# ABSTRACT

Several lines of evidence show that transplantation of osteoblastic cells or genetically engineered non-osteogenic cells expressing osteoblast-related genes into bone defects effectively promotes bone regeneration. To extend this possibility, we investigated whether oral mucosal fibroblasts are capable of differentiating into osteoblastic cells by conducting *in vitro* and *in vivo* experiments. We investigated the effects of bone morphogenetic protein-2 (BMP-2) on osteoblast differentiation of cultured fibroblasts isolated from canine buccal mucosa. We also transplanted green fluorescence protein (GFP)-expressing fibroblasts with gelatin/BMP-2 complexes into the subfascial regions of athymic mice, and investigated the localization of GFP-positive cells in the ectopically formed bones. The cultured canine buccal mucosal fibroblasts differentiated into osteoblastic cells by increasing their alkaline phosphatase (ALP) activity and *Osteocalcin*, *Runx2,* and *Osterix* mRNA expression levels in response to BMP-2. Transplantation experiments of GFP-expressing oral mucosal fibroblasts with gelatin/BMP-2 complexes revealed that 17.1% of the GFP-positive fibroblasts differentiated into ALP-positive cells, and these cells accounted for 6.2% of total ALP-positive cells in the ectopically formed bone. This study suggests that oral mucosal fibroblasts can differentiate into osteogenic cells in response to BMP-2. Thus, these cells are potential candidates for cell-mediated bone regeneration therapy in dentistry.

# INTRODUCTION

Bone formation and regeneration are mediated by the coordinate action of various factors. Among these factors, bone morphogenetic protein (BMP) plays crucial roles in bone formation and regeneration (Yamaguchi et al., 2000; Hirata et al., 2007). We have previously shown that BMP-2 not only stimulates osteoblastic differentiation of osteoprogenitors (Yamaguchi et al., 1991; Yamaguchi et al., 1996) but also transdifferentiates non-osteogenic mesenchymal cells to osteoblast lineage cells (Katagiri et al., 1990; Katagiri et al., 1994). Runx2 is essential for osteoblast differentiation and bone formation, because *Runx2*-deficient mice completely lacked bone formation ability due to maturational arrest of osteoblasts (Komori et al., 1997; Otto et al., 1997). Because BMP stimulates Runx2 expression during osteoblast differentiation (Yamaguchi et al., 2000; Ryoo et al., 2006), BMP is considered to play a crucial role in osteoblast differentiation, and hence bone formation and bone regeneration.

Several lines of evidence obtained from animal experiments have shown that transplantation of osteoblastic cells or genetically engineered non-osteogenic cells expressing osteoblast-related genes into bone defects effectively promotes bone regeneration (Breitbart et al., 1999; Franceschi et al., 2000; Krebsbach et al., 2000; Tamura et al., 2001; Gysin et al., 2002; Hirata et al., 2003). Although the use of such cells is a powerful therapeutic approach to cell-mediated gene therapy for critical-sized bone defects, several issues should be resolved for developing this technique and its potential clinical application. The first challenge is to identify the source of cells for genetic engineering and transplantation. Bone marrow stromal cells (Riew et al., 1998;

Musgrave et al., 2000; Cheng et al., 2001; Turgeman et al., 2001; Gysin et al., 2002; Partridge et al., 2002), periosteal cells (Breitbart et al., 1999), muscles (Riew et al., 1998; Lee et al., 2000; Cheng et al., 2001; Lee et al., 2001; Pelinkovic et al., 2001; Lee et al., 2002; Musgrave et al., 2002), and fibroblasts (Takayanagi et al., 1999; Franceschi et al., 2000; Krebsbach et al., 2000) have been successfully used as the source of transplanted cells in animal experiments. These cell types are strong candidates for clinical application, but these cause considerable challenge when applied in the dental clinics. Therefore, periodontal ligament cells (Seo et al., 2004; Hiraga et al., 2009) and dental pulp cells (Gronthos et al., 2000; Gronthos et al., 2002; Hosoya et al., 2007; Otaki et al., 2007) are being considered as candidates in the dental field. Second, before clinical application, the fate of the transplanted cells during bone regeneration should be traced in the preclinical experiments. For this purpose, green fluorescent protein (GFP) can be used to tag the source cells.

We have reported that skin fibroblasts (Hirata et al., 2003; Kadowaki et al., 2004) and osteoblastic cells (Kadowaki et al., 2004) isolated from GFP transgenic mice, which over express *BMP-2* by using an adenovirus vector, are good sources for cell-mediated bone repair. Furthermore, these cells are effective tools for tracing the fate of the transplanted cells during bone regeneration. Although the use of skin fibroblasts is a powerful therapeutic approach to cell-mediated gene therapy for critical-sized bone defects, dentists usually cannot easily procure skin fibroblasts by themselves. Therefore, we thought of oral mucosal fibroblasts as a candidate for cell-mediated bone regeneration therapy in dentistry.

In the present study, we performed *in vitro* and *in vivo* experiments to investigate whether oral mucosal fibroblasts are available as source cells for

cell-mediated bone regeneration therapy by using canine oral mucosal fibroblasts (COFs). Our *in vitro* study showed that COFs are capable of differentiating into osteoblastic cells in response to BMP-2. Furthermore, we transplanted the COFs expressing GFP with gelatin sponge/BMP-2 complexes into the subfascial regions of athymic mice; we found that oral mucosal fibroblasts retain the potency to differentiate into osteogenic cells in response to BMP-2. Thus, this study indicates that oral mucosal fibroblasts are a potential candidate for cell-mediated bone regeneration therapy in the dentistry.


## MATERIALS AND METHODS

### Cell culture and infection with a retrovirus expressing GFP

Canine buccal mucosal tissues, approximately $5 \times 5 \times 1$mm, were obtained from healthy 12-month-old beagle dogs (weight approximately 10kg). To allow the COFs to adhere to the surface of culture dishes, the samples were placed on the surface of 6-well dishes for 60 min at 37°C without any culture medium. The COFs were then cultured for 7 to 10 days in α-modified minimum essential medium (α-MEM; Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Gibco) and antibiotics (100 μg/ml penicillin G and 100 IU/ml streptomycin) at 37°C in 5% $CO_2$/95% air. The outgrown fibroblasts from the explants were harvested by digestion with 0.1% trypsin and 0.02% ethylenediaminetetraacetic acid (trypsin/EDTA) in calcium- and magnesium-free phosphate buffered saline (PBS(−)). Next, the harvested cells were inoculated on 10-cm dishes with α-MEM containing 10% FBS and antibiotics, and eventually transfected with retrovirus vectors.

COFs were transfected with replication-deficient retroviral vectors carrying the GFP gene (pLP-LNCX-GFP). The cells at the growing phase in the 10-cm dishes were incubated with media containing retrovirus (MOI, 200) for 2h at 37°C, and the culture media were then replaced with fresh media. The transfected cells were cultured for up to 6 days to analyze their osteoblastic differentiation potential *in vitro*. For transplantation experiments, the transfected COFs with GFP were immediately removed by trypsin/EDTA digestion after washing with PBS(−), and then washed with fresh medium. These cells were used for making transplantation complexes as described below. We used 4-8 passages cells for our *in vitro* and *in vivo* experiments.

**Measurement of alkaline phosphatase activity and histochemical staining**

The cells were inoculated at $4 \times 10^3$ cells/cm$^2$ in 48-well or 24-well plates, and treated for 3 or 6 days with various concentrations of recombinant human bone morphogenetic protein-2 (rhBMP-2), which was purchased from Osteogenetics GmbH (Wuerzburg, Germany). The cultured cells were sonicated in a radioimmunoprecipitation assay (RIPA) buffer to obtain cell lysate. Alkaline phosphatase (ALP) activity was determined using *p*-nitrophenylphosphate solution (Wako Pure Chemicals, Osaka, Japan) as the substrate. The amount of *p*-nitrophenylphosphate released was estimated by measuring the absorbance at 405nm after 30 minutes of incubation at 37°C. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo, Waltham, MA). For histochemical detection of ALP activity, the cultured cells were fixed in 10% phosphate-buffered formalin for 5 minutes, washed twice with 10mM Tris-HCl (pH 7.5), and then stained with ALP. ALP staining was performed at 37°C for 20 minutes by using nitrobluetetrazolium chloride (NBT) as theformazan dye

and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the enhancer (Roche, Mannheim, Germany). To assess a capability to generate mineralized bone nodules *in vitro*, we cultured COFs with osteogenic medium ($\alpha$-MEM containing 10% FBS, 10 mmol/L of $\beta$-glycerophosphate, 50 $\mu$g/ml of ascorbic acid, and antibiotics) in the presence of rhBMP-2 (200 ng/ml or 500 ng/ml) for 14 days, and then the cells were dual-stained with ALP and von Kossa.


**Reverse transcriptase-polymerase chain reaction analyses**

For reverse transcriptase-polymerase chain reaction (RT-PCR) analyses, total RNA was extracted from the cultured cells by using NucleoSpin (Macherey-Nagel, Duren, Germany). RNA aliquots were reverse transcribed to complementary DNAs by using an oligo(dT) primer (Roche), deoxynucleotide triphosphate (dNTP; Promega), and Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Fermentas, Hanover, MD). The complementary DNA products were subjected to PCR amplification with gene-specific primers for canine *Osteocalcin*, *Runx2*, and *Osterix* (Table 1). Real-time RT-PCR amplification was performed using the Light Cycler System (Roche) with a Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen, Carlsbad, CA). The relative amount of each mRNA was normalized to *β-2-microglobulin* mRNA (Brinkhof et al., 2006).


**Preparation and transplantation of gelatin sponge/BMP-2/COFs Complexes into athymic mice**

We used biodegradable gelatin sponges containing $\beta$-tricalcium phosphate (GS-$\beta$; MedGelCo.Ltd., Kyoto, Japan) (Takahashi et al., 2005) for a carrier of cell

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transplantation. Each GS-β disk was cut into a circular fragment (diameter, 4 mm; thickness, 2 mm). After the application of 10μl of rhBMP-2 solution (200ng/μl) on the GS-β disk (GS-β/BMP-2), they were freeze-dried and kept at −4°C until further use. As control, we prepared GS-β fragments that similarly absorbed only the buffer used for BMP-2 solution (GS-β alone). All procedures were carried out under sterile conditions.

After removing the retrovirus-infected cells from culture dishes by trypsin/EDTA digestion, they were re-suspended in the culture medium, and 1.5 ml aliquots of the culture medium containing $4 \times 10^6$ cells were transferred into a sterile serum tube containing GS-β/BMP-2 or GS-β alone. Each serum tube containing the cells and these carriers was shaken on a rotator (BR-40LF, Taitec, Japan) at 37°C for 2 hours. To ensure adhesion of the cells on GS-β, each GS-β fragment was allowed to stand for 1 hour at 37°C in 5% $CO_2$/95% air without any culture medium. Then, the GS-β and COFs complexes with rhBMP-2 (GS-β/BMP-2/COFs) or without rhBMP-2 (GS-β/COFs) were transplanted into the subfascial region of the back muscle of BALB/c Slc-nu/nu nude mice (8-week-old males) under anesthesia with pentobarbital (40 mg/kg). We also transplanted GS-β/BMP-2 as a positive control. For histological examination, we used 7 samples of GS-β/BMP-2/COFs group (3 for 1 week after transplantation and 4 for 2 weeks after transplantation), 6 samples of GS-β/COFs group (3 for 1 week after transplantation and 3 for 2 weeks after transplantation) and 3 samples of GS-β/BMP-2 group for 2 weeks after transplantation.

**Examination of the transplanted GS-β/BMP-2/COFs complexes**

The transplanted complexes of each group were dissected from each athymic mice at 1 or 2 weeks after the transplantation, and fixed in 4% paraformaldehyde solution for 4

hours at 4°C. These samples were subjected to radiographic and histological analyses. Soft X-ray photographs were obtained for radiographic analyses.

For histological analyses, the fixed samples were washed with PBS containing 6.8% sucrose for 12 hours at 4°C, and then directly immersed in acetone for 1 hour at 4°C. This quick dehydration process prevented a decrease in the intensity of GFP fluorescence. The dehydrated samples were embedded in Technovit 8100 (Heraeus Kulzer GmbH & Co., Germany), and 4-μm-thick undecalcified sections were prepared. These sections were tested for GFP fluorescence by conventional fluorescence microscopy. Some sections containing GFP-positive cells were processed for both ALP and von Kossa staining after recording the morphology of GFP fluorescence.

The number of GFP-positive cells was counted in selected areas by using enlarged photographs that were obtained before staining, and the total cell number in the same areas was counted using photographs obtained after dual staining of ALP and von Kossa with counterstaining with nuclear first red. The 1000-$\mu m^2$ square area used for cell counting was randomly chosen from the GS-$\beta$/BMP-2/COFs complexes, and the cell number was counted in 3 squares in each case.

All animal experiments were performed with the approval of the Animal Study Committee of Tokyo Medical and Dental University and conformed to relevant guidelines and laws.

**Statistical analyses**

Statistical analyses were performed using Student's *t*-test. *P*-values of less than 0.05 were considered significant. The data are presented as mean ± standard error of mean (SEM) of independent replicates.

## RESULTS

**Isolation of canine COFs and transfection with GFP**

Numerous fibroblastic cells had migrated from the canine buccal mucosa explants after

7-10 days of culture. We isolated these outgrowing fibroblastic cells by trypsin/EDTA

digestion, and cultured these cells in different culture dishes. These cultured cells

showed spindle-shaped cytoplasm with elongated nuclei, which appearances typical of

fibroblasts. We examined the efficiency of GFP transfection of COFs. As shown in Fig.

1A, numerous cultured COFs (over 90%) expressed GFP under the fluorescence

microscope. We also investigated GFP expression in the GS-β/COFs complexes before

transplantation. As shown in Fig. 1B, though GS-β exhibited strong fluorescence,

GFP-positive COFs were present in the GS-β/COFs complex even before

transplantation. These results indicate that the transfection efficiency of GFP is suitable

for further *in vivo* transplantation experiments.


**COFs differentiate into osteoblastic cells in response to BMP-2 in *in vitro***

To investigate whether BMP-2 stimulates the COFs to differentiate into osteoblastic

cells, we first tested the effects of rhBMP-2 on ALP activity using GFP-transfected

COFs. Treatment with various concentrations of rhBMP-2 for 3 days showed a

significant increase in ALP activity at concentration of 500 ng/ml, compared to the

control culture (Fig. 2A), and the 6-day treatment showed significant increase its

activity over 250 ng/ml of rhBMP-2 (data not shown). Histochemical analysis for ALP

also showed apparent increases in the number of ALP-positive cells after 6-day

treatment with over 250 ng/ml of rhBMP-2 (Fig. 2B-G). To further confirm the effects

of rhBMP-2 on osteoblast differentiation, the expression of mRNAs related to osteoblast differentiation was examined by real time RT-PCR analyses. Treatments with rhBMP-2 (500 ng/ml) for 3 days and 6 days significantly increased *Runx2*, *Osterix* and *Osteocalcin* mRNA expression, respectively (Fig. 3A, B, C), as compared to the expression level observed in cultures without BMP-2 treatment. These results indicated that COFs differentiate into osteoblastic cells in response to BMP-2. To further confirm a capability of COFs to generate mineralized bone nodules *in vitro*, we cultured these cells for 14 days with osteogenic medium. This treatment, however, failed to generate the mineralized bone nodules in the presence of rhBMP-2 (250 or 500 ng/ml)(data not shown).

**Transplantation of GS-β/BMP-2/COFs complex induces ectopic bone formation**

Figure 4 summarizes the typical features of the 3 types of transplanted complexes (GS-β/COFs, GS-β/BMP-2/COFs and GS-β/BMP-2) as seen on soft X-ray radiographs. The control complex (GS-β/COFs) showed weak radiopaque structures after 1 and 2 weeks of transplantation (Fig. 4A, C), which reflects the β-tricalcium phosphate incorporated in the GS-β. Transplants in GS-β/BMP-2/COFs group showed more extensive radiopaque area after 1 week of transplantation (Fig. 4B), and exhibited more extensive and higher radiopaque regions after 2 weeks of transplantation (Fig. 4D), as compared to the areas observed in the GS-β/COFs group. The radiopaque areas observed in GS-β/BMP-2/COFs group were similar to those in GS-β/BMP-2 group after 2 weeks of transplantation (Fig. 4E).

We next performed histological analyses of the 3 types of complexes (GS-β/COFs, GS-β/BMP-2/COFs and GS-β/BMP-2) after transplantation into athymic

mice. We found scattered foci of ALP-positive cells along with GS-β after 1 week of transplantation in the GS-β/BMP-2/COFs group (Fig. 5B). Some of these foci were associated with mineralized bone formation. After 2 weeks of transplantation, extensive bone formation associated with ALP-positive cells, which covered the bone surface, was observed in the GS-β/BMP-2/COFs group (Fig. 5F). Although histological features of transplants between GS-β/BMP-2 (Fig. 5C and D) and GS-β/BMP-2/COFs (Fig. 5E and F) groups were basically similar, extensive accumulation of ALP-positive cells was observed at some areas in GS-β/BMP-2/COFs group (Fig. 5E). Decalcified paraffin sections more clearly showed numerous osteocytes embedded in bone matrix (Fig. 5G). We found a small amount of cartilage foci in GS-β/BMP-2 (Fig. 5H) and GS-β/BMP-2/COFs (Fig. 5I) groups after 2 weeks of transplantation. Histological examination of the transplanted GS-β/COFs complex revealed neither apparent ALP-positive osteoblasts nor cartilage formation after 1 and 2 weeks of transplantation (Fig. 5A).

**Localization of COFs in BMP-2-induced ectopic bone formation**

Next, we investigated the localization of GFP-positive cells to prove the possible role of transplanted COFs in ectopic bone formation. We first recorded the localization of GFP-positive cells under a fluorescence microscope by using Technovit 8100-embedded undecalcified sections. Then, the same sections were stained with both ALP and von Kossa, and pictures of the fields corresponding to those recorded by fluorescence microscopy were captured. We compared the 2 picture sets and confirmed the localization of GFP-positive COFs in the ectopically formed bone tissues. Because GS-β showed strong fluorescence and mineralized bone and red blood cells exhibited

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weak fluorescence, we carefully observed and compared the 2 picture sets to definitively evaluate GFP-positive COFs.

GFP-positive cells were detected around bone tissues (Fig. 6 B, D). We could not find apparent GFP-positive mature osteoblasts directly covering the bone surface, but we did find cells positive for both GFP and ALP in the areas adjacent to the bones (Fig. 6, red arrows). We also observed GFP-positive and ALP-negative cells around the bones (Fig. 6, yellow arrows). To confirm these observations, we counted the number of ALP-positive and GFP-positive cells. As summarized in Table 2, ALP-positive cells comprised 59% of the total cells, and GFP-positive cells accounted for 23% of the total cells. Among the GFP-positive cells, 17.1% were also positive for ALP. Among the ALP-positive cells, 6.2% were also positive for GFP. Although a small amount of cartilage foci was observed in GS-β/BMP-2 and GS-β/BMP-2/COFs groups after 2 weeks of the transplantation (Fig. I and H), we found no GFP-positive cells in these foci.

## DISCUSSION

We first investigated whether oral mucosal fibroblasts could transdifferentiate into osteoblastic cells *in vitro* in response to BMP-2. For this purpose, we choose COFs isolated from the buccal mucosa of beagle dogs, because this species is a suitable model for preclinical experiments (Ducy et al., 1997; Neff and Rine, 2006). Our study showed that BMP-2 increased the ALP activity, which is an early-stage marker of osteoblast differentiation, and the *Osteocalcin* mRNA expression, which is a late-stage marker of osteoblast differentiation. Furthermore, BMP-2 treatment increased the mRNA expression levels of *Runx2* and *Osterix*, which encode essential transcription factors

(Runx2 and Osterix) for osteoblast differentiation and bone formation (Ducy et al., 1997; Komori et al., 1997; Nakashima et al., 2002). These results suggest that COFs can differentiate into osteoblast lineage cells in response to BMP-2 by *in vitro* assay system. In addition to these osteoblast-related markers, mineralization capability is another important character to identify osteogenic potential *in vitro*. We reported successful generation of the mineralized bone-like nodules in rat calvarial primary osteoblastic cells (Wada et al., 1998). Unfortunately, we could not reproduce such typical mineralized nodules in COFs culture even in the presence of additional BMP-2 supplement. Although the exact reason why mineralized bone-like nodules were not generated in COFs culture is obscure, the differentiation capability into osteogenic lineage cells from canine oral mucosa might be more restricted than that in rodent in culture system. This phenomenon is similar to fibroblastic cells isolated from human subcutaneous and muscle tissues as we previously reported (Kawasaki et al., 1998).

To confirm whether transplanted COFs can differentiate into osteoblast lineage cells in response to BMP-2 *in vivo*, we transplanted GFP-expressing COFs by using the GS-β/BMP-2 complex as the carrier (GS-β/BMP-2/COFs complex) into the back muscle of athymic mice. We found that transplantation of the GS-β/BMP-2/COFs complex induced ectopic bone formation. We carefully examined the localization of GFP-positive cells and ALP-positive cells by using undecalcified sections. We found that 17.1% of GFP-positive COFs differentiated into ALP-positive cells, and that these cells accounted for 6.2% of the total ALP-positive cells in the ectopically formed bones. We also found that all cells positive for both GFP and ALP were located in the area adjacent to bone but not on the bone surface. This distribution pattern suggests that the transplanted COFs could differentiate into osteoprogenitor cells at ectopically formed

bones, but not into mature osteoblasts. The present study also suggested that mature

bone forming osteoblasts at the ectopic bone formation regions in GS-β/BMP-2/COFs

group were originated from mesenchymal stem cells of host animals as well as in the

case of GS-β/BMP-2 group.

In our previous study (Hirata et al., 2003), which was conducted using the

same technique applied in this study, we showed that mouse skin fibroblasts isolated

from GFP transgenic mice, which overexpressed *BMP-2* by adenoviral transfection,

participated in bone repair when these cells were transplanted into bone defects in

wild-type mice. Transplantation of these cells also facilitated bone repair of

critical-sized bone defects created in parietal bones. In the previous study (Hirata et al.,

2003), we used *BMP-2*-overexpressing skin fibroblasts, but in the present study, we did

not use COFs that overexpressed this gene because we wanted to investigate whether

COFs could differentiate into osteoblastic cells in response to exogenous BMP-2. This

study revealed that COFs could differentiate into osteoblastic cells in response to

exogenous BMP-2, but failed to differentiate into mature osteoblasts. These results

suggest that simple application of non-osteogenic cells is not enough to involve these

cells in active bone formation, and overexpression of BMP-2 in transplanted COFs is

necessary to effectively induce more mature osteoblasts that possess the bone forming

ability after transplantation. Our study to further test this possibility is underway.

BMP-2 is a strong inducer for bone formation as well as cartilage formation. In

the present study, a small amount of cartilage foci was observed in GS-β/BMP-2 and

GS-β/BMP-2/COFs groups after 2 weeks of the transplantation (Fig. I and H). We

previously reported that transplantation of skin fibroblasts transduced with *BMP-2* gene

generated similar cartilage foci (Hirata et al., 2003), and showed that a small number of

chondrocytes in these foci were originated from the transplanted fibroblasts by assessment of GFP expression. In this study, however, we could not found GFP signals in cartilage foci, suggesting that chondrocytes observed in the present study were originated from the host cells as observed in GS-β/BMP-2 group. The differences between our previous study and the present study were an application technique of BMP-2; we overexpressed *BMP-2* gene in skin fibroblasts by using adenovirus vector in the former study and we applied exogenous recombinant BMP-2 using GS-β as a carrier in the latter study. Taken together, it is suggested that overexpression of BMP-2 in transplanted COFs might be necessary to directly induce the trans-differentiation of COFs to chondrocytes.

In summary, we demonstrated that COFs could differentiate into osteoblastic cells in response to exogenous BMP-2 *in vitro* and *in vivo*. Our results suggest that oral mucosal fibroblast is a potential candidate for cell-mediated bone regeneration therapy. Currently, the most popular cell source for cell-mediated bone regeneration therapy is mesenchymal stem cells isolated from bone marrow; however, the application of these cells is inconvenient for dentists because the cells are usually isolated from iliac bones or vertebrae. We believe that the application of oral mucosal fibroblasts to cell-mediated bone regeneration therapy will provide a unique and convenient opportunity to develop new bone regeneration therapies in the field of dentistry.

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**FIGURE LEGENDS**

Figure 1

A: Fibroblasts isolated from canine buccal mucosa (COFs). These cells show spindle-shaped cytoplasm with elongated nuclei. Numerous cultured cells expressed GFP signals under the fluorescence microscopic observation. B: Localization of GFP-positive COFs in GS-β/COFs complex before transplantation. GFP-positive COFs (white arrows) are observed in the GS-β/COFs complex. GS-β (pink asterisks) shows strong fluorescence. Bars: 50μm in A and B.


Figure 2

A: Effects of rhBMP-2 on ALP activity in COFs. The cells were cultured for 3days with various concentrations of rhBMP-2, and ALP activity was measured as described in Materials and Methods. $*p<0.05$. B-G: Effects of rhBMP-2 on histochemical detection of ALP in COFs. The cells were cultured for 6 days with various concentrations of rhBMP-2, and they were processed for histochemical staining of ALP as described in Materials and Methods. Concentrations of rhBMP-2: B; 0 ng/ml (control), C; 1 ng/ml, D; 50 ng/ml, E; 100 ng/ml, F; 250 ng/ml, G; 500 ng/ml.


Figure 3

Effects of rhBMP-2 on mRNA expression of *Runx2* (A), *Osterix*(B), and *Osteocalcin* (*OCN*) (C) in COFs. The cells were cultured for 3 or 6 days with or without rhBMP-2 (500 ng/ml), and mRNA expression was determined as described in Materials and Methods. Relative expression level of each mRNA shown in Figure 3 was normalized