台灣口腔醫學科學雜誌
ISSN 1560-1587

Expression of DSPP mRNA in Human Dental Pulp-derived Cells Initiating Odontogenesis in Subcutaneous Transplantation of Nude Mice

Fumiko Aikawa[1], Michiko Nakatsuka[1], Shun-Te Huang[2], Hiroyuki Hayashi[3] and Yasutomo Iwai[1]

1 Department of Oral Anatomy, Osaka Dental University, Japan
2 Graduate Institute of Oral Health Sciences, College of Dental Science Medicine, Kaohsiung Medical University, Taiwan
3 Department of Endodontics, Osaka Dental University, Japan

We isolated, cultured and transplanted odontogenetic human dental pulp-derived (HDP) cells for the dental tissue regeneration study. In this study, we observed that the differentiation of 13^{th} passage HDP cells was induced by glycerol 2-phosphate disodium salt hydrate (β-GP) to initiate calcification in two-dimensional (2-D) monolayer cultures *in vitro*. Immunohistological and fine-structure studies of transplantation of the nude mouse backs by 3-D culture of HDP cells with an alginate scaffold identified synthesis of a specific matrix resembling dentin in the transplants *in vivo*. The expression of dentin sialophosphoprotein (DSPP) mRNA on day 13^{th} passage HDP cells added with β-GP was observed by the reverse transcription polymerase chain reaction (RT-PCR). The results indicated that the sub-cultured 13^{th} passage HDP cells were able to differentiate into odontoblast-like cells to initiate dentinogenesis in the transplants with the alginate scaffold.

Key words：dentin sialophosphoprotein, odontoblast-like cell, dental pulp, human, cell culture, transplantation.

Correspondence: Shun-Te Huang
Address: 100, Shih-Chuan 1st Road, San Ming District, 807 Kaohsiung City, Taiwan
Kaohsiung Medical University
TEL: 07-3121101#2272
FAX: 07-3233752
Submitted: December, 23, 2005
Accepted: January, 23, 2006

Introduction

There have been several studies on tissue stem cells for the regenerative medicine. It was elucidated that cultured mesenchymal cells differentiated the bone, cartilage, muscle, tendon, fatty tissue and the bone marrow stroma [1~5]. On the other hand, some studies have demonstrated the appearance of odontoblast-like cells in the human dental pulp (HDP) cell culture [6]. Previously we subcultured dental pulp-derived cells for several passages, transplanted the cells with alginate scaffold under the skin of nude mice, and observed the hard tissue formation in the transplants [6, 7].

Dentin phosphoprotein (DPP), a highly acidic protein and the major non-collagenous component of dentin, is expressed by the ectomesenchymal derived odontoblast cells of the tooth. It is suggested that deficiency of the DPP is a causative factor in *dentinogenesis imparfecta*. DPP is the major noncollagenous dentin extracellular matrix (DECM) protein, while dentin sialoprotein (DSP) is a 95-kDa glycoprotein in the DECM [8]. It has been elucidated that both the two major non-collagenous dentin matrix proteins - DSP and DPP (also known as phosphophoryn)- were encoded by a single gene the dentin sialophos-phoprotein (DSPP) [9,10]. DSPP is in particular mapped to human chromosome 4 using a somatic cell hybrid panel [8]. Whereas most of the previous regenerative medicine studies were designated on primary and young subcultured cells, in the present study we examined the expression of DSPP mRNA, the relation between the expression and the cell differentiation to identify the property of the sub-cultured HDP cells. We studied cultures and transplants containing HDP cells for further elucidation of the mechanisms of odontoblast differentiation and the mineralization process [11].

Material and Method

Preparation of human dental pulp-derived (HDP) cells

The human dental pulp was sterilely dissected from teeth removed for treatment purpose at the Hospital of Osaka Dental University. The protocol was approved by the Ethics Review Board at Osaka Dental University (No. 050355: culture of human dental pulp stem cells). The cells were isolated by collagenase type Ⅰ (4mg/ml; Wako Pure Chemical Industries, Osaka Japan) and dispase (4mg/ml; Gibco Laboratories, Grand Island, NY, USA) at 37℃ for 40 minutes. The solution with isolated cells was filtrated with the Cell Strainer (70 μm; BD Falcon, Bedford, MA, USA), centrifuged (1,500rpm, 3min) and added with Dulbecco's Modified Eagle Medium (DMEM, Nacalai Tesque, Inc., Kyoto, Japan) containing penicillin (100 units/ml; Gibco), streptomycine (100 μ g/ml; Gibco) and Fetal Bovine Serum (20%; FBS, Hyclone, Logan, UT, USA). The primary culture was seeded on dishes (35mm; BD Falcon) and cultured under 5% CO_2 gas at 37℃. While confluent occurred, the HDP cells were seeded consequently at 5,000/cm^2 cell concentration by gradient reduction of 10% FBS every 3rd day in every succeeding passage. After 2 months of cell subculture, the 13th passage HDP cells were obtained.

Examination of HDP cell differentiation

Glycerol 2-phosphate disodium salt hydrate (β-GP; Sigma-Aldrich GmbH, Steinheim, Germany) was added to the experimental group cultures. Each culture was seeded with 10,000 cells/cm^2 using 24-well plates (Asahi Technoglass Co., Chiba, Japan).

1. Alkaline phosphatase activity

The alkaline phosphatase (ALP) enzyme activity, of 3, 7, 14, and 21 days subcultured HDP cells was examined using an Alkaline Phosphatase Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA) following the directions. Afterward, the specimens were stained with fluorescence dye Bisbenzimide H 33258 (Wako), and the fluorescent intensity (excitation wave length: 355nm, emission wave length: 460nm) was scored using the multilabel counter (Wallacoy). The ALP activity was corrected by the DNA amount.

2. Formation of calcifying loci

For examination of calcification loci on the 3, 7, 14, and 21 days subcultures, they were fixed with formalin (10%) and then stained with alizalin red S (1%; Sigma-Aldrich Japan).

Transplantation

13th passage HDP cells (1.5 × 10^7 cells/well) were made to be a cluster by mixture with alginate solution (1.5%, 0.3ml; Protanal FL 10/60 Sodium Alginate; FMC Bio Polymer, Drammen, Norway) and $CaSO_4$ (21%, 40 μl). The cell clusters were transplanted subcutaneously and epi-fascically into the dorsolumbar portion of KSN/Slc-nu/nu nude mice (7week-old, male; Japan SLC, Inc., Hamamatsu, Japan). After 6 weeks of the transplantation, Softex (soft X-ray) photography was conducted for detection of hard tissue formation (25kVp, 20mA, 10sec; Industrial X-Ray Film FR, Fuji Film Co., Ltd.). Subsequently, the mice were fixed by intracardiac perfusion with paraformaldehyde (4%), the back tissue with implants were dissected and processed for light and transmission electron microscopy purpose, respectively.

Histological examination

H-E staining of the samples was routinely processed. For the immunological staining, mouse monoclonal collagen type Ⅰ antibody (COL-1) (Abcom Limited, Cambridgeshire, UK) and Rabbit Anti (bovine) type ⅢCollagen (LSL Co., LTD., Tokyo, Japan) were used. Tunel method was conducted on paraffin sections using ApopTag Plus Fluorescein in Situ Apoptosis Detection Kit (Chemicon Intl, Inc., Temecula, CA, USA) and observed under a confocal laser scanning microscopy (CLSM). The transplants with surrounding tissues were dissected, embedded with epon-812, ultrathin-sectioned and observed with conventional transmission electron microscope (CLSM).

Property of 13th passage HDP cells

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The examination was performed on the 10th day after adding β -GP. First-strand cDNA synthesis was performed by using SuperScript™ Ⅲ CellsDirect cDNA Synthesis System (Invitrogen life technologies, Cattahad, CA, USA). Specific primers were designed according to the previous study (Table 1) [12, 13]. First-strand cDNA (concertration) was diluted in a PCR reaction mixture (10 × PCR ExTaq Buffer, 1.5mM $MgCl_2$, 0.2mM each of dNTP, 0.25units of TaKaRa ExTaq™ ; TaKaRa BIO, 10pmol of each human specific primer sets; Sigma-Aldrich Japan). Amplification was performed in a thermal cycler (TaKaRa PCR Thermal Cycler Personal).

Table 1

	Size	Primer	
dentin sialophosphoprotein (DSPP)	248bp	forward	5'-GAT GAT CCC AAT AGC CA-3'
		reverse	5'-CCT TTG CCA CTG TCT G-3'
glyceraldehydes-3-phosphate dehydrogenase (G3PDH)	450bp	forward	5'-ACC ACA GTC CAT GCC ATC AC-3'
		reverse	5'-TCC ACC ACC CTG TTG CTG TA-3'

This amplification system for DSPP included 94℃ (2 min), 40 cycles of 94℃ (45 sec), 55℃ (45 sec) and 72℃ (1 min), followed by 10 min at 72℃. Amplified sample (5 μl) was analysed by 1.5% agarose gel electrophoresis at 100V for 30 min. After electrophoresis, the gel was stained with ethidium bromide solution (0.2 μg/ml) for 30 min. Stained gels were observed under ultraviolet light (ATTO Printgraph; ATTO, Tokyo, Japan).

Results

Examination of the monolayer cultures

1. ALP activity

No significant ALP activity was detected in the 3 and 7 days subcultured human dental pulp-derived (HDP) cells between control (β-GP(-)) and experimental (β-GP(+)) groups. However, ALP activity was observed in both groups on the 14^{th} day. Furthermore, we observed that the ALP activity of β-GP (+) group became distinct as twice as higher than the β-GP(-) group on the 21^{st} day of subculture (Fig.1).

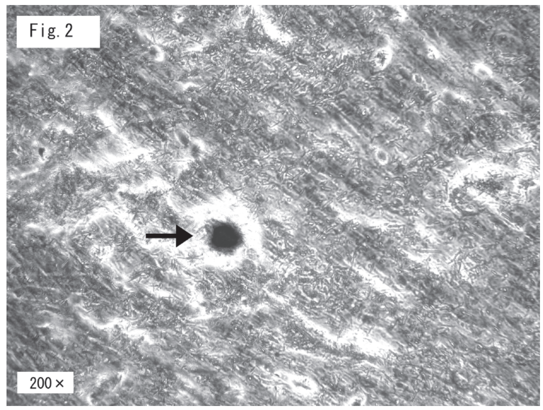

Fig.2 Calcification loci of the cultured cell (Alizarin Red staining)
Occurrence and concrescence of calcification loci (arrow) was observed since day 14 of subculture.

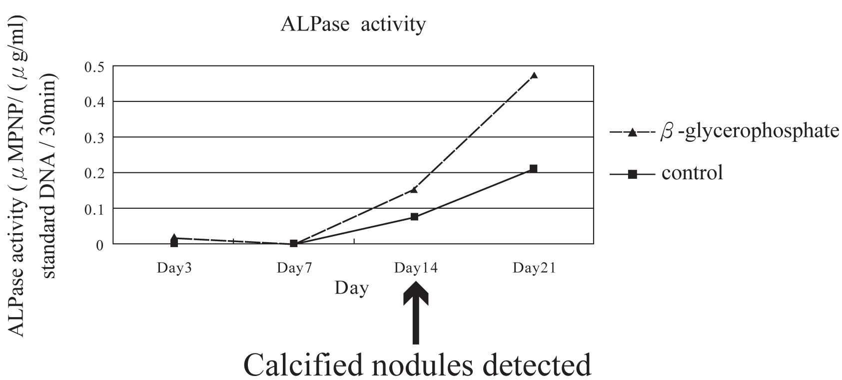

Fig.1 ALP activity of the cultured cells
On the day 21 of subculture, ALP activity in β-GP(+) group is twice as high as in β-(-) group.

Taiwan J Oral Med Sci 2006; 22:15-28

2. Observation of calcification loci

Occurrence and concrescence of calcification loci had been observed since the 14th day of subculture composed of isolated proliferating and differentiating HDP cells (Fig.2).

Examination of the transplant in situ

1. Softex radiography revealed radio-opaque calcified bodies in the subcutaneously transplanted cell/alginate clusters. We dissected the transplant with surrounding tissue and observed some whitish hard granules in the transplant.
2. Histology of the tranplants
   1) Light microscopy
      a. Control group: transplantation of alginate scaffold into the backs of nude mice
      Histological findings of the H-E stained specimens showed a network of fibroblast strands extending between the cutis and transplant. By using mouse monoclonal collagen type I antibody, distribution of Type I collagen immunoreactivity was evident in the cell-strand network in the transplants, based on mouse monoclonal collagen type I antibody staining.
      b. Experimental group: transplantation of the cell cluster (HDP cells with the alginate scaffold) into the backs of nude mice
      a) Routine H-E staining of the specimens showed extracellular matrix (ECM) formation in transplants containing sub-cultured 13th passage HDP cells and alginate scaffold (Fig. 3).
      b) Immuno-reactivities of mouse monoclonal collagen type I antibody and Rabbit Anti (bovine) Type III Collagen was evident in the transplants (Fig.4).
      c) Monoclonal Antibody to Bovine Osteocalcin localized osteocalcin in the ECM. No vascular system ex-

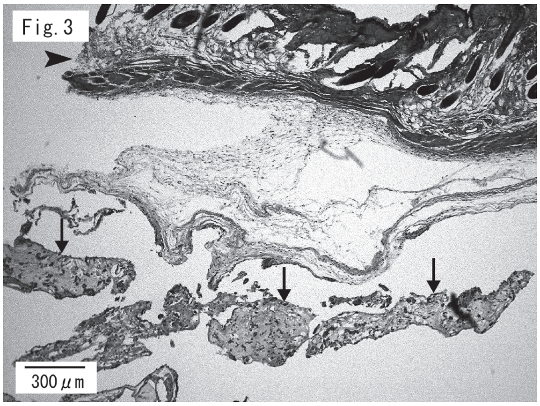

Fig.3 Photomicrograph of a transplant and surrounding tissue (H-E staining; an arrow head: cutis) Extracellular matrix (ECM) formation is identified in the subcutaneous transplant containing 13th passage cells/alginate scaffold in situ (arrows).

tension between the cutis and the ECM was demonstrated by Polyclonal Rabbit anti-human Von Willebrand factor (factor VIII), which stains endothelial cells.

2) CLSM

Cell turnover by the occurrence of Tunel (+) cells in the experimental group was evident in the calcifying transplant containing HDP cells and the alginate scaffold (Fig.5). Non-collagenous protein DSP (+) reaction was observed in the transplants (Fig.6).

3) TEM

Fine structure study of the ultrathin-sectioned demineralized specimens revealed isolated spindle-shaped, elongated and polarized fibroblast-like cell in the transplants; the intercellular junctional apparatuses of the cells were not well

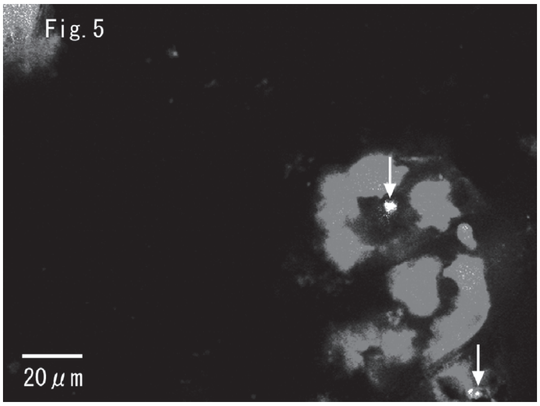

Fig.5 DSP (+) reaction was observed in the transplant by confocal laser scanning microscopy
DSP (+) reaction suggests the dentin-like tissue formation.

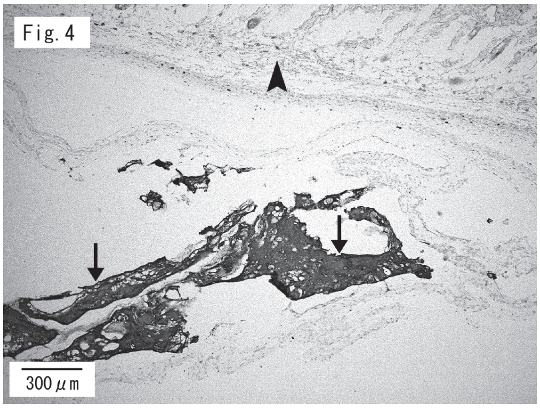

Fig.4 Immunohistochemistry of type Ⅰ collagen (arrow head: epidermis)
Type Ⅰ collagen is observed in the transplant (arrows)

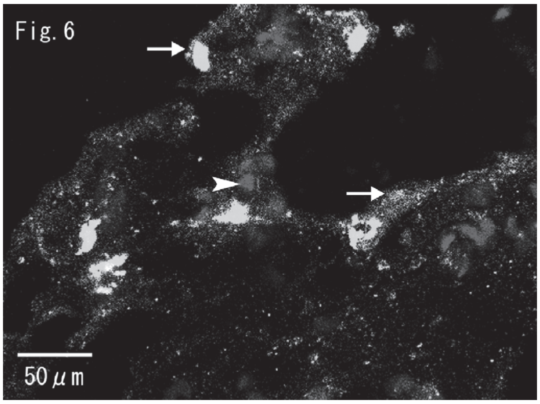

Fig.6 The transplant in situ Tunel (+) cells were observed in the calcifiying transplant by confocal laser scanning microscopy.

developed. The polarized odontoblast-like cells were abundant in cytoplasm having a distal extending main cellular process. They were morphologically pre-odontoblasts. Some scattered autolyzing cells were found in the transplants (Fig. 7).

Examination of the property of the transplanted cells

1. RT-PCR

No expression of DSPP mRNA was found in the β -GP (-) primary passage cells or succeeding 12 passages of HDP cells on the 10th day culture. However, in the β -GP (+) group expression of DSPP mRNA was detected in the 13th passage HDP cells on the 10th day of culture.

In the present RT-PCR study, positive control amplifications were performed using a primer set for the housekeeping gene, glyceraldehydes 3-phosphate dehydrogenase (G3PDH). Negative controls were performed using each specific primer set with the added cDNA target being replaced by sterile water. Expression of DSPP mRNA was not found in the β -GP(-) primary and succeeding 13 passages. On the contrary, by adding β -GP into the media, DSPP mRNA expression was

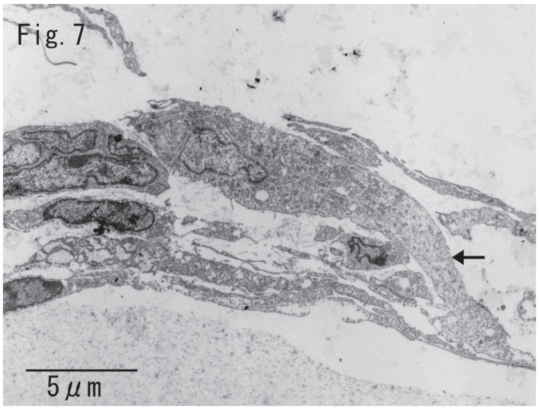

Fig.7 Transmission electron microscopic appearance of a transplant
Isolated and polarized cells with a distal cell process (arrow) contain abundant cytoplasm; the intercellular junctional apparatuses are not well developed.

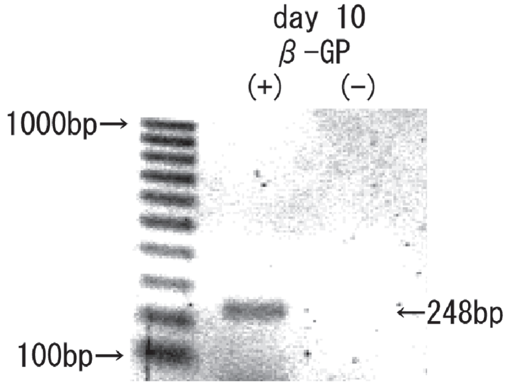

Fig.8 RT-PCR analysis of expression of DSPP mRNA on day 10
DSPP mRNA was expressed in the β -GP (+) group, but not in the β -GP (-) group.

observed on the 10th day after subculture (Fig. 8). In the control group, DSPP mRNA expression was observed on the 23rd day after subculture. But in the β-GP(+) group, DSPP mRNA expression was not observed on the 23rd day after subculture (Fig.9).

Discussion

Several studies have shown that the differentiation of odontoblast-like cells with matricial calcification by culture of dental pulp-derived cells from the rat, human and cattle teeth (11, 14~16). The odontoblast-like cells were characterized by cell polarization and distal extension of the main cell process. Additionally, the expression of DSPP that encodes DSP and DPP relating to the dentin specific protein was observed in these differentiated cells found in dentin (14, 17, 18). On the other hand, many studies have conducted on factors inducing mesenchymal stem cells to initiate calcifying tissue. The studies indicated that the bone formation was induced by dexamethasone, β-GP, vitamin D, ascorbic acid and bone morphogenetic proteins (BMP), while the cartilage formation was induced by dexamethasone, ascorbic acid, TGF-β (11). A previous study has reported that β-GP was particularly essential in inducing the cultured dental pulp-originated cells to acquire the odontoblast property (14).

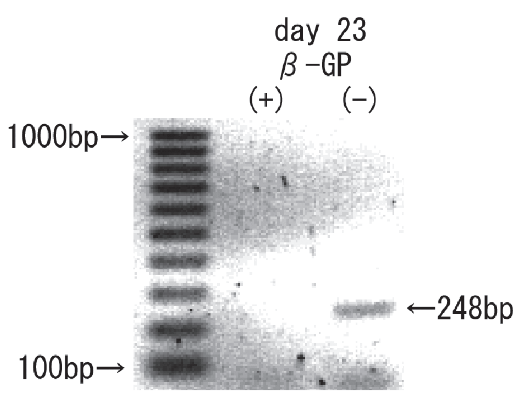

Fig.9 RT-PCR analysis of expression of DSPP mRNA on day 23
DSPP mRNA was expressed in the β-GP (-) group, but not in the β-GP (+) group.

In tooth formation, the odontoblasts, highly specialized cells aligned in a single layer at the periphery of the dental pulp, are responsible for secretion and mineralization of the fibrillar extracellular matrix of dentin. They originated from mesenchymal dental papilla cells showing different degrees of differentiation. Some of the cells withdraw from the cell cycle, proliferate to show cellular polarization with formation of a main cellular process, and contribute to synthesis and secretion of specific proteins. Among those proteins, DSP and DPP are dentin specific and others like type Ⅰ and type Ⅰ trimer collagen, osteocalcin, osteopontin, dentin matrix protein (DMP1) are common to both dentin and bone [19].

DSPP gene encodes two dentin-specific proteins: DSP and DPP. In a previous study of rodent incisors, a small amount of DSPP mRNA has been detected in young odontoblasts secreting predentin. Immunolo-calization of DSP and DPP in either young or mature odontoblasts and the dentin suggested that DSP and DPP are important in initial dentinogenesis and dentin mineralization [19]. Some experiments *in vitro* has found that DPP appeared to be secreted at the mineralization front, bond to collagen fibers in the gap regions, and initiated apatite crystal formation, but its function *in vivo* remains unclear [11]. We performed RT-PCR to investigate DSPP mRNA expression, as a differentiation marker of odontoblasts, in HDP cells in the presence of β -GP. The expression of DSPP mRNA was detected on the 10th day of culture. Also, DSP was detected within the transplants with a dentin-like hard tissue formation. The findings indicate the dentin-like formation in the transplants containing cultured HDP cells.

There are studies showing that bone morphogenetic protein (BMP) and dexamethasone induce tissue formation and up-regulate the expression of DSPP mRNA [20, 21]. In the present study, we cultured 13 passages of HDP cells and obtained certain fibroblast-like cells. ALP activity was not found except in succeeding β -GP (+) cultures. We also noted that ALP activity and the expression of DSPP mRNA induced by β -GP were indicative for the differentiation of odontoblast-like cells in the 13th passage HDP cells. The expression of DSPP mRNA on the 23rd day in the β -GP (-) group suggested that β -GP accelerated the differentiation of odontoblast-like cells.

Furthermore, we found that the ALP activity expressed in both β -GP (-) and β -GP (+) groups between the 10th and 14th day of HDP cell cultures, and in the experimental β -GP (+) group, it became evident on the 21st day of culture. On the other hand, calcifying loci were demonstrated in the day 14 monolayer cultures; they showed similar histological findings as previously described [21]. Additionally, in the present results, we observed that transplants 3-dimensionally contained strands of intermingled HDP cells in different turn-over phases; the cell proliferated, differentiated to odontoblast-like cells and some of them died.

In summary, the present study observed differentiation of odontoblast-like cells and dentin-like tissue formation in the transplants. The results indicated that alginate scaffold supported the differentiation of cultured cells in transplants. However, the cultured HDP cells initiated dentin-like tissue formation in a specific environment -transplantation of the nude mice back skin- which is so different from the usual dentinogenesis. Further studies to clarify the mechanism of dentin-like tissue formation and succedent remolding of the transplants are next question we should address.

References

1. Tuan RS, Boland G. and Tuli R. Adult mesnchymal stem cells and cell-based tissue engineering. Arthritis Res Ther Ther 2003; 5: 32-45.
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, and Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999; 284: 143-147.
3. Awad HA, Butlar DL, Harris MT, Ibrahim RE, Wu Y, Young RG, Kadiyala S, and Bovin GP. In vitro characterization of mesenchymal stem cell-seeded collagen scaffolds for tendon repair: Effects of initial seeding density on contraction kinetics. J Biomed Mater Res 2000; 51: 233-240.
4. Wakitani S, Saito T, and Caplan AI. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle & Nerve 1995; 18: 1417-1426.
5. Majumdar MK, Yhiede MA, Haynesworth SE, Bruder SP, and Gerson SL. Human marrow-derived mesenchymal stem cells (MSCs) express hemetopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages. J Hematother Stem Cell Res 2000; 9: 841-848.
6. Kim GS, Kumabe S, and Iwai Y. An experimental study on transplantation of human dental pulp-derived cells using alginate scaffold. J Jpn Assoc Regenerative Dent 2005; 3: 41-56.
7. Gronthos S, Mankani M, Brahim J, Robey PG, and Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo, PNAS 2000; 97: 13625-13630.
8. MacDougall M, Simmons D, Luan X, Nydegger J, Feng J, and Gu TT. Dentin phosphoprotein and dentin sialoprotein are cleavage products expressed from a single transcript coded by a gene on human chromosome 4: dentin phosphoprotein DNA sequence determination. J. Biol Chem 1997; 272: 835-842
9. Papagerakis P, Berdal A, Mesbah M, Peuchmaur M, Malaval L, Nydegger J, Simmer J, and

Macdougall M. Investigation of osteocalcin, osteonectin, and dentin sialophosphoprotein in developing human teeth. Bone 2002; 30: 377-385.

10. Narayanan K, Ramachandran A, Peterson MC, Hao J, Kolsto AB, Friedman AD, and George A. The CCAAT enhancer -binding protein(C/EBP) β and Nrf1 interact to regulate dentin sialophosphoprotein(DSPP) gene expression during odontoblast differentiation. J Biol Chem 2004; 279: 45423-45432.
11. Couble ML, Farges JC, Bleicher F, Perrat-Mabillion B, Boudeulle M, and Magloire H. Odontoblast differentiation of human dental pulp cells in explant cultures. Calcif Tissue Int 2000; 66: 129-138.
12. Kamata N, Fujimoto R, Tomonari M, Taki M, Nagayama M, and Yasumoto S. Immortalization of human dental papilla, dental pulp, periodontal ligament cells and gingival fibroblasts by telomerase areverse transcriptase. J Oral Pathol Med 2004; 33: 417-23.
13. Fujimoto R, Kamata N, Yokoyama K, Ueda N, Satomura K, Hayashi E, and Nagayama M. Expression of telomerase components in oral keratinocytes and squamous cell carcinomas. Oral Oncology 2001; 37: 132-140.
14. Kasugai S, Shibata S, Suzuki S, Susami T, and Ogura H. Characterization of a system of mineralized-tissue formation by rat dental pulp cells in culture. Arch Oral Biol 1993; 38: 769-77.
15. Seux D, Couble ML, Hartmann DJ, and Gauthier JP, Magloire H. Odontoblast-like cytodifferentioation of human dental pulp cells in vitro in the presence of a calcium hydroxide-containing cement. Arch Oral Biol 1991; 36: 117-128.
16. Nakashima M. Establishment of primary cultures of pulp cells from bovine permanent incisors. Arch Oral Biol 1991; 36: 655-663.
17. James MJ, Järvinen E, and Thesleff I. Bono 1: A gene associated with regions of deposition of bone and dentin. Gene Expression patterns 2004; 4: 595-599.
18. Bègue-Kirn C, Krebsbach PH, Bartlett JD, and Butler WT. Dentin sialoprotein, dentin phosphoprotrin, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. Eur J Oral Sci 1998; 106: 963-970.
19. Saito T, Ogawa M, Hata Y, and Bessho K. Acceleration effect of human recombinant bone morphogenetic protein-2 on differentiation of humanPulp cells into odontoblasts. J Endod, 2004; 30(4); 205-208.
20. Ritchie HH, Park H, Liu J, Bervoets TJ, and

Bronckers AL. Effects of dexamethasone, vitamin A and vitamin D3 DSP-PP mRNA expression in rat organ culture. Biochem Biophys Acta 2004; 1679: 263-271.

21. Tsukamoto Y, Fukutani S, Shin-Ike T, Kubota T, Sato S, Suzuki Y, and Mori M. Mineralized nodule formation by cultures of human dental pulp-derived fibroblasts. Arch Oral Biol 1992; 37: 1045-1055.

Taiwan J Oral Med Sci 2006; 22:15-28