The effect of *in vitro* osteogenic induction on *in vivo* hard tissue forming potential of the dental pulp stromal cells from deciduous teeth

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Directed by Professor Jae-Ho Lee

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Seunghye Kim

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This certifies that the dissertation of Seunghye Kim is approved.

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감사의 글

남편을 따라 간 독일에서 준이 유모차를 끌고 다니며 학위 논문을 완성 할 수 있을지 막막했었습니다. 이 논문을 완성하게 된다면 그건 기적이라고 생각했습니다. 그리고 잘 끝낼 수 있게 되길 간절히 기도했습니다. 오늘 박사 논문을 마무리 하며 이 논문이 끝날 수 있도록 물심 양면으로 도와주시고 인도해주신 분들께 깊은 감사의 마음을 전하고 싶습니다.

수련 과정 수료 후 Fellow 과정을 거쳐 이렇게 박사 과정까지 임상과 공부를 병행할 수 있도록 지도해 주시고 큰 그림을 그려주신 손흥규 교수님, 소아치과 의사로서의 자세와 마음을 가르쳐주신 최병재 교수님, 석사와 박사 지도교수가 되어주셨고 항상 부족한 저를 믿어주시고 기다려주시고 방향을 제시해주신 이제호 교수님, 빈틈없는 임상을 가르쳐 주시고 학문적 깊이를 더해주신 최형준 교수님, Fellow 2년의 생활 동안 셀 수 없이 많은 밥을 사주시고 추억을 만들어 주신 김성오 교수님, 그리고 연구에 눈을 뜨도록 이끌어 주시고 마지막까지 함께 달려주신 송제선 교수님께 깊은 감사를 전합니다.

연구실 장비와 공간을 사용할 수 있도록 사용할 수 있도록 배려해주시고 학부 때부터 관심을 가지고 든든하게 인도해 주신 정한성 교수님께 감사 드립니다. 2010년과 2011년 중견 연구자가 되어주셔서 연구비를 받을 수 있도록 힘을 실어 주시고 좀 더 좋은 논문이 나올 수 있도록 지도해주신 신동민 교수님께 감사 드립니다.

처음 만났을 때 Pipetting 조차 서투르던 실험 초보자인 저에게 비법도 알려주시고 헌신적으로 도와주신 전미정 선생님께 감사 드립니다. 조직학 연구실에서 바쁜 와중에도 저의 질문들에 답해 주시고 실험 중 막힌 부분들을 뛰어넘을 수 있도록 도와주신 은정언니, 혁제, 김은정 선생님과

신정호 선생님께 감사 드립니다. 그리고 연구를 도와주었던 지은혜, 김재은 선생님을 비롯하여 소아치과 의국원들에게도 감사를 드립니다. 소아치과 수련기간과 fellow 기간 동안 손이 되어주시고 저의 부족함을 묵묵히 채워주신 이호정 직원, 강선영 위생사, 이철준 위생사, 이화진 위생사를 비롯한 소아치과 직원분들에게도 감사를 드립니다.

그리고 지금까지 항상 옆에서 묵묵히 저의 모든 필요를 채워주시고 준이와 현이를 키워주신 부모님께 감사를 전합니다. 신앙의 유산과 더불어 살아갈 힘과 용기를 주셨습니다. 공부를 지속할 수 있도록 깊은 배려와 기도로 응원해 주신 연변 부모님께 감사를 드립니다. 논문을 쓸 수 있도록 주말에는 육아와 집안일을 담당해주고 독일에서 많은 시간을 기다려준 남편에게 감사를 전합니다. 그리고 엄마 공부로 인해 어린 나이에 독일 유아원에 다니며 고생한 준이, 그리고 할아버지 할머니 품에서 돌까지 잘 자라준 현이에게도 고마움을 전합니다. 마지막으로 제가 박사를 생각지도 못했을 때 승혜는 김박사가 되게 해달라고 내가 기도하고 있다고 말씀해 주셨던 이원도 집사님과 강한 기도 후원자가 되어주신 이옥수 권사님께 감사를 전합니다.

부족한 제가 이 논문을 쓸 수 있었던 것은 여러분을 통해 이루신 하나님의 기적이었습니다. 감사합니다.

2012년 12월

김승혜 드림

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The effect of *in vitro* osteogenic induction on *in vivo* hard tissue forming potential of the dental pulp stromal cells from deciduous teeth

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(Directed by Professor Jae-Ho Lee)

Human dental pulp tissue of deciduous teeth has been considered as a source of multipotent stem cells, and there have been many attempts for its application in dentin or bone regeneration. The objective of this study was to examine the effect of *in vitro* osteogenic induction of deciduous dental pulp stromal cells (DDPSCs) on their *in vivo* hard tissue forming potential. DDPSCs were isolated from extracted deciduous teeth

using outgrowth method. During *ex vivo* expansion, DDPSCs were exposed to osteogenic stimuli for different time periods (4 and 8 days) while the control group was expanded without osteogenic stimuli. First, *in vitro* differentiation pattern was investigated using alkaline phosphatase (ALP) staining and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Then the *ex vivo* expanded and differentiated DDPSCs were subcutaneously transplanted into immune-compromised mice with macroporous biphasic calcium phosphate (MBCP) as a carrier. The results in *in vivo* transplantation were analyzed by quantitative RT-PCR, ALP activity assessment, histological analysis, and immunohistochemical staining. The amount of hard tissue was greatest in Day 4 group, followed by Day 8 and the control group. The hard tissue generated in Day 8 group presented most bone-like morphology among the three groups, while the control group featured more dentin-like characters. 4-day induction period in the osteogenic media was related to greater potential to form hard tissue, whereas 8-day induction period was related to formation of more bone-like hard tissue. In conclusion, *in vitro* osteogenic induction of DDPSCs enhanced hard tissue formation *in vivo* and also altered character of the newly generated hard tissue.

Keywords: deciduous teeth, dental pulp stromal cell, *in vitro* osteogenic induction, *in vivo* transplantation, hard tissue formation

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I. Introduction

The presence of multipotent postnatal stem cells have been identified in various types of tissues, such as bone marrow, adipose tissue, umbilical cord blood, and dental tissues (Campagnoli et al., 2001, Gronthos et al., 2000, Mareschi et al., 2001, Miura et al., 2003, Owen and Friedenstein, 1988, Seo et al., 2004, Zuk et al., 2002). Currently, the most well defined and clinically applied multipotential postnatal stem

cell is BMMSCs. Osteogenic potential of bone marrow mesenchymal stem cells (BMMSCs) has been extensively investigated, and there are several studies reporting favorable bony repair potential of BMMSCs in clinical application (Marcacci et al., 2007, Quarto et al., 2001). However, postnatal stem cells isolated from dental tissues, such as human dental pulp tissue and periodontal ligament, have several advantages over BMMSCs in cases of large bony defect area or orofacial bony defect: higher incidence of clonogenic cells, higher proliferation rate, and matched ectomesenchymal origin with the orofacial bones (Gronthos et al., 2000, Miura et al., 2003, Seo et al., 2004). In addition, human dental pulp tissue can be obtained with easy surgical access and low morbidity.

The dental pulp stem cells from human permanent teeth (DPSC) and stem cells from human exfoliated deciduous teeth (SHED) presented *in vitro* multilineage differentiation potential in previous studies, such as osteogenic, chondrogenic, adipogenic, myogenic, and neurogenic potential (Gronthos et al., 2002, Kerkis et al., 2006, Laino et al., 2005, Miura et al., 2003, Zhang et al., 2006). However, DPSC and SHED demonstrated different *in vivo* characteristics when transplanted into immunocompromised mice. DPSCs exhibits odontogenic features, generating dentin pulp-like complex, whereas SHEDs produced both dentin-like and bone-like structures. However, SHEDs did not produce dentin-pulp-like complex like DPSCs (Batouli et al., 2003, Gronthos et al., 2002, Gronthos et al., 2000, Miura et al., 2003). Seo et al. (Seo et al., 2008) showed potential of SHED as a source of bony regeneration in orofacial region: they reported bony repair of critical-size cranial defect in immunocompromised mice using SHED.

Researchers have tried to determine the ideal expansion condition best for the cultivation of mesenchymal stem/stromal cells (MSCs) intended for bony repair. One of the interesting recent modifications is the use of scaffolds seeded with pre-differentiated MSCs (Castano-Izquierdo et al., 2007, Scotti et al., 2010). In these studies, such pre-differentiation procedure imposed intrinsic capacity to undergo specific differentiation pathway more efficiently compared to MSCs without differentiation stimuli. SHEDs are known to form both dentin-like and bone-like structure in ectopic subcutaneous *in vivo* model, but there are no studies about in vivo characterization of SHEDs after pre-differentiation in osteogenic media (Miura et al., 2003).

The purpose of this study was to examine *in vivo* hard-tissue forming potential of dental pulp stromal cells from deciduous teeth (DDPSCs) upon pre-differentiation in osteogenic media. *In vitro* osteogenic induction was executed for different time periods, and DDPSCs were transplanted into immunocompromised mice at various stages of osteogenic differentiation.

II. Materials and Methods

1. Subjects and Cell Culture

Human deciduous incisors (n=4), extracted for dental treatment, were collected from 4 children (aged 7-11; 2 males and 2 female), under approved guidelines set by the Institutional Review Board of the Dental Hospital, Yonsei University (#2-2011-0008). The pulp tissue was separated from teeth and subjected to primary culture by outgrowth method. Briefly the separated pulp tissue was minced into about 1.0 mm^3 size and pressed gently unto 60mm culture dish (BD Falcon, Franklin Lakes, NJ, USA) using cover glass (Superior, Lauda-Königshofen, Germany). The explants were cultured in alpha minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100U/ml penicillin and 100ug/ml streptomycin (Invitrogen) at 37 ℃ in 5% CO_2. The isolated cells from 4 samples were blended at passage 1, and passages 3 to 5 were used in this study.

2. *In Vitro* Osteogenic Induction

The prepared cells were seeded at a density of $3.0x10^3$ cells/cm^2 and were exposed to osteogenic media for different time periods of 4 or 8 days. Osteogenic media was made by adding osteogenic factors, 100nM dexamethasone (Sigma, St. Louis, MO, USA), 50 mM L-ascorbic acid 2-phosphate (Sigma), and 2mM β-glycerophosphate (Sigma) to the α-MEM culture media. In 4-day group, the cells were cultured in α-MEM culture media

for 4 days and exposed to the osteogenic media for following 4 days. In 8-day group, the cells were cultured in the osteogenic media for 8 days. The control group was cultured in the α-MEM culture media for 8 days without exposure to osteogenic factors. Media was changed every two days, and all groups reached full confluency after 8 days.

In Vitro Study

3. Alkaline Phosphatase (ALP) Staining

After in vitro culture for 8 days, each group underwent ALP staining. After fixation with 10% neutral buffered formalin (Sigma) at 4℃ for 1 hour, the cells were washed and stained with ALP stain solution [100 mM Tris/HCl (pH 8.4; WelGENE Inc., Daegu, Korea), 0.01% naphthol AS-MX phosphate (Sigma) and 0.06% fast red violet LB salt (Sigma)], a modified composition from the one previously introduced (Kamon et al., 2010), for 30 minutes. The cells were washed with distilled water for three times and observed for color change.

4. Gene Expression Analysis by Quantitative Reverse Transcription Polymerase Chain reaction (RT-PCR)

Quantitative RT-PCR was performed to examine relative gene expression level of DSPP, Runx2, osteopontin (OPN), and osteocalcin (OC). After completion of each culture period, total RNA of cells were extracted by using RNeasy Mini Kit (Qiagen, Valencia,

CA, USA) according to the manufacturer's instruction. Its integrity and concentration were measured using a spectrophotometer (Nanodrop ND-1000; Thermo Scientific, Waltham, MA, USA). Reverse transcription of 1 μg of total RNA was performed using Maxime RT premix kit (Oligo $d(T)_{15}$ primer; iNtRON Biotechnology, Seoul, Korea), according to the manufacturer's instructions. The reaction was set at 45℃ for 1 hour and terminated by incubation at 95℃ for 5 min. KAPA SYBR FAST Master Mix Prism (KAPA Biosystems, Woburn, MA, USA) and ABI 7300 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) were used for PCR reaction. It was initiated by activation at 95℃ for 3 min and performed with 40 cycles of denaturation at 95℃ for 3 sec, annealing at 60℃ for 60 sec, and extension at 60℃ for 60 sec. The primer sequence and size of the target genes are shown in Table 1. Quantitative PCR was performed in duplicate for each target gene. For relative comparison of each target gene, the threshold cycle (C_T) was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Target gene transcript levels were normalized against GAPDH gene expression, an endogenous control. The relative expression level of each gene was calculated in respect to the value obtained for the control group.

Table 1. Primer sequence and size used for quantitative RT-PCR.

Gene	Primer Sequence (5'-3')	Size (bp)	Reference
DSPP	F: GGGATGTTGGCGATGCA R: CCAGCTACTTGAGGTCCATCTTC	70	(Wei et al., 2007)
Runx2	F: CACTGGCGCTGCAACAAGA R: CATTCCGGAGCTCAGCAGAATAA	127	(Qian et al., 2010)
OPN	F: ACCTGAACGCGCCTTCTG R: CATCCAGCTGACTCGTTTCATAA	66	(Dyson et al., 2007)
OC	F: CAAAGGTGCAGCCTTTGTGTC R: TCACAGTCCGGATTGAGCTCA	150	(Garlet et al., 2007)
BSP	F: CTGGCACAGGGTATACAGGGTTAG R: ACTGGTGCCGTTTATGCCTTG	182	(Fujii et al., 2008)
Col I	F: CGATGGCTGCACGAGTCACAC R: CAGGTTGGGATGGAGGGAGTTTAC	180	(Dehne et al., 2009)
GAPDH	F: TCCTGCACCACCAACTGCTT R: TGGCAGTGATGGCATGGAC	100	(Fujii et al., 2008)

* Quantitative RT-PCR. Annealing procedures were performed at 60℃ for all primers.

In Vivo Study

5. Transplantation

These procedures were performed in accordance with protocol approved by the Institutional Animal Care and Use Committee of Yonsei University (#2011-0176). Cells were prepared according to the same condition as in *in vitro* study, differing the culture period in osteogenic media. *Ex vivo* differentiated $3.0x10^6$ cells were mixed with 40 mg of macroporous biphasic calcium phosphate (MBCP; Biomatlante, Vigneux de Bretagne, France) and subcutaneously transplanted into the dorsal surface of 5-week male immunocompromised mice (BALB/c-nu, SLC, Shizuoka, Japan), as described in the previous study (Kuznetsov et al., 1997). Four pockets were made per each mouse (n=20), and four different types of transplants were individually inserted; cell-loaded MBCP particles of the 4-day, 8-day, and control groups and MBCP particles only without cell-loading. All transplants were retrieved after 8 weeks post-transplantation.

6. Gene Expression Analysis by Quantitative RT-PCR in the Transplants

The relative gene expressions of DSPP, Runx2, BSP, Col I, OPN, and OC were evaluated by quantitative RT-PCR. Total RNA was extracted from the retrieved transplants by using RNeasy Mini Kit (Qiagen) and cDNA was synthesized as previously described. After retrieval, the transplants were immediately immersed in buffer RLT, a component of RNeasy Mini Kit (Qiagen) and homogenized by using

stainless steel beads of 0.5mm mean diameter (Next Advance, Averill Park, NY, USA) and Bullet blender (Next Advance). Quantitative RT-PCR was performed by the same procedure as in *in vitro* study. The sequence and size of the primers are shown in table 1. The value of each gene was normalized to the expression level of GAPDH. The expression level of each gene was calculated relatively to their expression level in the MBCP transplants without cells.

7. ALP Activity in the Transplants

The level of ALP activity in the retrieved transplants was measured using SensoLyte® *p*NPP Alkaline Phosphatase Assay Kit (AnaSpec, Fremont, CA, USA). The retrieved transplants were rinsed and soaked in PBS (pH 7.4; Invitrogen) overnight. Then, it was lysed with Trinton-X-100, provided in the kit, according to the manufacturer's instructions. The supernatant of the cell lysate was used for detection of alkaline phosphatase activity. Then, *p*-Nitrophenylphosphate (*p*NPP) was added to the tissue extract, and ALP activity was measured by colorimetric change caused by dephosphorylation of *p*NPP. The absorbance at 405nm was measured using Benchmark Plus Microplate Spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA). The quantity of ALP activity was normalized against the total protein quantity in the supernatant of the same cell lysate using Thermo Scientific Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

8. Histology

Transplants were fixed with 10% buffered formalin (Sigma) overnight, decalcified with 10% EDTA (pH 7.4; Fisher Scientific, Houston, TX, USA) for 2 weeks, embedded in paraffin, and sectioned at a thickness of 5 ㎛. Sections were deparaffinized and stained with hematoxylin and eosin (HE) and Masson's trichrome (MT). They were invested under optical microscope (Olympus BX40, Olympus Co., Tokyo, Japan). HE stained sections were captured with a CCD digital camera (Infinity 2.0, Lumenera Co., Ottawa, Ontario, Canada) and digitized using image analyzer software (InnerView 2.0, iNNERViEW Co., Seongnam-Si, Gyeonggi-do, Korea). On HE sections, the areas of newly formed hard tissue and of MBCP were measured by using ImageJ program (National Institute of Health, Bethesda, Maryland, USA). For comparison of hard tissue forming potential, the total area of newly formed hard tissue was divided by the total area of MBCP on the section.

9. Immunohistochemistry

The sections were deparaffinized in xylene, rehydrated, and rinsed with distilled water. For antigen retrieval, protease K (Dako, Carpinteria, CA, USA) was used in OC staining while no treatment was performed in dentin sialoprotein (DSP) staining. The sections were immersed in 3% hydrogen peroxide for 10 min to inactivate endogenous peroxidase activities and incubated with primary antibody overnight. For OC staining, a 1:2500 dilution of the anti-human OC (rabbit polyclonal antibody, sc-33586; Santa Cruz

Biotechnology, Santa Cruz, CA, USA) was used. For DSP staining, a 1:3000 dilution of the anti-human DSP (rabbit polyclonal antibody, #AB10911; Millipore, Temecula, CA, USA) was used. Sections were subsequently incubated with HRP labeled polymer conjugated with secondary rabbit antibody in EnVision+ system kit (Dako), for 20 min. The color was developed using 3,3'-diaminobenzidine (DAB) substrate (Dako) and counterstained with Gill's hematoxyline solution (Merck, Darmstadt, Germany). Negative control section was stained in the same manner, but without primary antibody reaction procedure.

10. Statistical Analysis

Statistical analysis was performed with SPSS (19.0, Chicago, IL, USA). Multiple comparison test was performed using Kruskal-Wallis test ($p<0.05$), followed by Mann–Whitney U test (Bonferroni correction; $p<0.017$).

III. Result

1. *In Vitro* Effects of Osteogenic Induction

Dyeing analysis by ALP staining method showed increased ALP production in the group with longer culture period in osteogenic media. As shown in Figure 1, increased ALP staining is detected in Day 8 group compared to Day 4, and no detection in the control group.

The gene expression pattern according to different culture period in osteogenic media is shown in Fig. 2. DSPP, a dentin-specific marker, was expressed in similar level among Day 4, Day 8, and the control groups. The level of Runx2, a key transcription factor for osteoblast differentiation, slightly increased in Day 4 group compared to Day 8 group. OPN and OC, the late markers for mineralization, showed gradual increase as culture period in osteogenic media elongated (Fig. 2).

2. *In Vivo* Effects of *In Vitro* Osteogenic Induction on DDPSCs

2-1. Gene Expression Pattern of the Transplanted Tissues

Sharp decrease in the level of DSPP was detected in Day 4 and Day 8 groups in comparison to the control group ($p<0.05$; Fig. 3). The level of DSPP further decreased in Day 8 group compared to Day 4 group. In contrast, the expression of BSP, Col I, and OPN was significantly up-regulated in Day 4 group compared to the control ($p<0.05$). As

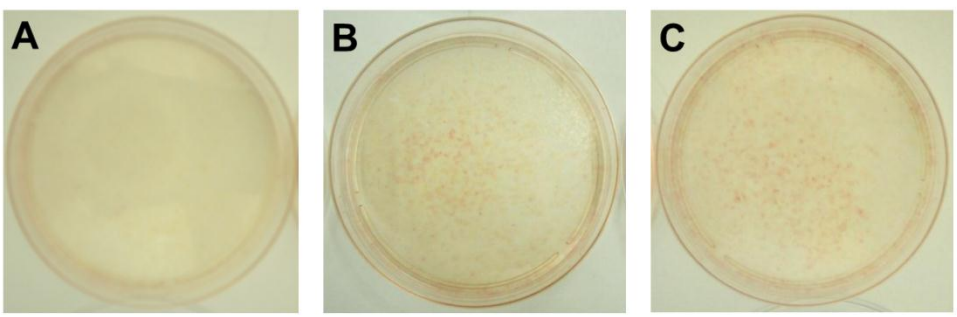

Figure 1. Alkaline phosphatase (ALP) staining after exposure to osteogenic induction for 0 day **(A)**, 4 days **(B)** and 8 days **(C)**.

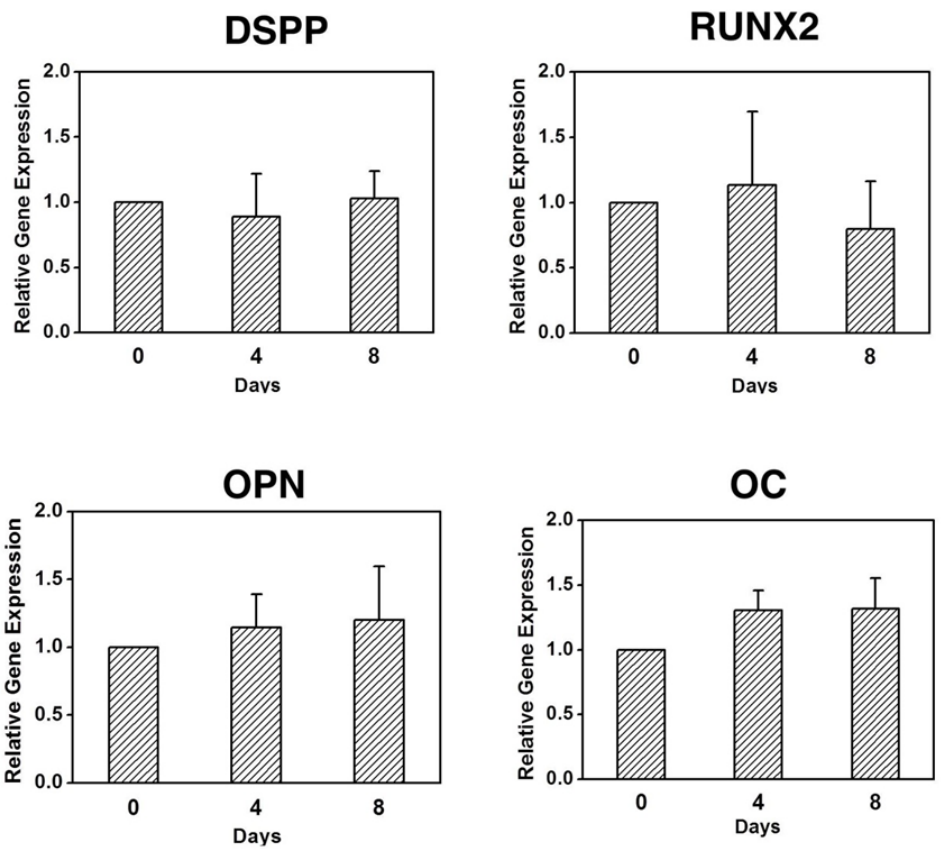

Figure 2. Relative gene expression level of dentin sialophosphoprotein (DSPP), Runt-related transcription factor 2 (Runx2), osteopontin (OPN), and osteocalcin (OC) after pre-culture in osteogenic media for different time periods. Data represents mean value ± standard deviation (SD). There was no statistical significance in Kruskal-Wallis test (p<0.05, n=6).

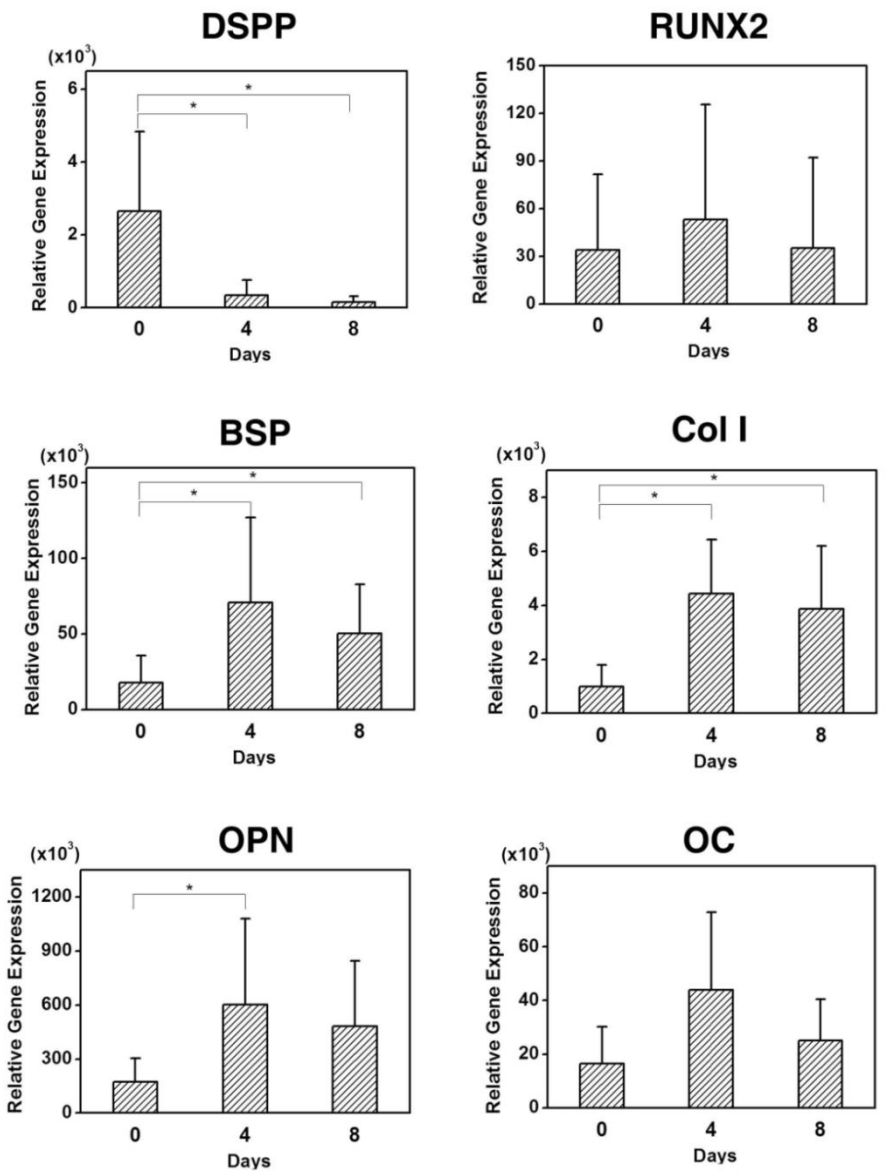

Figure 3. Relative gene expression level of DSPP, Runx2, Bone sialoprotein (BSP), Collagen I (Col I), OPN, and OC in retrieved transplants according to different period of in vitro differentiation in osteogenic media. Data represents mean value ± SD.

*Kruskal-Wallis test ($p<0.05$, $n=10$)

shown in Fig. 3, the genes related with hard tissue formation, Runx2, BSP, Col I, OPN, and OC, showed their peak in Day 4 group, followed by Day 8 and the control group.

2-2. Relative Amount of the Newly Formed Hard Tissue

The ALP activity in the transplants and area of the newly formed hard tissue estimated on the HE sections were used to compare relative amount of the newly formed hard tissue. As shown in Fig. 4, the ALP activity was greatest in Day 4, followed by Day 8 group and the control group. The difference between Day 4 group and the control was statistically significant ($p<0.05$). This result was in accordance with estimated hard tissue area on the histologic sections, as illustrated in Fig. 5. The amount of the newly formed hard tissue was greatest in Day 4 group, followed by Day 8 group and the control group.

2-3. Histomorphologic Features of the Newly Formed Hard Tissue

On HE sections, different histomorphologic characteristics were observed among the groups (Fig. 6, 7). In the control group, cultured without osteogenic stimuli, two types of hard tissue appearance were observed. Dentin-like appearance (Fig. 6A, D) was characterized by more linear alignment of mineral matrix, perpendicular to lining cell layer, and the absence of embedded cells. Another type presented irregular organization of mineralized matrix with cells entrapped within the matrix (Fig. 7A). In Day 4 group, increased deposition of mineralized matrix was observed compared to the thin layers in the control group, accompanying increased number of embedded cells. Its mineralized

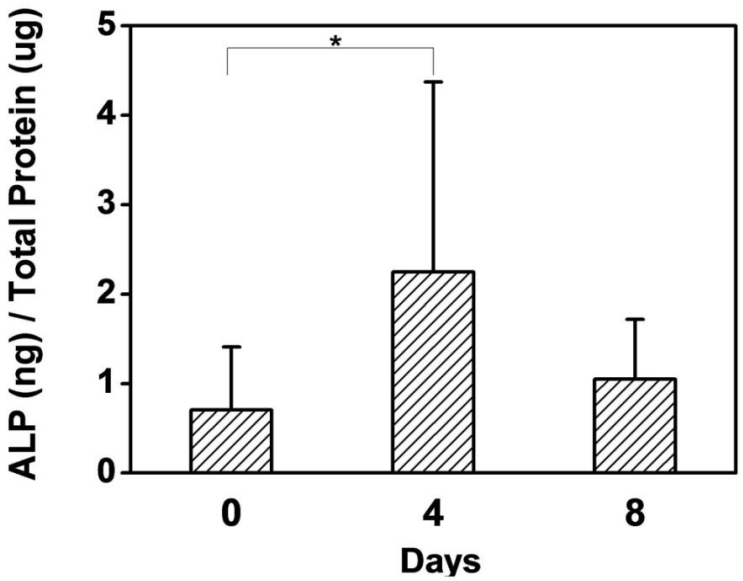

Figure 4. ALP activity in the retrieved transplants. Y-axis indicates relative amount of ALP, which was calculated by dividing ALP (ng) by the total protein (ug) in the transplants. Data represents mean value ± SD. *Kruskal-Wallis test (p<0.05, n=15).

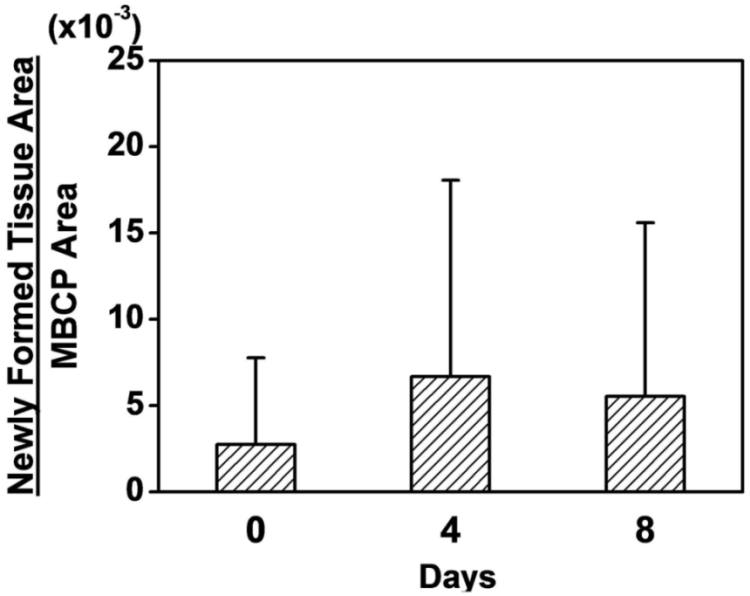

Figure 5. Comparison of the newly formed hard tissue area in respect of the pre-culture period. Relative area was calculated by dividing the total of newly formed hard tissue area by the total MBCP area. Data represents mean value ± SD. Kruskal-Wallis test (p>0.05, n=9).

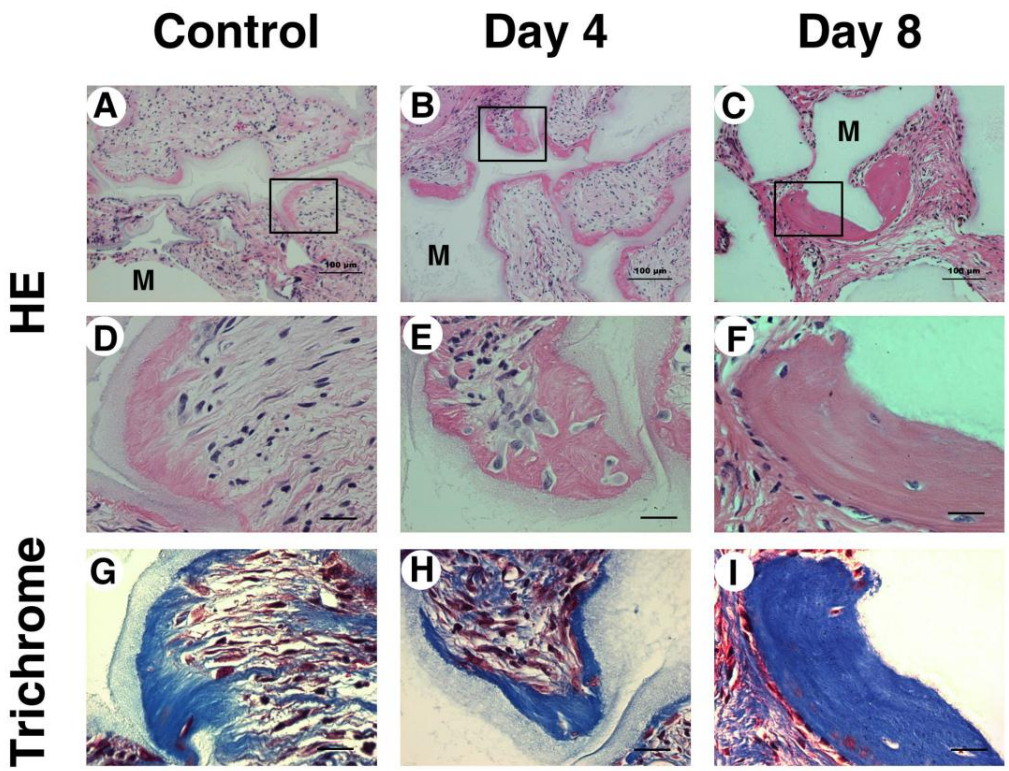

Figure 6. Histomorphologic characteristics of the newly formed hard tissue **(A-I)**. HE staining **(A-F)**. Masson's Trichrome staining on the consequent section **(G-I)**. Sections are representative of the control transplant **(A, D, G)**, Day 4 **(B, E, H)**, Day 8 **(C, F, I)**. Scale bars: 100 ㎛ (A-C), 20 ㎛ (D-I). Abbreviation: M, MBCP.

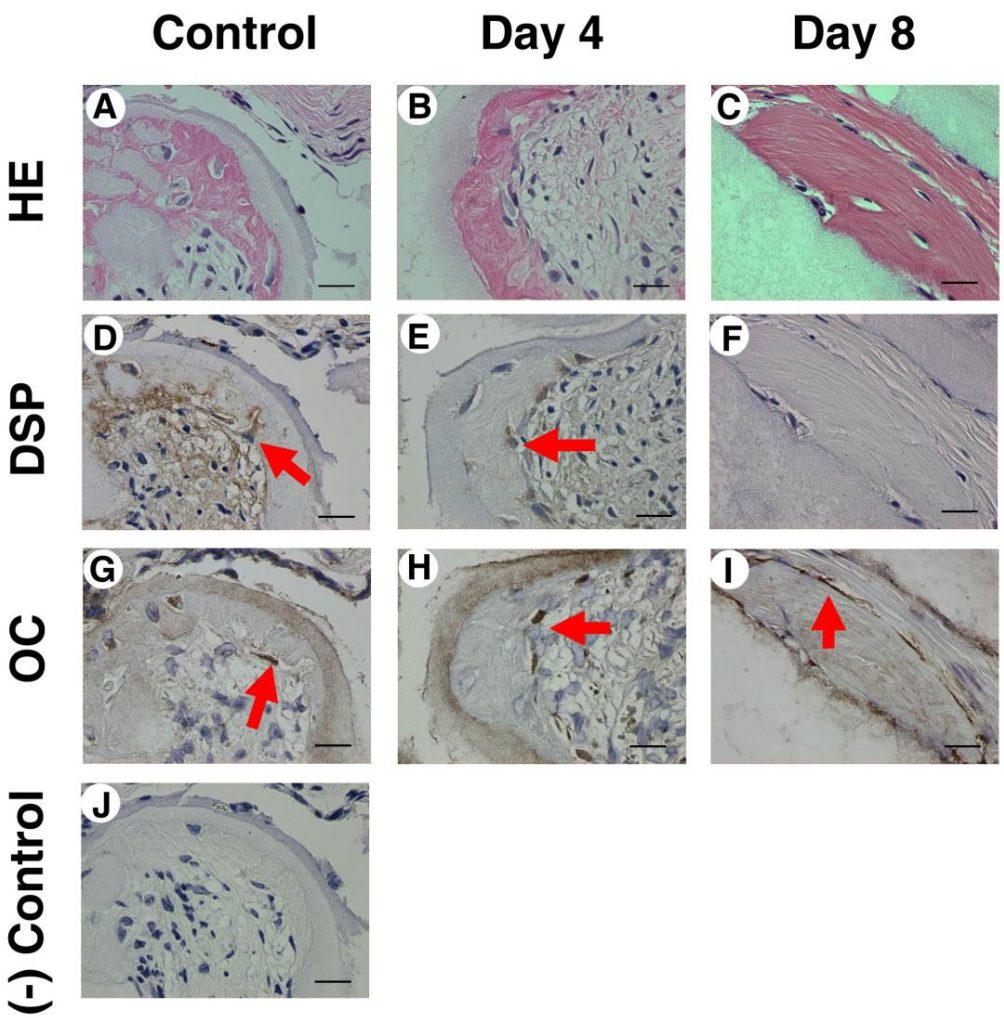

Figure 7. Immunohistochemical staining of human DSP and human OC in transplants. IHC staining was performed in consequent sections of HE sections **(A-C)**. **(D-F)** Human DSP staining. **(G-I)** Human OC staining. **(J)** Negative control for DSP staining. **(A, D, G, J)** The control group. **(B, E, H)** Day 4 group. **(C, F, I)** Day 8 group. Arrowheads indicate the cell having positive signal. Scale bars: 20 ㎛ in all sections.

matrix presented poor level of organization, featuring intermingled alignment of matrix and embedded cells with large cytoplasmic space and large nuclei (Fig. 6B, E). In Day 8 group, most of the newly formed hard tissue resembled typical appearance of bone (Fig. 6D). Compact matrix with lamellar pattern of alignment, parallel to the lining of osteoblast-like cells, was apparent. There were embedded cells within the matrix, which had reduced cytoplasmic space and condensed nuclei like osteocytes in lacunae. The lining cells were more apparent along the margin of the newly formed hard tissue.

2-4. Characterization of the Newly Formed Hard Tissue

Immunohistochemical staining of human DSP and human OC was performed to characterize the newly formed hard tissue in each group. DSP was highly expressed in the control group, and the intensity of DSP staining significantly reduced in Day 8, in accordance with the result of quantitative RT-PCR (Fig. 7D-F). OC expression was detected in all samples where mineralized hard tissue was present, but its expression was more obvious along the lining cells in Day 4 and 8 groups (Fig. 7 G-I).

IV. Discussion

In previous studies, SHED has been reported to differentiate and produce dentin-like and bone-like structures (Miura et al., 2003, Seo et al., 2008). Miura et al. (Miura et al., 2003) showed various morphology patterns of newly formed hard tissue, some resembling characteristics of dentin and some of bone. Their *in vitro* study results demonstrated the potential of SHED to differentiate into functional odontoblast-like cells, but SHED failed to generate dentin-pulp-like complex in *in vivo* study. SHED generated only dentin-like hard tissue, and interestingly SHED induced formation of bone-like hard tissue (Miura et al., 2003). In other studies, *in vivo* osteogenic potential of SHED was confirmed. In a mouse calvarial defect model, Seo et al. reported repair of the defect area by regeneration of bone by SHEDs without detectable amount of dentin formation (Seo et al., 2008). Bony repair of cranial defect in nonimmunosuppressed rats using dental pulp stem cell from a nonexfoliated deciduous tooth has been also reported (de Mendonca Costa et al., 2008). These results indicate presence of osteogenic subpopulations in multipotent postnatal stem cells harvested and isolated from dental pulp tissue of deciduous teeth.

Human dental pulp tissue of deciduous teeth can be obtained by two different methods, and the resulting population present different characteristics. Miura et al. (Miura et al., 2003) isolated multipotent stem cells from exfoliated deciduous teeth using enzymatic method and named them SHED. There is another population of deciduous dental pulp stem cells, called immature dental pulp stem cells (IDPSCs), which was isolated by using

outgrowth method (Kerkis et al., 2006). There are controversies over efficacy between enzymatic method and outgrowth method regarding the stemness and osteo/odontogenic differentiation potential of the isolated deciduous dental pulp stem cells. Kerkis et al. reported higher STRO-1 expression percentage (24%) in IDPSCs compared to 9% in SHEDs as well as the expression of ES cell markers, such as Oct-4, Sox2, Nanog (Kerkis et al., 2006, Miura et al., 2003). In contrast, another study reported superiority of enzymatic method regarding higher expression level of surface markers, STRO-1 and CD34, and biomineralization potential (Bakopoulou et al., 2011). In this study, outgrowth method was used, and the isolated and harvested cell population was named as deciduous dental pulp stromal cells (DDPSCs) for two reasons: the stem cell properties of this population were not examined, and it seemed to gradually lose its proliferation potency and differentiation potential in repeated cultivation.

In this study, DDPSCs were pre-cultured in the osteogenic media in order to evaluate the effect of *in vitro* osteogenic induction on *in vivo* odontogenic or osteogenic capacity of DDPSCs. Previous studies modified expansion conditions in order to manufacture MSCs intended for bony formation, such as by altering cell seeding density (Sotiropoulou et al., 2006), supplementation to culture media (Chadipiralla et al., 2010, Cheng et al., 1994), and low oxygen tension(Carrancio et al., 2008). Recent development deals with scaffolds seeded with pre-differentiated MSCs (Castano-Izquierdo et al., 2007, Cowan et al., 2005, Scotti et al., 2010). In these studies, transplantation of pre-differentiated stem cells resulted in enhanced regeneration of bone-like tissue compared to transplantation of undifferentiated stem cells, and pre-

culture period was an important factor affecting the amount and morphology of the newly generated hard tissue.

The pre-culture period in differentiation media determines the stage of differentiation at time of *in vivo* transplantation. In a previous study, rat MSCs were expanded *in vitro* in osteogenic media for different time periods (4, 10, 16 days), seeded on sintered titanium, and implanted in the rat cranium. Implants seeded with cells that have been cultured in the osteogenic media for 4 days revealed the highest bone formation, followed by 10-day group, the control group, and 16-day group (Castano-Izquierdo et al., 2007). In other studies, rat MSCs were expanded in osteogenic media for 7 days and seeded on titanium fiber mesh for additional 1, 4, and 8 days. In this case, cell/scaffold constructs cultured for 1 day showed the highest bone formation in both subcutaneous and cranial transplantation (Sikavitsas et al., 2003, van den Dolder et al., 2002). These two previous studies showed highest osteogenicity of the pre-differentiated MSCs exposed to osteogenic media for short time periods. There is an inverse relationship between proliferation and differentiation (Owen et al., 1990). Osteoblastic differentiation accompanies a decrease in the proliferation rate, changes in gene and protein expression pattern, and a deposition of minerals. MSCs at early differentiation stage have stronger proliferation potency, therefore increasing their number after transplantation and resulting in greater amount of regenerated hard-tissue.

Osteoblast differentiation is defined by three stages: proliferation, extracellular matrix maturation, and mineralization (Lian and Stein, 1992). Each stage can be characterized by different gene expression pattern, expression of proteins, and appearance of a mineralized

nodule formation. As differentiation progresses, proliferation decreases. Expression of Runx2 is observed in the early stage of osteogenic differentiation. Runx2 is the 'master gene' for osteoblast differentiation: it regulates the differentiation of MSCs to pre-osteoblast s and it is required for the expression of BSP and OC (Ducy et al., 1997). The matrix maturation stage is defined by increased expression of ALP and simultaneous development of a collagen matrix. The peak of ALP expression level coincides with their commitment to become osteoblast (Tenenbaum, 1987). OPN and OC are expressed in high level along with consistent calcium accumulation during mineralization stage (Lian and Stein, 1992). In this *in vitro* study, Runx2, ALP, OPN, and OC were used as markers to assess the differentiation stages in *in vitro* study. In addition, dentin-specific marker, DSPP, was used to examine the differentiation potential of DDPSCs into odontoblast-like cells. During odontoblast differentiation and maturation, DSPP expression increases as Runx2 is downregulated (Chen et al., 2005). In this study, upon *in vitro* induction in osteogenic media, DDPSCs initiated differentiation as illustrated by timely gradual increase in mRNA expression level of ALP and OC level and increased area of ALP staining. Timely increase in the expression of marker genes and ALP-positive cells indicate DDPSCs in Day 8 group were most differentiated at time of *in vivo* transplantation among the three groups. However, the absence of significant increase in the mRNA level of later differentiation markers, such as ALP, OPN, OC, or DSPP indicates that DDPSCs are in early stage of differentiation, despite of relative difference in differentiation progress.

In contrast to *in vitro* data, significant increase in the expression level of odontogenic and osteogenic markers in the retrieved transplants was observed, which indicate

continued differentiation of DDPSCs and mineralization after *in vivo* transplantation. The relative amount of the newly formed hard tissue was evaluated by comparing expression level of Col I, BSP, OC, OPN, and DSPP. Col I is actively expressed during proliferation stage and early differentiation stage, produced by osteoprogenitor cells (Boskey et al., 1999, Owen et al., 1990). BSP and OC are non-collagenous protein, expressed by fully differentiated osteoblasts, and they are involved in mineralization (Chenu et al., 1994, Oldberg et al., 1988). BSP binds to collagen I and nucleates hydroxyapatite crystal formation, initiating mineralization of bone (Tye et al., 2005). OC is the most abundant osteoblast-specific non-collagenous protein, and it is involved in binding of calcium and hydroxyapatite during mineralization stage (Chenu et al., 1994). Osteopontin also increases during matrix mineralization period (Franzen and Heinegard, 1985). Higher mRNA expression level of Col I, BSP, OC, and OPN in the retrieved transplants suggest greater amount of newly formed hard tissue. In particular, high expression level of DSPP distinguishes the dentin-like character of the hard tissue. Significant increase in the expression level of Col I, BSP, and OPN implies most active hard tissue formation occurred in Day 4 group, followed by Day 8 and the control group. The relative hard-tissue forming potential suggested by mRNA expression level of marker genes was in accordance with ALP activity assessment and also hard tissue area estimated on the histologic sections.

The results of this study are in accordance with other previous study results that shorter osteogenic induction period results in greater amount of hard tissue formation (Castano-Izquierdo et al., 2007, Sikavitsas et al., 2003, van den Dolder et al., 2002). The

enhancement in hard tissue forming potential may be attributed to shorter induction period that results in maintenance high proliferation potential. The induced cells may keep proliferating after implantation and differentiate into specific lineage as they have been stimulated before implantation. In fact, Castano-Izquierdo et al. (Castano-Izquierdo et al., 2007) showed that transplantation of fully differentiated osteoblast resulted in very low bone formation. This shows the importance of differentiation stage at time of transplantation.

The character of newly formed hard tissue was different between the pre-cultured group and the control. The expression level of DSPP, dentin-specific protein, in the retrieved transplants was significantly high in the control group compared to Day 4 and Day 8 groups. It suggests loss of odontogenic character in the newly generated hard tissue when pre-differentiated in osteogenic media prior to transplantation. The distinctive Histomorphologic features of the three groups well reflect the result of in vivo quantitative RT-PCR. The control group, where DSPP was expressed in the highest level, presented dentin-like features on histologic sections and was stained positive for anti-human DSP and OC. In contrast, Day 8 group featured more bone-like histomorphologic characteristics compared to the control and was negative to anti-human DSP.

While 4-day pre-culture period resulted in greatest amount of hard-tissue formation, 8-day pre-culture period in osteogenic media resulted in generation of hard tissue with more bone-like histomorphologic features and loss of dentinogenic character. Osteogenic induction of DDPSCs prior to in vivo transplantation guided the differentiation pattern of DDPSCs towards osteogenic lineage instead of odontogenic lineage.

Deciduous teeth provide easily accessible and immature source of stem cells for tissue engineering in various fields. The findings of this study can be utilized in future studies to increase the efficiency of target tissue engineering, especially in bone tissue engineering using DDPSCs.

V. Conclusion

The objectives of this study were to investigate the effect of pre-osteogenic induction on in vivo differentiation pattern of DDPSCs. Shorter osteogenic induction period was effective in increasing the amount of the newly formed hard tissue due to greater proliferation potential, whereas longer osteogenic induction period was related to formation of more bone-like structure. The proliferation potential and degree of differentiation of DDPSCs at time of *in vivo* implantation were important determinants for quantity and quality of the newly generated hard tissue.

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국문요약

유치에서 분리된 치수기질세포의 골유도 전처치 과정이 생체 내 경조직 형성능에 미치는 영향

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지도교수: 이제호

인간 유치의 치수 조직에서 다분화 줄기 세포가 분리될 수 있는 것으로 알려졌으며, 이를 상아질 및 골조직 재생에 응용하려는 시도가 계속 이루어지고 있다. 이 연구의 목적은 유치에서 분리한 치수기질세포의 골유도 전처치 과정이 생쥐의 생체 내에서 경조직 형성능에 어떤 변화를 가져오는지를 검사하는 것이었다. 유치 치수기질세포는 발거된 유치에서 outgrowth 방법을 사용하여 분리되었다. 시험관내 증식 과정에서 유치 치수기질세포는 골분화 유도 배지에서 4일 (Day 4 군) 또는 8일 (Day 8 군) 간 배양되었고, 대조군의 경우 일반 배양 배지에서 배양되었다. 시험관내에서 증식 및 분화된 유치 치수기질세포를 MBCP와 함께 면역 억제된 쥐의 등에 이식한 후 생성된 조직을 조직학적, 면역조직학적 방법으로 조사하고 정량적 역전사 중합효소 연쇄반응

(RT-PCR)을 사용하여 분석하였다. 이식 후 생성된 조직에서 형성된 경조직 양은 Day 4에서 가장 많았고, Day 8과 대조군 순으로 감소하였다. 형성된 경조직의 조직형태학적 특성은 세 군에서 상이하였는데, Day 8 군의 경우 정상 골의 형태와 가장 비슷한 특성을 보였으며 골유도 전처치를 시행하지 않은 대조군의 경우 상아질과 비슷한 형태의 경조직을 형성하였다. 4일의 전처치 기간은 더 많은 경조직 형성에 유리하였으며, 8일의 전처치 기간은 좀 더 골과 비슷한 특성을 가진 경조직 형성을 가능하게 하였다. 결론적으로 유치 치수기질세포의 골유도 전처치 과정을 통해 생체 내 경조직 형성능을 향상시킬 수 있으며, 전처치 기간은 새로 형성되는 경조직의 양과 질을 결정하는 중요한 요소이다.

핵심되는 말: 유치, 치수기질세포, 골유도 전처치, 생체내 이식, 경조직 형성