Jansen, J. A. (2003) In vivo evaluation of gene therapy vectors in ex vivo-derived marrow stromal cells for bone regeneration in a rat critical-size calvarial defect model. *Human Gene Therapy* **14**, 1689–1701.

Breitbart, A. S., Grande, D. A., Mason, J. M., Barcia, M., James, T. & Grant, R. T. (1999) Gene-enhanced tissue engineering: applications for bone healing using cultured periosteal cells transduced retrovirally with the BMP-7 gene. *Annals of Plastic Surgery* **42**, 488–495.

Dunn, C. A., Jin, Q., Taba Jr., M., Franceschi, R. T., Rutherford, R. B. & Giannobile, W. V. (2005) BMP gene delivery for alveolar bone engineering at dental implant defects. *Molecular Therapy* **11**, 294–299.

Ferrari, A., Hannouche, D., Oudina, K., Bourguignon, M., Meunier, A., Sedel, L. & Petite, H. (2001) *In vivo* tracking of bone marrow fibroblasts with fluorescent carbocynine dye.

Journal of Biomedical Materials Research **56**, 361–367.

Franceschi, R. T., Wang, D., Krebsbach, P. H. & Rutherford, R. B. (2000) Gene therapy for bone formation: in vitro and in vivo osteogenic activity of an adenovirus expressing BMP7. *Journal of Cell Biochemistry* **78**, 476–486.

Franceschi, R. T., Yang, S., Rutherford, R. B., Krebsbach, P. H., Zhao, M. & Wang, D. (2004) Gene therapy approaches for bone regeneration. *Cells Tissues Organs* **176**, 95–108.

Gazit, D., Turgeman, G., Kelley, P., Wang, E., Jalenak, M., Zilberman, Y. & Moutsatsos, I. (1999) Engineered pluripotent mesenchymal cells integrate and differentiate in regenerating bone: a novel cell-mediated gene therapy. *The Journal of Gene Medicine* **1**, 121–133.

Gelse, K., Jiang, Q. J., Aigner, T., Ritter, T., Wagner, K., Poschl, E., von der Mark, K. & Schneider, H. (2001) Fibroblast-mediated delivery of growth factor complementary DNA into mouse joints induces chondrogenesis but avoids the disadvantages of direct viral gene transfer. *Arthritis and Rheumatism* **44**, 1943–1953.

Giannobile, W. V. (2002) What does the future hold for periodontal tissue engineering? *The International Journal of Periodontics and Restorative Dentistry* **22**, 6–7.

Griffith, L. G. & Naughton, G. (2002) Tissue engineering-current challenges and expanding opportunities. *Science* **295**, 1009–1014.

Gugala, Z., Olmsted-Davis, E. A., Gannon, F. H., Lindsey, R. W. & Davis, A. R. (2003) Osteoinduction by ex vivo adenovirus-mediated BMP2 delivery is independent of cell type. *Gene Therapy* **10**, 1289–1296.

Jin, Q., Anusaksathien, O., Webb, S. A., Rutherford, R. B. & Giannobile, W. V. (2003) Gene therapy of bone morphogenetic protein for periodontal tissue engineering. *Journal of Periodontology* **74**, 202–213.

Krebsbach, P. H., Gu, K., Franceschi, R. T. & Rutherford, R. B. (2000) Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone in vivo. *Human Gene Therapy* **11**, 1201–1210.

Lee, J. Y., Musgrave, D., Pelinkovic, D., Fukushima, K., Cummins, J., Usas, A., Robbins, P., Fu, F. H. & Huard, J. (2001) Effect of bone morphogenetic protein-2-expressing muscle-derived cells on healing of critical-sized bone defects in mice. *The Journal of Bone and Joint Surgery (Am)* **83**, 1032–1039.

Lou, J., Xu, F., Merkel, K. & Manske, P. (1999) 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. *Journal of Orthopaedic Research* **17**, 43–50.

Mason, J. M., Grande, D. A., Barcia, M., Grant, R., Pergolizzi, R. G. & Breitbart, A. S. (1998) Expression of human bone morphogenic protein 7 in primary rabbit periosteal cells: potential utility in gene therapy for osteochondral repair. *Gene Therapy* **5**, 1098–1104.

Musgrave, D. S., Bosch, P., Lee, J. Y., Pelinkovic, D., Ghivizzani, S. C., Whalen, J., Niyibizi, C. & Huard, J. (2000) Ex vivo gene therapy to produce bone using different cell types. *Clinical Orthopaedics and Related Research* **11**, 290–305.

Nussenbaum, B., Rutherford, R. B., Teknos, T. N., Dornfeld, K. J. & Krebsbach, P. H. (2003) Ex vivo gene therapy for skeletal regeneration in cranial defects compromised by postoperative radiotherapy. *Human Gene Therapy* **14**, 1107–1115.

Reddi, A. H. (1994) Bone and cartilage differentiation. *Current Opinion in Genetics and Development* **4**, 737–744.

Rutherford, R. B., Moalli, M., Franceschi, R. T., Wang, D., Gu, K. & Krebsbach, P. H. (2002) Bone morphogenetic protein-transduced human fibroblasts convert to osteoblasts and form bone in vivo. *Tissue Engineering* **8**, 441–452.

Schmitz, J. P. & Hollinger, J. O. (1986) The critical sized defect as an experimental model for cranio-mandibulofacial nonunions. *Clinical Orthopaedics and Related Research* **205**, 299–308.

Schmitz, J. P., Schwartz, Z., Hollinger, J. O. & Boyan, B. D. (1990) Characterization of rat calvarial nonunion defect. *Acta Anatomica* **138**, 185–192.

Tang, L., Ebara, S., Kawasaki, S., Wakabayashi, S., Nikaido, T. & Takaoka, K. (2002) FK506 enhanced osteoblastic differentiation in mesenchymal cells. *Cell Biology International* **26**, 75–84.

Voggenreiter, G., Assenmacher, S., Kreuzfelder, E., Wolf, M., Kim, M. R., Nast-Kolb, D. & Schade, F. U. (2000) Immunosuppression with FK506 increases bone induction in demineralized isogeneic and xenogeneic bone matrix in the rat. *Journal of Bone and Mineral Research* **15**, 1825–1834.

Voggenreiter, G., Siozos, P., Hunkemoller, E., Heute, S., Schwarz, M. & Obertacke, U. (2005) Immunosuppression with FK506 has no influence on fracture healing in the rat. *Bone* **37**, 227–233.

Wang, E. A., Rosen, V., D'Alessandro, J. S., Bauduy, M., Cordes, P., Harada, T., Israel, D. I., Hewick, R. M., Kerns, K. M. & LaPan, P. (1990) Recombinant human bone morphogenetic protein induces bone formation. *Proceedings of the National Academy of Sciences of the USA* **87**, 2220–2224.

Weir, C., Morel-Kopp, M.-C., Gill, A., Tinworth, K., Ladd, L., Hunyor, S. N. & Ward, C. (2008) Mescenchymal stem cells: isolation, characterization and *in vivo* fluorescent dye tracking. *Heart Lung Circulation* **17**, 395–403.

Yan, L., Han, Y., He, Y., Xie, H., Liu, J., Zhao, L., Wang, J., Gao, L. & Fan, D. (2007) Cell tracing techniques in stem cell transplantation. *Stem Cell Reviews* **3**, 265–269.

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Clinical Relevance

Scientific rationale for the study: Gene therapy has been considered as an alternative method for topical delivery of BMPs. This study was carried out to investigate bone regeneration following ex vivo BMP-2 gene delivery using HGFs in rat calvarial defects.

Principal finding: Transplantation of BMP-2 gene-transduced HGFs promoted osseous healing of rat calvarial defects.

Practical implication: Ex vivo BMP-2 gene therapy with HGFs might be a possible therapeutic approach for the regeneration of bone defects. In addition, HGFs that are easily obtained with gingival biopsy could be used in gene therapy to deliver BMP-2.