Biochemical and Biophysical Research Communications 371 (2008) 90–93

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hard tissue regeneration capacity of apical pulp derived cells (APDCs) from human tooth with immature apex

Shigehiro Abe [a], Satoshi Yamaguchi [a,*], Akihiko Watanabe [b], Keiichi Hamada [a], Teruo Amagasa [a]

[a] Maxillofacial Surgery, Maxillofacial Reconstruction and Function, Division of Maxillofacial and Neck Reconstruction, Graduate School, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8549, Japan

[b] Division of Organic Materials, Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, 2-3-10, Kanda-surugadai, Chiyoda-ku, Tokyo 101-0062, Japan

ARTICLE INFO

Article history:
Received 24 March 2008
Available online 11 April 2008

Keywords:
Dental pulp
Bone
Dentin
Hydroxyapatite
Tissue engineering

ABSTRACT

Recent studies indicate that dental pulp is a new source of adult stem cells. The human tooth with an immature apex is a developing organ, and the apical pulp of this tooth may contain a variety of progenitor/stem cells, which participate in root formation. We investigated the hard tissue regeneration potential of apical pulp derived cells (APDCs) from human tooth with an immature apex. APDCs cultured with a mineralization-promoting medium showed alkaline phosphatase activity in porous hydroxyapatite (HA) scaffolds. The composites of APDCs and HA were implanted subcutaneously in immunocompromised rats and harvested at 12 weeks after implantation. In histological analysis, the APDCs/HA composites exhibited bone- and dentine-like mineralized tissues in the pore areas of HA. This study suggests that the human tooth with an immature apex is an effective source of cells for hard tissue regeneration.

An adult stem cell is an undifferentiated cell that can differentiate into all the specialized cell types of the tissue from which it originated. The multilineage differentiation potential of adult stem cells has been extensively studied not only in basic research but also in therapeutic use. The most studied type of adult stem cell is hematopoietic stem cells and mesenchymal stem cells [1] in bone marrow, and multilineage cells were actually isolated from adipose tissue [2], artery wall [3], and umbilical cord blood [4]. The dental pulp is also thought to be a source of adult stem cells. Multilineage populations of cells were isolated from the dental pulp of human permanent teeth [5] and human exfoliated deciduous teeth [6].

In our study, the focus of interest is human open-apex tooth. The human tooth with an immature apex is a developing organ. The root is formed by the dynamic biological reactions, including proliferation and differentiation of stem cells in the apical pulp. We previously reported that cells derived from the tip of apical pulp of human tooth with immature apex, apical pulp derived cells (APDCs), had a high proliferation activity and multilineage differentiation potential [7]. APDCs were able to differentiate into mineralized cells, adipocytes, chondrocytes, and neural cells under appropriate conditions in vitro. However, it was not clear whether mineralized cells differentiated from APDCs in vitro were osteoblasts or odontoblasts. In this study, we examined the potential of APDCs to form hard tissue in vivo. This paper describes scanning electron micrographic, histological, and immunohistochemical analyses of the mineralized tissue regeneration potential of APDCs in porous hydroxyapatite (HA).

Materials and methods

Tooth and cell culture. The present study was approved by the Institutional Review Board of faculty of Dentistry, Tokyo Medical and Dental University, and all donors gave informed consent. Human impacted third molars with open apex (Fig. 1B), freshly extracted for orthodontic reason or treatment were obtained at the oral and maxillofacial surgery clinic of Tokyo Medical and Dental University.

The APDCs were isolated as previously described [7]. For the primary outgrowth cell culture, apical pulp tissue not framed by dentin was separated from the tip of the root and cut into samples about 1 mm^3 in size. The apical pulp tissue samples were plated on a 60-mm culture dish with Iscove's modified Dulbecco's medium (IMDM) (Sigma Chemical Co., St. Louis, USA) supplemented with 10% fetal bovine serum (FBS) (Moregate FCS, Buckingham, UK) and 2 mM L-glutamine (Wako Pure Chemical Co. Ltd., Osaka, Japan), and maintained at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2 for 3 weeks. The medium was changed every 2 days. After 3 weeks of primary outgrowth culture, the apical pulp derived cells (APDCs) were detached by exposure to 0.25% trypsin and 0.05% EDTA for 60 min at 37 °C. The cells were then seeded at a density of 2.0×10^5 cells on a 100-mm culture dish, and cultured with IMDM supplemented with 10% FBS and 2 mM L-glutamine (Wako) at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2. When the cells approached confluence, they were re-plated under the same culturing conditions. The APDCs at third passage was used for each experiment.

In vitro mineralized cell differentiation of APDCs on HA scaffold. The APDCs at third passage was seeded at a density of 2.5×10^5 cells/15 μL into porous HA scaffold consisting of approximately 1/8-size ($5 \times 2.5 \times 2$ mm) pieces of Interpore-200 (Interpore). Cells were then cultured in alpha-modified essential medium

* Corresponding author. Fax: +81 3 5803 5500.
E-mail address: yamachan.mfs@tmd.ac.jp (S. Yamaguchi).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.04.016

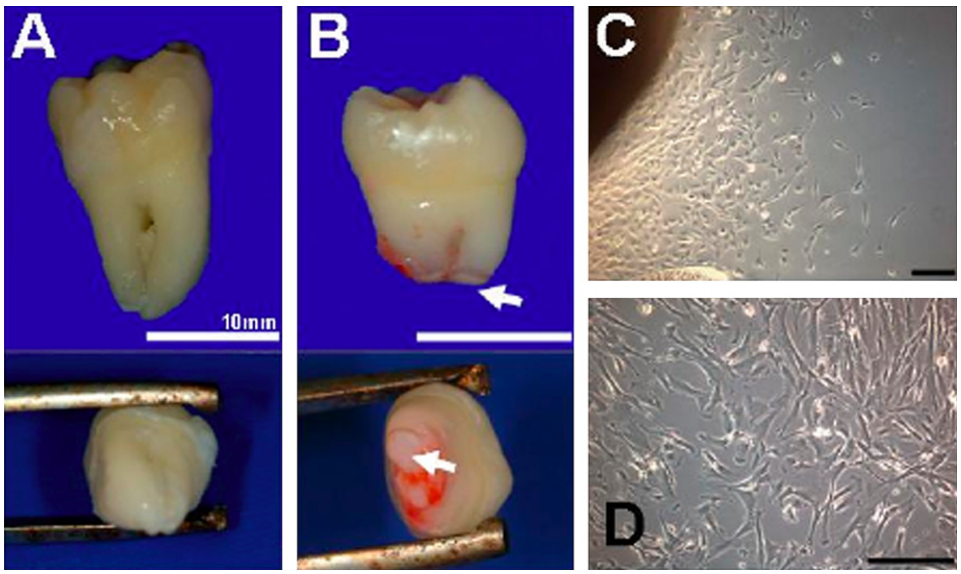

Fig. 1. APDCs from an open-apex tooth. Photographs of human teeth with mature apex (A) and immature apex (B). (C) APDCs surrounding explanted tissue in primary outgrowth culture. (D) Morphology of 3rd passage APDCs. Original magnifications: (C) 10×, (D) 20×; scale bars: (A,B) 10 mm, (C,D) 100 μm.

(α-MEM) (Sigma) supplemented with 10% FBS, 2 mM L-glutamine (Wako), 0.2 mM ascorbic acid (Wako), 1 mM β-glycerophosphate (Sigma), 100 nM dexamethasone (Sigma) and 300 ng/ml rh-BMP2 (R&D system) for 10 days. Then the cells were cultured in the same medium without rh-BMP2 for up to 3 weeks. This medium was changed every 2 or 3 days for up to 3 weeks. To evaluate the alkaline phosphatase activity, each sample was fixed with 4% formaldehyde for 10 min and soaked in 0.01% naphthol AS-MX phosphate (Sigma), 0.06% red violet LB salt (Sigma) in 0.2 M Tris/HCl buffer for 15 min at 37 °C.

Scanning electron micrograph analysis (SEM analysis). The samples were fixed with 2.5% glutaraldehyde in PBS for 24 h at 4 °C and washed with PBS. Then, they were, freeze-dried, and coated with gold. The cells were examined with a SEM (Joel JSM-5400, Tokyo, Japan). The control group was cell-free HA scaffold.

Implantation of the composites of APDCs and HA scaffold. The APDCs at third passage were seeded at a density of 5.0×10^5 cells/30 μL into porous HA scaffolds consisting of approximately 1/4-size ($5 \times 5 \times 2$ mm) pieces of Interpore-200 (Interpore). The cells on scaffold were differentiated into mineralized cells with same culture protocol as mentioned above. The composites of APDCs and HA scaffold were implanted in subcutaneous pouches of the back of Null rats (Nihon Krea Co., Japan). The composites were recovered at 12 weeks after implantation.

The Animal Care and Use Committee of Tokyo Medical and Dental University approved the protocols used in this study.

Histological analysis of implanted APDCs/HA composites. The recovered composites were fixed immediately in 4% paraformaldehyde at 4 °C for 48 h and decalcified in 5% formic acid for 3 weeks. The decalcified samples were embedded in Tissue-TEK® O.C.T. compound (Sakura Finetechnical Co., Tokyo, Japan). Frozen sections were used for hematoxylin-eosin (HE), masson trichrome and immunohistochemical staining.

Immunofluorescence. The sections used for immunohistological analysis were washed with TBST. The slides were then incubated in TBST/5% skim-milk solution for 30 min to prevent non-specific binding of antibodies. The slides were incubated with mouse primary antibodies for human specific mitochondria (1:50) (Chemicon) for 1 h, followed by fluorescein-coupled goat anti-mouse IgG secondary antibodies for 1 h. Between incubations, the slides were washed with PBS.

Result

In vitro mineralized cell differentiation of APDCs in porous hydroxyapatite (HA) scaffold

The cells cultured in porous hydroxyapatite scaffold with a mineralization-promoting medium showed time-dependent alkaline phosphatase activity (Fig. 2A).

In order to show cell adhesion to the porous hydroxyapatite scaffolds, the APDCs cultured on the scaffolds were examined using SEM at 1, 2, and 3 weeks after seeding. It was evident that the APDCs had disseminated well on the scaffolds (Fig. 2B–M).

Histological analysis of implanted APDCs/HA composite

To investigate the potential of the APDCs to form hard tissue in vivo, the composite of APDCs and HA were implanted subcutaneously into an immunocompromised rat. The APDCs generated ectopic bone-like tissue and dentin-like tissues on the border of the porous HA at 12 weeks after implantation (Fig. 3A). The bone-like tissues contained osteocyte-like cells embedded within the calcified matrix, and osteoblast-like cells were observed along the surface of lining the bone surface (Fig. 3B and C). Dentin-like tissues that did not contain osteocytes-like cells and did not form clearly tubular structures had a dentin-like matrix, pulp-like tissue (Fig. 3D and E), and masson trichrome staining revealed odontoblast-like cells having odontoblastic processes (Fig. 3F). Most of the generated tissue had a bone-like structure, and only a small portion of the generated hard tissues showed dentin-like structure.

Immunohistochemical analysis

To examine the origin of the generated tissue, we performed immunohistochemical analyses. The generated tissues in the scaffold pores stained positive for anti human specific mitochondria (Fig. 4B). These data suggest that the generated tissue was derived from human cells.

Discussion

The protective and reparative function of dental pulp is well known. Odontoblasts are responsible for this function, and produce physiological secondary dentin. When odontoblasts are irreversibly damaged, they are replaced by a second generation of newly differentiated odontoblasts that give rise to a reparative dentin matrix. This suggests the presence of resting progenitor or stem cell of odontoblast in dental pulp. For biological phenomenon mentioned above, it is likely that transplantation of cultured dental pulp cells generate dentin tissue in vivo. In fact, previous reports showed that the cells derived from human dental pulp have a dentin-forming capability [5].

On the other hand, bone does not occur naturally in dental pulp, therefore, it is unlikely that osteoblasts or preosteoblasts are included in dental pulp. However, dental pulp is assumed to possess

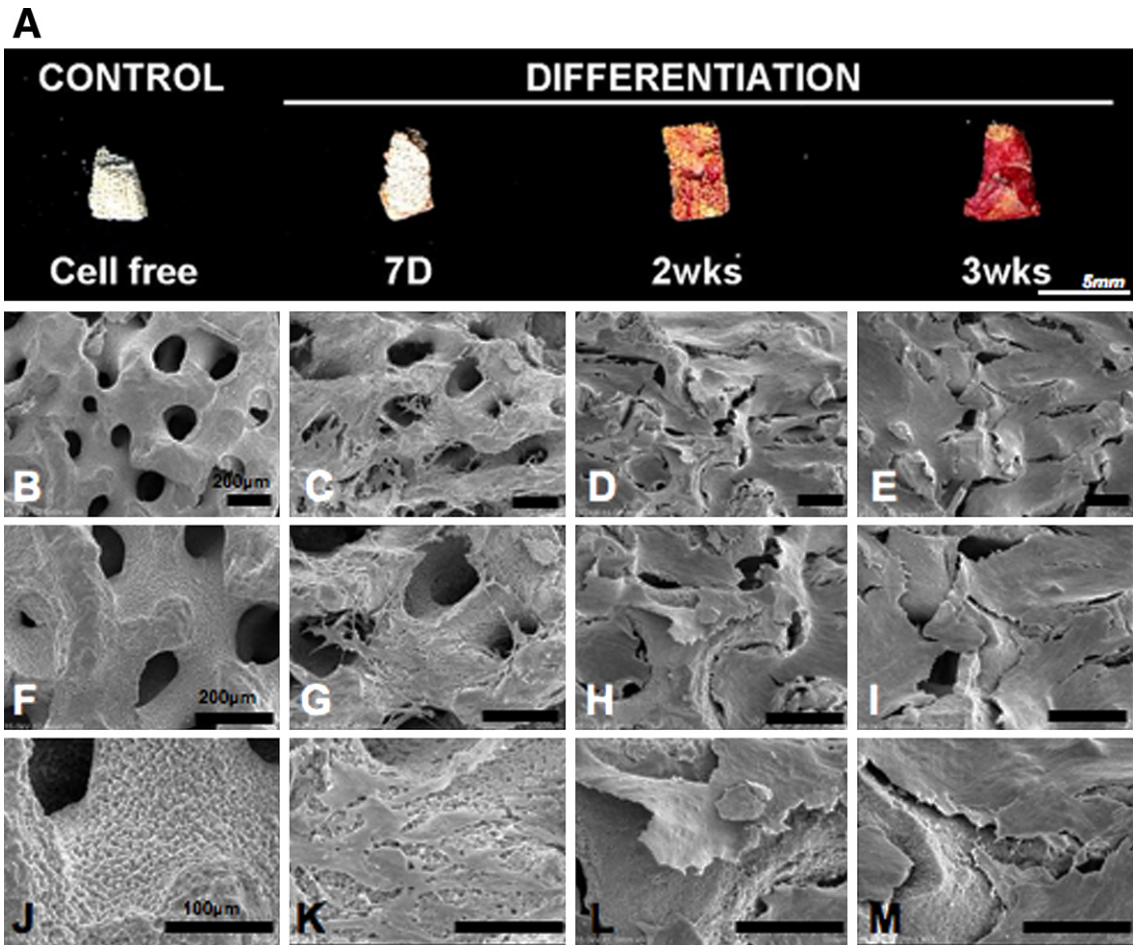

Fig. 2. In vitro mineralized cell differentiation and cell adhesion of APDCs in porous HA scaffold. (A) Alkaline phosphatase staining of differentiated APDCs in porous HA scaffold. Scale bar: 5 mm. (B–M) Scanning electron micrographs (SEM) of cell adhesion on porous HA scaffold. HA scaffold without cells (B, F, and J). One week (C, G, and K), 2 weeks (D, H, and L), and 3 weeks (E, I, and M) after seeding. Original magnifications: (B–E) 100×, (F–I) 200×, (J–M) 500×; scale bars: (B–I) 200 μm, (J–M) 100 μm.

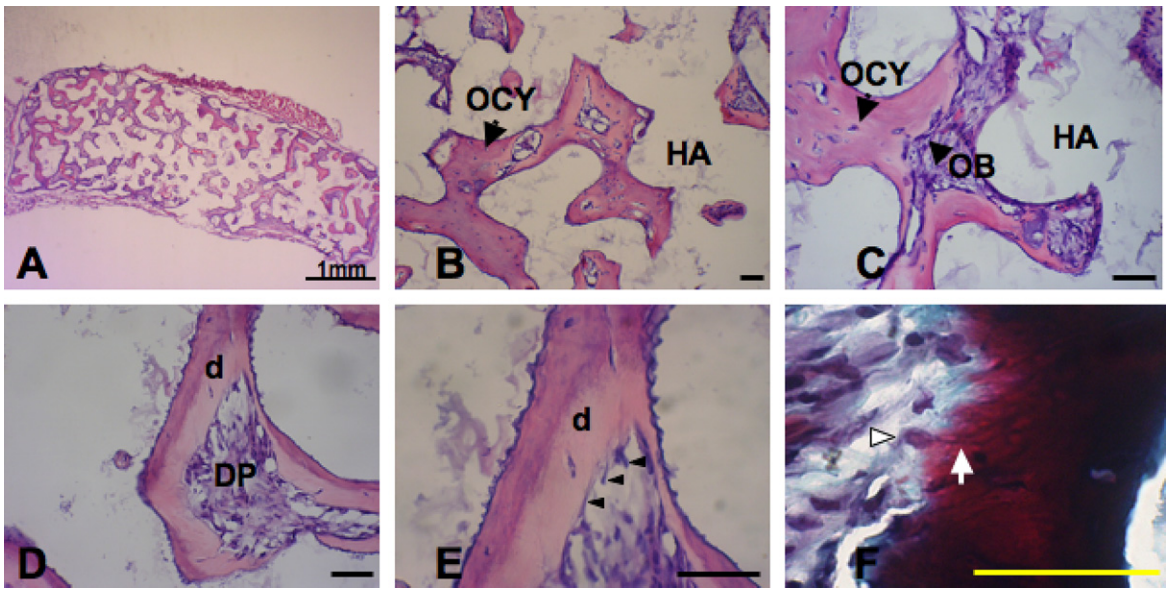

Fig. 3. In vivo regeneration capacity of APDCs/HA composite after transplantation. (A) APDCs/HA composite stained with HE (B,C). Bone-like tissues contained osteocyte-like cells (OCY) embedded within the calcified matrix and osteoblast-like cells (OB) lining the bone surface. (D,E) Dentin-like tissues (d) had a dentin-like matrix, pulp-like tissue (DP), and odontoblast-like cells (triangles). (F) Masson Trichrome staining demonstrates odontoblast-like cell (triangle) having odontoblastic processes (arrow). Original magnifications: (A) 1×, (B) 10×, (C,D) 20×, (E) 40×. (J) 80×. Scale bars: (A) 1 mm, (B–F) 50 μm.

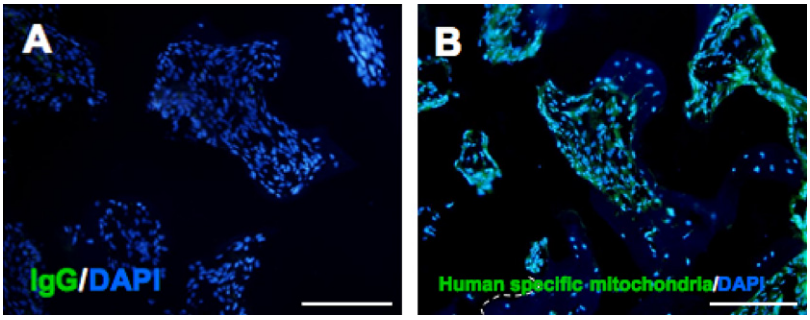

Fig. 4. Immunohistochemical analysis of APDCs/HA composite transplants. (A) Negative control. (B) Cells stained positive for anti human specific mitochondria within the scaffold pores. Original magnifications: (A,B) 10×. Scale bar: 100 μm.

the capacity to elaborate both bone and dentin matrix under the pathological conditions following tooth injury [8]. The mechanism determining divergent healing process remains unclear. Recent study demonstrated that the appearance of TRAP-positive cells and CK-positive cells is involved in the induction of bone tissue formation in dental pulp [9]. And it is reported that cells derived from dental pulp have a bone-forming capability in vivo [10] and [11]. The presence of stem cell differentiating into osteoblast under appropriate condition is assumed from these previous results.

In this study, APDCs formed both bone and dentin in vivo after transplantation into immunocompromised rats. APDCs are a heterogeneous cell population, and include several kinds of cells. There is a possibility that APDCs include two kinds of cells; one is odontoblast or preodontoblast, the other is stem cell which can differentiate into osteoblast. Dental pulp is composed of various cell populations, including resident mesoderm-derived cells at the site of tooth development, paraxial mesenchyme-derived cells and cells derived from cranial neural crest cell [12]. Odontoblasts are derived from the dorsal cranial neural crest. The origin of cells to form dentin in APDCs, therefore, may be cranial neural crest cells. The origin of cells to form bone in APDCs is interesting, but remains to be proved. It is well known that mesenchymal stem cell is able to differentiate into osteoblast in vitro under appropriate conditions [1], and form bone tissue in vivo [13]. The occurrence of mesenchymal stem cells in dental pulp is likely, but not supported by unequivocal evidence.

The cells derived from dental pulp are extensively studied for hard tissue engineering. Definitive strategy to generate bone or dentin by dental pulp cells, however, is not established. There are several differences in culture condition, selection of cells with surface marker, scaffold or differentiation-inducing factor to generate bone or dentin by cultured dental pulp cells between previous reports. It was reported that different culture methods give rise to different populations or lineage cells during cell culture in vitro [14]. Cell culture condition is to be noted when the character of cultured cell is evaluated. The scaffold is also important for tissue engineering. Hydroxyapatite is the commonly used ceramic in the study of regeneration of bone [15]. However, it may not be the ideal scaffolds, because the ceramics are solid but brittle and not biodegradable. The better scaffolds for hard tissue engineering should be developed.

In this study, we showed the potential of APDCs to generate bone or dentin in vivo. Further studies are required to develop ideal hard tissue engineering with APDCs.

References

[1] M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, Multilineage potential of adult human mesenchymal stem cells, Science 284 (1999) 143–147.
[2] P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick, Human adipose tissue is a source of multipotent stem cells, Mol. Biol. Cell 13 (2002) 4279–4295.
[3] Y. Tintut, Z. Alfonso, T. Saini, K. Radcliff, K. Watson, K. Bostrom, L.L. Demer, Multilineage potential of cells from the artery wall, Circulation 108 (2003) 2505–2510.
[4] O.K. Lee, T.K. Kuo, W.M. Chen, K.D. Lee, S.L. Hsieh, T.H. Chen, Isolation of multipotent mesenchymal stem cells from umbilical cord blood, Blood 103 (2004) 1669–1675.
[5] S. Gronthos, J. Brahim, L.W. Fisher, N. Cherman, A. Boyde, P. DenBesten, P.G. Robey, S. Shi, Stem cell properties of human dental pulp stem cells, J. Dent. Res. 81 (2002) 531–535.
[6] M. Miura, S. Gronthos, M. Zhao, B. Lu, L.W. Fisher, P.G. Robey, S. Shi, SHED: stem cells from human exfoliated deciduous teeth, Proc. Natl. Acad. Sci. USA 100 (2003) 5807–5812.
[7] S. Abe, S. Yamaguchi, T. Amagasa, Multilineage cells from apical pulp of human tooth with immature apex, Oral Sci. Int. 4 (2007) 45–58.
[8] A. Shimizu, K. Nakakura-Ohshima, T. Noda, T. Maeda, H. Ohshima, Responses of immunocompetent cells in the dental pulp to replantation during the regeneration process in rat molars, Cell Tissue Res. 302 (2000) 221–233.
[9] H. Tsukamoto-Tanaka, M. Ikegame, R. Takagi, H. Harada, H. Ohshima, Histochemical and immunocytochemical study of hard tissue formation in dental pulp during the healing process in rat molars after tooth replantation, Cell Tissue Res. 325 (2006) 219–229.
[10] G. Laino, R. d'Aquino, A. Graziano, V. Lanza, F. Carinci, F. Naro, G. Pirozzi, G. Papaccio, A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB), J. Bone Miner. Res. 20 (2005) 1394–1402.
[11] E. Ikeda, M. Hirose, N. Kotobuki, H. Shimaoka, M. Tadokoro, M. Maeda, Y. Hayashi, T. Kirita, H. Ohgushi, Osteogenic differentiation of human dental papilla mesenchymal cells, Biochem. Biophys. Res. Commun. 342 (2006) 1257–1262.
[12] M. Goldberg, A.J. Smith, Cells and extracellular matrices of dentin and pulp, Crit. Rev. Oral. Biol. Med. 15 (2004) 13–27.
[13] H. Petite, V. Viateau, W. Bensaid, A. Meunier, C. de Pollak, M. Bourguignon, K. Oudina, L. Sedel, G. Guilleman, Tissue-engineered bone regeneration, Nat. Biotechnol. 18 (2000) 959–963.
[14] G.T.J. Huang, W. Sonoyama, J. Chen, S.H. Park, In vitro characterization of human dental pulp cells, Cell Tissue Res. 324 (2006) 225–236.
[15] H. Ohgushi, A.I. Caplan, Stem cell technology and bioceramics: from cell to gene engineering, J. Biomed. Mater. Res. 48 (1999) 913–927.