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ABSTRACT

Regenerative medicine is based on stem cells, signals, and scaffolds. Dental pulp tissue has the potential to regenerate dentin in response to noxious stimuli, such as caries. The progenitor/stem cells are responsible for this regeneration. Thus, stem cell therapy has considerable promise in dentin regeneration. Culture of porcine pulp cells, as a three-dimensional pellet, promoted odontoblast differentiation compared with monolayers. The expression of *dentin sialophosphoprotein (Dspp)* and *enamelysin/matrix metalloproteinase 20 (MMP20)* mRNA confirmed the differentiation of pulp cells into odontoblasts and was stimulated by the morphogenetic signal, bone morphogenetic protein 2 (BMP2). Based on the *in vitro* experiments, an *in vivo* evaluation of pulp progenitor/stem cells in the dog was performed. The autogenous transplantation of the BMP2-treated pellet culture onto the amputated pulp stimulated reparative dentin formation. In conclusion, BMP2 can direct pulp progenitor/stem cell differentiation into odontoblasts and result in dentin formation. Abbreviations: BMP2, bone morphogenetic protein 2; *Dspp, dentin sialophosphoprotein; Dmp1, dentin matrix protein 1; ALPase, alkaline phosphatase; MMP20, matrix metalloproteinase 20; Phex, phosphate-regulating gene with homologies to endopeptidases on X-chromosome.*

KEY WORDS: dentin regeneration, stem cell therapy, BMP2, dental pulp-capping, pellet culture.

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INTRODUCTION

The dental pulp contains progenitor/stem cells, which can proliferate and differentiate into dentin-forming odontoblasts (Nakashima *et al.*, 1994; Gronthos *et al.*, 2000, 2002). Damaged odontoblasts can be replaced by newly generated populations of odontoblasts derived from stem cells from pulp (Tziafas *et al.*, 2000). Following physiological stimulation or injury, such as caries and operative procedures, stem cells in pulp may be mobilized to proliferate and differentiate into odontoblasts by morphogens released from the surrounding dentin matrix (Tziafas *et al.*, 2000). Tissue engineering with the triad of dental pulp progenitor/stem cells, morphogens, and scaffolds may provide a useful alternative method for pulp-capping and root canal treatment (Nakashima and Reddi, 2003). In pulp cell therapy, the technique for manipulation of the growth of the isolated pulp progenitor/stem cells and induction of three-dimensional tissue formation *in vitro* needs to be developed. Naturally derived collagen or synthetic materials such as polyglycolic acid (PGA) are used as a scaffold for attachment and guidance of cells (Putnam and Mooney, 1996). The pulp-derived fibroblasts adhering to the PGA fibers can proliferate and form a new tissue similar to that of native pulp (Mooney *et al.*, 1996). The synthetic matrices, however, must undergo degradation simultaneously with the new tissue formation by the cultured cells. An alternative to the use of synthetic matrix with variable degradation rate is the use of three-dimensional cultures with an assembly of endogenous extracellular matrix or scaffold. A three-dimensional *in vitro* culture system has been developed for chondrocytes (Kato *et al.*, 1988; Ballock and Reddi, 1994), bone marrow stromal cells (Yoo *et al.*, 1998), and intervertebral disc cells (Lee *et al.*, 2001). This system involves formation of cell pellets or aggregates by a one-step centrifugation method, allowing for three-dimensional interaction between the neighboring cells, and is followed by synthesis of extracellular matrix in the pellet. Cell-cell interactions and environmental cues are important in modulation of the phenotype of cells grown *in vitro*.

Bone morphogenetic proteins (BMPs) have been implicated in tooth development, and the expression of BMP2 is increased during the terminal differentiation of odontoblasts (Nakashima *et al.,* 1994; Nakashima and Reddi, 2003). Beads soaked in human recombinant BMP2 induce the mRNA expression of *Dspp*, the differentiation marker of odontoblasts after implantation onto dental papilla in organ culture. BMP2 also induces a large amount of reparative dentin on the amputated pulp *in vivo* (Nakashima, 1994a). It has been suggested that BMP2 may regulate the differentiation of pulp cells into odontoblastic lineage and stimulate reparative dentin formation (Nakashima and Reddi, 2003). We have compared three-dimensional pellet culture system with monolayer cultures. The efficacy of BMP2 on the differentiation of pulp cells into odontoblasts was also

examined with the use of this pellet culture system. In addition, we investigated cell therapy *in vivo* for dentin regeneration.

MATERIALS & METHODS

Three-dimensional Pellet Culture of the Pulp Cells

The porcine premolar pulp was isolated, and the pulp cells were separated enzymatically as previously described (Nakashima, 1991). The pellet culture of the porcine pulp cells was then performed (Kato *et al.*, 1988; Ballock and Reddi, 1994). Briefly, 1-mL aliquots containing 2 x 10^5 cells were centrifuged in a 15-mL conical polypropylene tube (Asahi techno glass corp., Tokyo, Japan) at 1000 rpm for 5 min. Pellets were maintained in Dulbecco's modified essential medium (Life Technologies, Rockville, MD, USA) supplemented with 10% heat-inactivated bovine calf serum (JRH Biosciences, Lenexa, KS, USA) and 50 μg/mL L-ascorbic acid phosphate (Wako pure chemical industries, LTD, Osaka, Japan), and penicillin-streptomycin. The medium was changed two times *per* wk. From day 28 on, the medium was supplemented with Pi to a 1-mM final concentration. For comparison, a traditional monolayer culture was performed at a density of 1 x 10^5 cells/mL in a 35-mm dish. In some experiments, recombinant human BMP2 (10, 25, 50, 100, and 200 ng/mL) (kindly provided by Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan) was added to the medium 1 hr before centrifugation after enzymatic isolation.

Total Cell Number and Alkaline Phosphatase Activity

The cells were dispersed by trypsin and counted at each time point during pellet culture and monolayer culture. For analysis of alkaline phosphatase activity, the pellet or the cells at each time points were sonicated and assayed by the method of Lowry *et al.* (1954).

Tissue Morphology

Pellets on days 7, 14, and 21 were fixed in 4% paraformaldehyde overnight and processed for paraffin-embedding. The sections of 4.5-μm thickness were stained in hematoxylin and eosin (H&E). The mineralization was confirmed by Alizarin Red staining.

Quantification of Collagen Type I and Type III Syntheses

To determine the effect of BMP on collagen type I and type III syntheses, we stained the paraffin-embedded sections of the pellet with Picro-sirius red (Junqueira *et al.*, 1979) on day 21 and observed them by light microscopy with polarizing filters. Surface areas of collagen fibers were measured, and quantitative analysis was performed with the use of Image J 1.30 software.

Real-time RT-PCR

For each time point on days 10, 14, and 21, total cellular RNA was isolated with the use of Trizol reagent (Invitrogen, Carlsbad, CA, USA). First-strand cDNA syntheses were performed by reverse transcription with the SuperScript pre-amplification system (Invitrogen). The design of the oligonucleotide primers was based on published cDNA sequences (Table). Real-time PCR for *β-actin, α1(I)collagen, dentin matrix protein 1, dentin sialophosphoprotein, Osterix, enamelysin/MMP20, phosphate-regulating gene with homologies to endopeptidases on the X-chromosome (Phex), Cbfa1*, and *Cbfa3* was performed with Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics, Tokyo, Japan) by Light Cycler 330 (Roche Diagnostics). Those RT-PCR products were subcloned into pCR2.1-TOPO vector (Invitrogen) and confirmed by sequencing.

Transplantation of the Pellet on the Canine Amputated Pulp

Twenty-four teeth from 6 young adult dogs were used. Surgical anesthesia was obtained in the dogs by intraveneous administration of pentobarbital sodium. The upper incisor pulp was extracted and autogenous pellets were prepared as described earlier for porcine pulp. A surgical exposure was made in the canine, and the amputation was carried out. Pellet cultures were applied to the amputated pulp on day 14, and the cavity was filled with glass-ionomer cement and composite resin. Four wks after transplantation, dentin formation was examined in the serial paraffin sections. Our animal use protocols (porcine and canine) were reviewed and approved by the Kyushu University Institutional Review Board.

RESULTS

Comparison of the Pellet Culture with the Normal Monolayer Culture

First, the sequential morphological changes in the pellet were examined. The pulp cells were oval or polygonal, and the nuclei contained a few round or ovoid nucleoli. The pellet progressively became spherical on days 14 and 21. On day

Table. Primers for Real-time RT-PCR

Name		5′ ← Sequence → 3′	Product Size	Accession Number
β-actin	Forward	CTGGGGCCTAACGTTCTCAC	198 bp	BI118314
	Reverse	GTCCTTTCTTCCCCGATGTT		
α1(I)collagen	Forward	AAGGACAAGAGGCACGTCTG	166 bp	BI233976
	Reverse	CGCTGTTCTTGCAGTGGTAG		
Dentin matrix protein 1	Forward	TGGGGATTATCCTGTGCTCT	177 bp	AY524986
	Reverse	GCTGTCACTGGGGTCTTCAT		
Dentin sialophosphoprotein	Forward	GGAATGGAGAGAGGACTGCT	174 bp	AF332578
	Reverse	AGGTGTTGTCTCCGTCAGTG		
Osterix	Forward	ACCAATGGGCTCCTGTCAC	163 bp	AY514037
	Reverse	CACTGGGCAGACAGTCAGAA		
Enamelysin/MMP20	Forward	CACTGTTGCTGCTCACGAAT	182 bp	SSU54825
	Reverse	CAGTGGGCTTTCCTGTGAAT		
Phex	Forward	GTGGATACTGCCGTGCTTTT	182 bp	AY514036
	Reverse	CAGTCGAACTGGGGAATCAT		
Cbfa1	Forward	AAGCTGAAACGGTTCCTCAC	188 bp	BE234439
	Reverse	GCCTTCAAAAATGGGATGAC		
Cbfa3	Forward	CAGAAGCTGGAGGACCAGAC	170 bp	AY515225
	Reverse	GGGTTCAGGTCCGAGGTG		

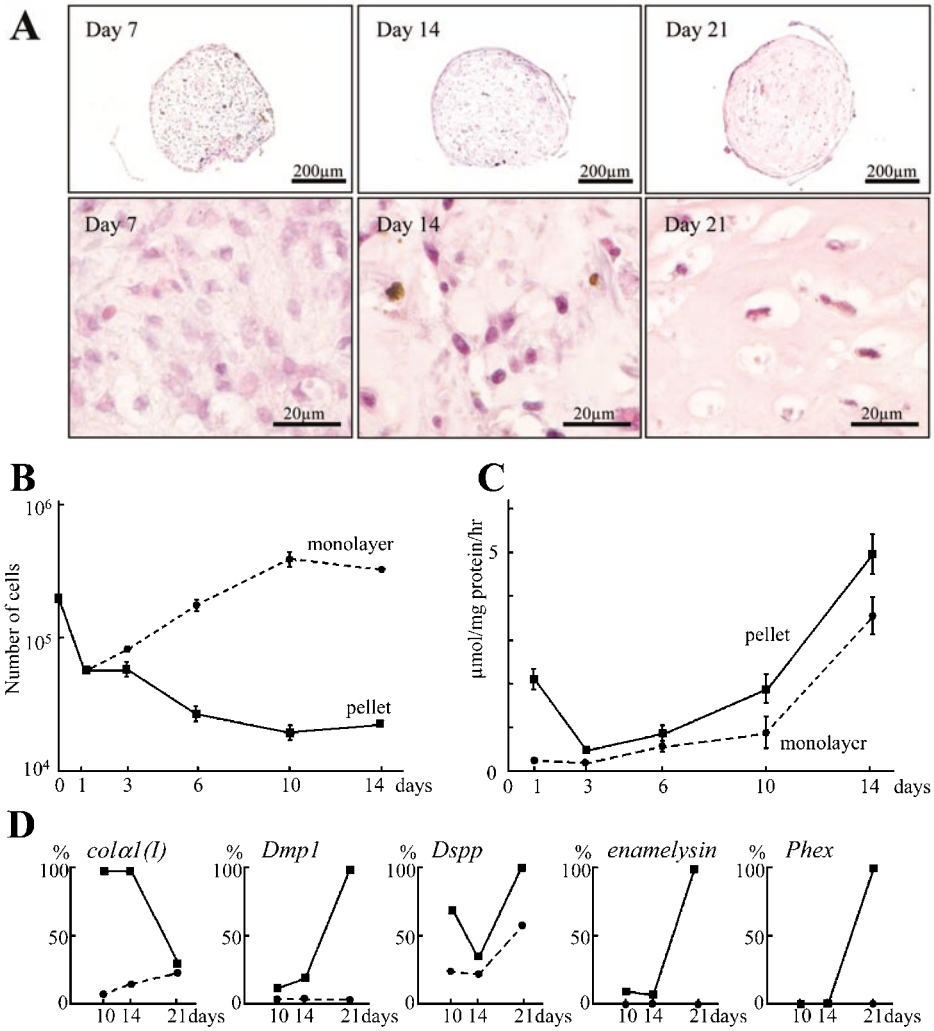

Figure 1. The three-dimensional pellet culture for porcine primary pulp cells compared with the monolayer culture. **(A)** Morphological changes in the pellet during culture on day 7, day 14, and day 21 (H-E stain). Note the decrease of cell number and increased matrix on day 21. **(B)** The changes in cell numbers during pellet cultures of porcine pulp cells compared with monolayer culture in 35-mm dish. Each point is expressed as the mean ± SD of 6 determinations. Note the lack of proliferation of cells in pellet cultures. **(C)** The changes in alkaline phosphatase activity. Each point is expressed as the mean ± SD of 6 determinations. **(D)** Real-time RT-PCR analysis of *α1(I)collagen, Dmp1, Dspp, enamelysin/MMP20,* and *Phex* expression in pellet cultures compared with monolayer cultures. The experiment was repeated 3 times, and 1 representative experiment is presented. The relative percentage of expression was shown in relation to the highest value as 100% after normalization against *β-actin.* Solid lines represent pellet cultures and dashed lines monolayer cultures.

14, the cell density decreased, the cells became basophilic, and the extracellular matrix accumulated. On day 21, pellet cultures contained cells surrounded by newly formed matrix, osteodentin. The cells were faintly basophilic, and their nuclei were round or oval and darkly staining (Fig. 1A).

In general, there was minimal cell proliferation in pellet cultures compared with monolayers as enumerated by cell numbers (Fig. 1B). In contrast, the cells in the monolayer culture proliferated. The alkaline phosphatase activity gradually increased and was significantly higher on day 10 and day 14 in the pellet compared with the monolayer culture (Fig. 1C). The mRNA expression of *α1(I)collagen* was much higher on days 10 and 14 in pellet cultures than that in monolayer cultures, indicating extracellular matrix accumulation. The expression of *Dmp1, Dspp, enamelysin*, and *Phex,* differentiation markers, was much increased on day 21, compared with expression in the monolayer culture (Fig. 1D).

Effect of BMP2 on Differentiation and Mineralization in the Pellet Culture

We performed morphological and molecular biological evaluations to examine the efficacy of BMP2 in pellet cultures. BMP2 did not affect cell proliferation (Fig. 2A). Alkaline phosphatase activity was increased by BMP2 in a dose-dependent manner (Fig. 2B) on day 14. Extensive osteodentin formation was observed in the recombinant human BMP2-treated pellet on day 21 (Fig. 2C). Collagenous matrix accumulation was more in the BMP2-treated pellet compared with control pellets on day 21 (Fig. 2D). The quantitative analyses demonstrated that the amount of collagen fibers was significantly higher in the BMP2-treated pellet compared with the non-treated pellet on day 21 (Fig. 2E). The expression of *α1(I)collagen, Osterix,* and *Cbfa1* was higher on day 10 in the BMP2-treated pellet than that in the non-treated pellet. The expression of *Dmp1, enamelysin,* and *Phex* was increased in the BMP2-treated pellet on day 14. Relative expressions of *Dmp1, Dspp, enamelysin, Phex,* and *Cbfa3* in the BMP2-treated pellet were 7.4, 2.4, 6.0, 11.7, and 5.5 times more, respectively, compared with expressions in the non-treated pellet on day 21 (Fig. 2F). The mineralization was intense in the BMP2-treated pellet on day 35 compared with that in the non-treated pellet (Fig. 2G). These findings demonstrated that BMP2 stimulated differentiation of pulp cells into the odontoblastic lineage.

Effect of BMP2 on Reparative Dentin Formation in the Pulp Cells

Autogenous transplantation of the pellet was performed on the canine amputated pulp. Pulp cells labeled by transfection with adenovirus delivering *CMV-lacZ* cDNA before the pellet was made showed transgene *lacZ* expression in the pulp cells 14 days after transplantation (Figs. 3A, 3B), indicating successful transduction. We examined the feasibility of therapeutic reparative dentinogenesis using the pellet on the amputated

pulp. In the control group, fibrodentin formation was not observed (Fig. 3C). Fibrous osteodentin matrix was observed in the cavity on the amputated pulp 4 wks after transplantation of the non-treated pellet (Fig. 3D). Tissue necrosis and inflammation were not observed. This suggested that the transplanted pellet was involved in reparative dentin formation *in vivo*. The osteodentinoblasts produced extracellular matrix around them (Fig. 3F). Large amounts of osteodentin were seen in the BMP2-treated pellet (Fig. 3E), compared with the non-treated pellet. A few osteodentinocytes were observed in the osteodentin (Fig. 3G). Odontoblast-like cells with long processes attached to the osteodentin to form tubular dentin (Fig. 3H).

DISCUSSION

Cell therapy utilizing pulp progenitor/stem cells has the potential to improve on conventional pulp-capping with calcium hydroxide or other artificial materials that can induce only a small amount of reparative dentin beneath the exposed or amputated site of the pulp. As a first step toward the goal of successful development of cell therapy in clinical dentistry, we developed the three-dimensional pellet culture system using porcine dental pulp cells. This method results in an endogenous scaffold of collagenous extracellular matrix after treatment with BMP2. When human pulp progenitor/stem cells with hydroxyapatite/tricalcium phosphate as a scaffold were implanted into immunocompromised mice, tubular dentin was formed (Gronthos *et al.*, 2000, 2002). The porcine pulp cells grown in the monolayer culture underwent a characteristic process of dedifferentiation and redifferentiation, marked by a loss of *α1(I)collagen* on day 10 and by an increase of *α1(I)collagen* and ALPase activity on day 14 following proliferation and differentiation. The expression of Dspp, the differentiation marker of odontoblasts, was increased later, but not that of *enamelysin/MMP20* and *Phex*. In this study, the dental pulp cells exhibited minimal proliferation in the pellet, as analyzed by total cell number, correlating well with previous studies with other cells (Chiba *et al.*, 1998; Lee *et al.*, 2001). The enhanced expression of *enamelysin/MMP20* and *Phex* on day 21 in pellet cultures, compared with monolayer cultures, suggested that the differentiation of pulp

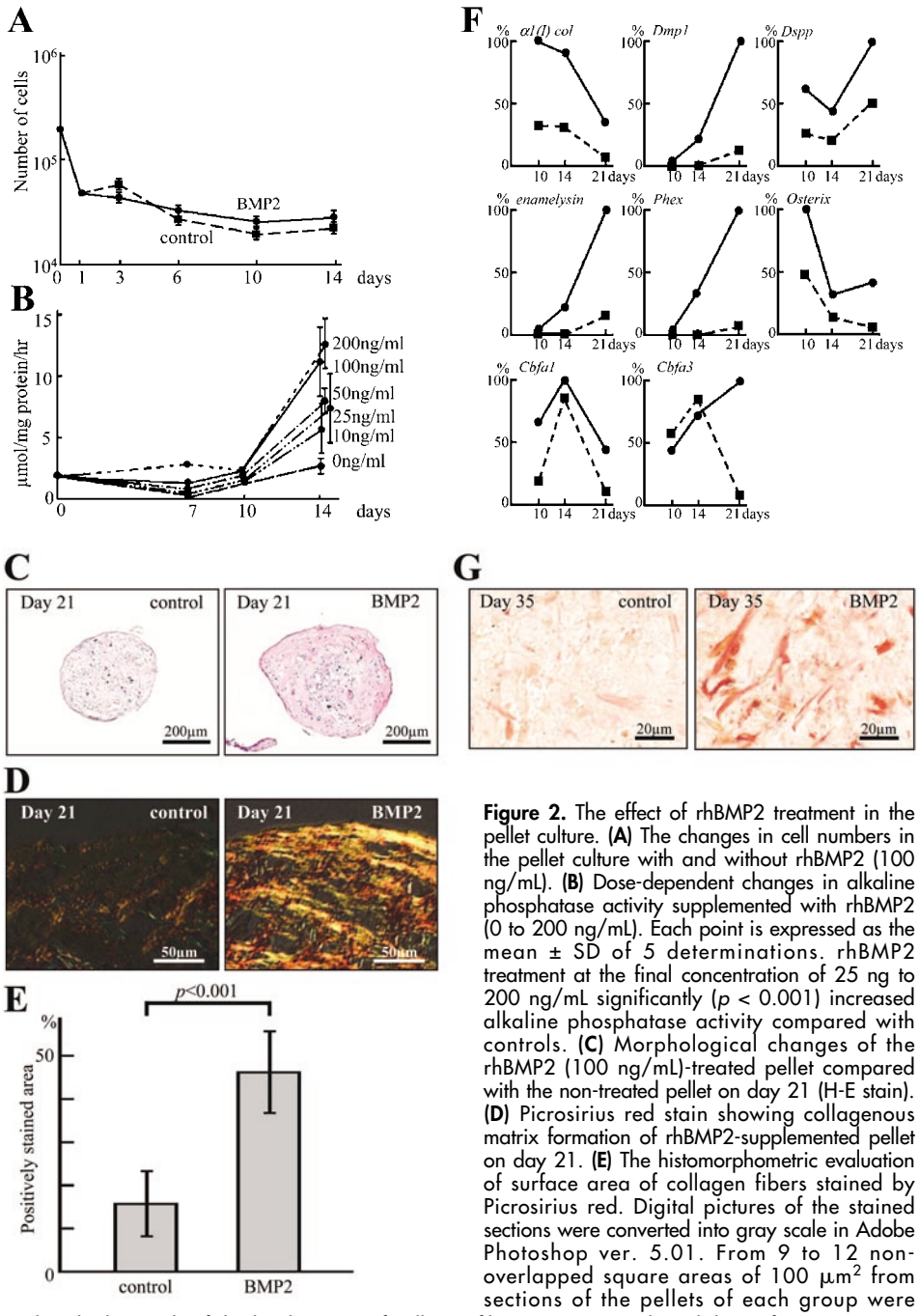

Figure 2. The effect of rhBMP2 treatment in the pellet culture. **(A)** The changes in cell numbers in the pellet culture with and without rhBMP2 (100 ng/mL). **(B)** Dose-dependent changes in alkaline phosphatase activity supplemented with rhBMP2 (0 to 200 ng/mL). Each point is expressed as the mean ± SD of 5 determinations. rhBMP2 treatment at the final concentration of 25 ng to 200 ng/mL significantly ($p < 0.001$) increased alkaline phosphatase activity compared with controls. **(C)** Morphological changes of the rhBMP2 (100 ng/mL)-treated pellet compared with the non-treated pellet on day 21 (H-E stain). **(D)** Picrosirius red stain showing collagenous matrix formation of rhBMP2-supplemented pellet on day 21. **(E)** The histomorphometric evaluation of surface area of collagen fibers stained by Picrosirius red. Digital pictures of the stained sections were converted into gray scale in Adobe Photoshop ver. 5.01. From 9 to 12 non-overlapped square areas of 100 μm^2 from sections of the pellets of each group were analyzed. The pixels of the bright areas of collagen fibers were counted, and the surface areas were calculated. The values are expressed as average ± standard deviations. Statistical analysis was performed by the non-paired *t* test. Note significant increase by rhBMP2 treatment. **(F)** Real-time RT-PCR analysis of *α1(I)collagen, Dmp1, Dspp, enamelysin/MMP20, Phex, Osterix, Cbfa1,* and *Cbfa3* expression in the pellet culture with rhBMP2 (100 ng) compared with cultures without rhBMP2. The experiment was repeated 4 times, and 1 representative experiment is presented. Solid lines represent pellet cultures and dashed lines monolayer cultures. **(G)** Alizarin Red staining showing mineralization of rhBMP2-supplemented pellet on day 35.

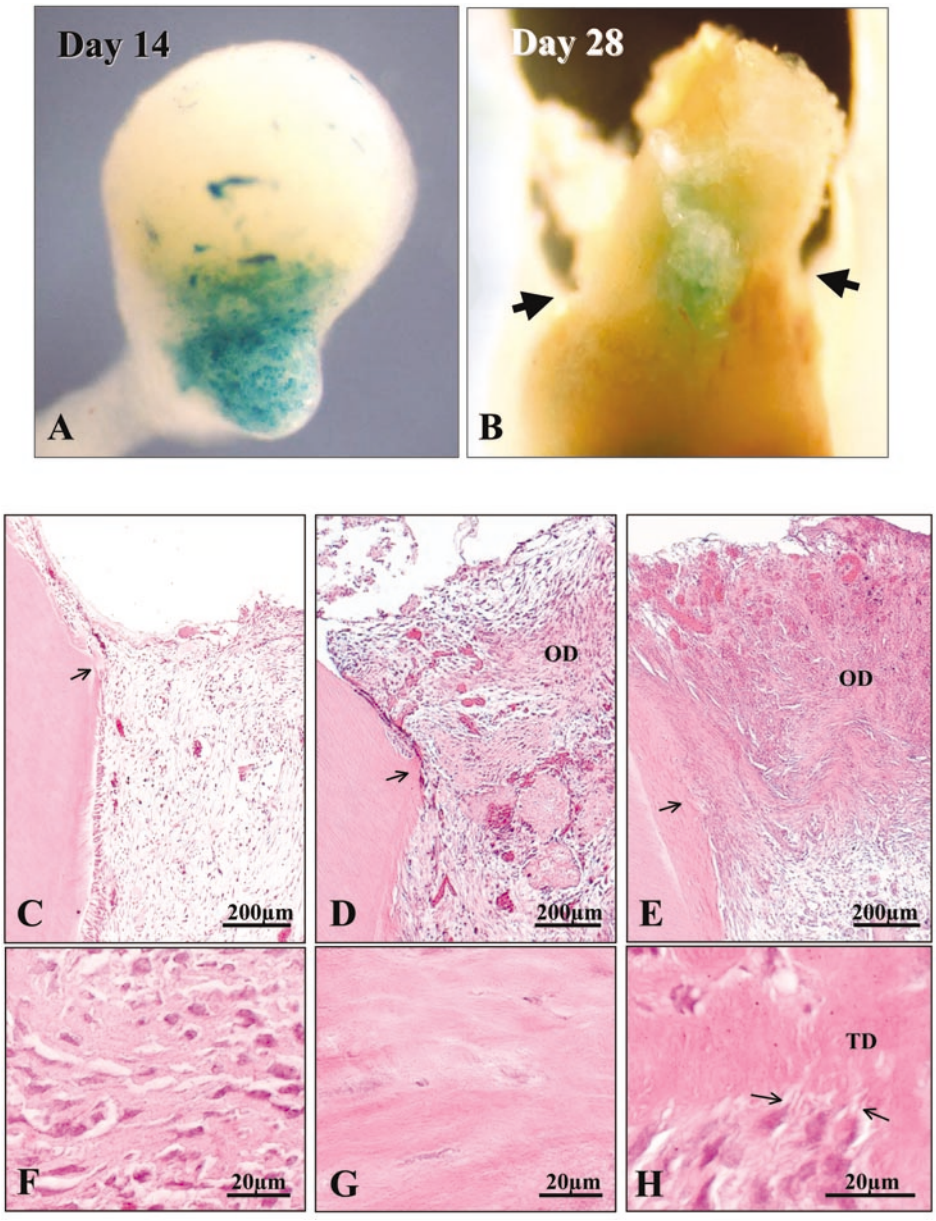

Figure 3. The autogenous transplantation of the pellet culture on the canine amputated pulp. **(A)** Canine pulp cells transduced with adenovirus *lacZ* before the pellet culture began and stained by β-galactosidase on day 14. **(B)** The implanted pellet on the amputated pulp showing *lacZ* transgene expression on day 28. The amputated site (arrow). **(C)** The amputated pulp without transplantation of the pulp cell pellet. Note no osteodentin matrix formation after 4 wks. **(D)** The *in vivo* transplantation of the pellet without rhBMP2. **(E)** The transplantation with rhBMP2. Note the formation of the thicker osteodentin matrix (OD) beneath the amputated site (arrows) in response to cell therapy with rhBMP2 compared with cultures without rhBMP2. **(F-H)** Higher magnification of osteodentin. Fewer cells and more homogenous matrix surrounding cells in rhBMP2-supplemented implantation (G) compared with that without rhBMP2 (F). Note the dentinal tubes (arrows) in the matrix (H).

cells into odontoblastic lineage was more advanced. Enamelysin/MMP20 is a matrix metalloprotease detected during predentin secretion by odontoblasts (Bègue-Kirn *et al.*, 1998). A phosphatase-regulating gene with homologies to endopeptidases on the X-chromosome (PHEX) is an enzyme involved in phosphate homeostasis during odontoblast differentiation (Ruchon *et al.*, 1998). Histological analysis of the pellet, however, has shown that these cells formed osteodentin-like structure but not tubular dentin. In contrast to other studies (Couble *et al.*, 2000; Yokose *et al.*, 2000), differentiation was enhanced without the addition of dexamethasone and organic phosphate/inorganic phosphate. A potential reason for this may be the result of the optimal cell-to-cell interaction and cell-to-matrix interaction, providing a favorable micro-environment/scaffold in pellet cultures.

Next, the effects of rhBMP2 on differentiation were examined. We have previously reported that treatment of monolayer cultures of bovine pulp cells with rhBMP2 significantly increased the expression of *α1(I)collagen* and ALPase activity (Nakashima *et al.*, 1994). The increase in ALPase activity by rhBMP2 was higher in pellet cultures than in monolayer culture on day 14. The expression of *Dmp1, Dspp, enamelysin,* and *Phex* was more increased by rhBMP2 in pellet cultures than in monolayer cultures. The pellet culture system allowed for better responsiveness of pulp cells to rhBMP2. This may be the result of optimal cell-to-cell interaction in the pellet culture system. The expression of *Cbfa3* transcription factor has been reported in the dental papillae of mouse tooth germ at cap and bell stages, and later is limited in the odontoblastic layer. *Cbfa1* is expressed in the dental papillae at the early stage, then disappears at the late bell stage (Yamashiro *et al.*, 2002). The decreased expression of *Cbfa1* and the enhanced expression of *Cbfa3* in the pellet of porcine pulp cells treated with rhBMP2 on day 21 in the present investigation are consistent with *in vivo* findings in mice.

Finally, the utility of this pellet culture system treated with BMP2 for dentin regeneration *in vivo* was investigated. Since the cells in the pellets were surrounded by collagenous matrix, it allowed for convenient manipulation and implantation for cell therapy. Human pulp cells with PGA which were cultured for 24 hrs and implanted into immunocompromised mice expressed BMP2, BMP4, and BMP7 mRNA (Buurma *et al.*, 1999). *In vivo* protein therapy with BMP2 (Nakashima, 1994a,b) and BMP7 (Rutherford *et al.*,

1993, 1994) and *in vivo* gene therapy with *Bmp11/Gdf11* by ultrasound-mediated gene delivery stimulated reparative dentin formation on the amputated dental pulp (Nakashima *et al.*, 2003). *Ex vivo* cell therapy may have an advantage, in that the cultured tissue stem/progenitor cells can be implanted after differentiation into odontoblasts and might result in copious amounts of reparative dentin formation. Skin fibroblasts transduced with BMP7-adenovirus induce reparative dentin formation (Rutherford, 2001). The present investigation demonstrated larger amounts of reparative dentin formation on the amputated pulp with a BMP2-supplemented pellet compared with a control pellet. The extracellular matrix of the pellet functions as a natural scaffold, which retains and releases BMPs. Techniques to isolate human pulp stem cells and manipulate their growth under defined *in vitro* conditions have to be established and optimized before cell therapy with BMP2 can become a clinical reality for caries and endodontic therapy. This investigation is a first step toward that long-term goal of biological regenerative endodontic therapy.

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