Effect of Passage Number on Cell Proliferation and Pluripotent Markers of Stem Cells Isolated from Human Exfoliated Deciduous Teeth

ผลของจำนวนรุ่นในการเพาะเลี้ยงต่อการแบ่งตัวและตัวบ่งชี้พลูริโพเทน ของเซลล์ต้นกำเนิดจากฟันน้ำนมมนุษย์

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ABSTRACT

The main purpose of this study was to evaluate the effect of passage number on the behaviors of stem cells isolated from human exfoliated deciduous teeth (SHED). SHEDs were maintained in culture and continued passage upon confluence. At passage (P) 5 and 10, cell proliferation, colony forming unit efficacy and the mRNA expression of pluripotent markers (Oct-4 and Rex-1) were determined. The results showed that cell proliferation and colony forming unit were lower at P10 compared to P5. Moreover, Rex-1 and Oct-4 mRNA levels were decreased in higher passage numbers. However, there was no statistically difference ($P<0.05$). The results indicated that high passage number may attenuate pluripotent properties of SHEDs, therefore it should be taken into account in SHEDs amplification for therapeutic application.

บทคัดย่อ

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของจำนวนรุ่นของการเพาะเลี้ยงต่อพฤติกรรมของเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันน้ำนมมนุษย์ โดยทำการเลี้ยงเซลล์เนื้อเยื่อในฟันน้ำนมมนุษย์และปลูกถ่ายลงในจานเลี้ยงใหม่เป็นรุ่นต่อไปเมื่อถึงความหนาแน่นที่ต้องการ เซลล์ในรุ่นที่ 5 และ 10 จะถูกวิเคราะห์การแบ่งตัวของเซลล์ การสร้างหน่วยโคโลนี และการแสดงออกของอาร์เอ็นเอนำรหัสของตัวบ่งชี้พลูริโพเทน (เร็กซ์-1 และอ็อกซ์-4) จากผลการทดลองพบว่าการเพิ่มจำนวนของเซลล์และการสร้างหน่วยโคโลนี ลดลงที่รุ่นที่10 เปรียบเทียบกับรุ่นที่ 5 นอกจากนี้ ระดับของอาร์เอ็นเอนำรหัสของยีนเร็กซ์-1 และอ็อกซ์-4 มีการลดลงเมื่อจำนวนรุ่นของการเพาะเลี้ยงเพิ่มขึ้นเช่นกัน อย่างไรก็ตามพบว่าไม่มีความแตกต่างกันทางสถิติ ($P<0.05$) จากผลการศึกษานี้แสดงให้เห็นว่าจำนวนรุ่นที่มากขึ้นอาจมีผลต่อคุณสมบัติพลูริโพเทนของเซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันน้ำนมมนุษย์ ดังนั้นจึงควรตระหนักในกระบวนการเพิ่มจำนวนเพื่อการประยุกต์ใช้ในการรักษา

Key Words: Stem cells from human exfoliated deciduous teeth, Cell proliferation, Pluripotency markers
คำสำคัญ: เซลล์ต้นกำเนิดจากฟันน้ำนม การแบ่งตัวของเซลล์ ตัวบ่งชี้พลูริโพเทน

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Introduction

Human deciduous dental pulp cells have been shown that they contained a subpopulation cells called stem cells from human exfoliated deciduous teeth (SHEDs) (Miura et al., 2003). SHEDs are unspecialized cells that have the capacity of both self-renewal and multipotency (Nakamura et al., 2009; Govindasamy et al., 2010). SHEDs were able to differentiate into osteogenic/odontogenic cells (Miura et al., 2003), adipocytes (Miura et al., 2003), neural cells (Miura et al., 2003; Nourbakhsh et al., 2011), endothelial cells (Sakai et al., 2010) and hepatocyte-like cells (Ishkitiev et al., 2010). SHEDs were also employed for wound healing promotion (Nishino et al., 2011) and calvarial bone defects repaired in immuno-compromised mice (Seo et al., 2008), suggesting the potential utilization in clinical application for tissue repair or regeneration (Cordeiro et al., 2008).

The main advantage of SHEDs is that these cells could be isolated noninvasively from physiologic exfoliated deciduous teeth (Miura et al., 2003). SHEDs exhibited higher proliferation rate compared to human dental pulp stem cells (DPSCs) and bone marrow-derived mesenchymal stem cells (BMMSCs) (Wang et al., 2012; Nakamura et al., 2009). SHEDs expressed various stem cell surface markers, including STRO-1, SSEA4, CD73, CD105, CD146 and CD166 (Yamaza et al., 2010). In comparison to DPSCs, SHED expressed higher levels of the pluripotent markers (Oct-4, SOX-2, Nanog and Rex-1) and presented a higher capability of mineralized hard tissue formation upon transplantation *in vivo* (Wang et al., 2012), suggesting that SHEDs maybe in a more immature state. However, the main concern of using SHEDs is the limited cells number after isolation therefore the long-term culture to increase cell quantities is unavoidable. Previous studies indicated that prolong expansion *in vitro* might decrease the differentiation potential of mesenchymal stem cells (Derubeis and Cancedda, 2004; Recquicha et al, 2012). Despite the reports that SHEDs showed no sign of degeneration or spontaneous differentiation even in high passage number (Suchanek et al., 2010), the effect of long-term cultivation on stemness properties is yet unknown. We hypothesize that increased passage numbers may have adverse effects on proliferation, colony forming unit efficacy and the mRNA expression of pluripotency markers of SHEDs.

Objectives of the study

The aim of the present study was to evaluate cell proliferation, colony forming unit efficacy and mRNA expression of pluripotency markers of SHEDs after the increase in passage numbers.

Methodology

SHEDs isolation and culture

Normal exfoliated deciduous teeth were collected from three healthy patients (6-12 years old) for cell isolation procedure. The experimental protocols were approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. SHEDs isolation and culture protocols were followed as previously described (Osathanon, Nowwarote, and Pavasant, 2011), in brief, dental pulp tissues were gently removed, washed with sterile phosphate buffer solution (PBS) and digested with type I collagenase (Gibco, USA). Cells were maintained in Dulbecco's Modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS, Gibco, USA), 2mML-glutamine (Invitrogen, USA), 100 U/ml penicillin (Invitrogen, USA), 100 mg/ml streptomycin

(Invitrogen, USA) and 5 mg/ml amphotericin B (Invitrogen, USA) in 100% humidity, 37°c and 5%carbon dioxide. Medium was changed every 48 hours. After reaching 90% confluence, cells were sub-cultured at 1:3 ratio. Cells culture at the fifth and tenth passage was used in the experiment.

Cell proliferation assay

Cell proliferation assay was performed as previously described (Osathanon et al., 2011). with modification. Cells were seeded at density of 12,500 cells/well in 24-well-plate and maintained in the medium described above. The (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; USB Corporation, USA) assay was performed after 1, 3, and 7 days of cultivation. The cells were treated with 1mg/ml MTT solution for 10 minutes at 37°C to allow formazan crystal formation. Further, the formazan was dissolved using dimethylsulfoxide (DMSO, Sigma-Aldrich, Germany) and glycine buffer. The solution was evaluated in optical density (OD) by a microplate reader (ELx800; BIO-TEK®, USA) at 540 mm. . Cell numbers were calculated using standard curve which OD values were plot against known cell numbers. The experiments were performed in triplicate.

Colony-forming unit assay

Colony-forming unit assay was performed according to the previous study (Osathanon et al., 2011). Cells were seeded into 60-mm-diameter culture dishes at density of 500 cells per dish and maintained in the medium described above. After 14 days, cells were fixed with 10% buffer formalin (MERCK, Germany) for 10 min, washed twice with PBS and stained with methylene blue (Sigma, USA). Aggregates of approximately 50 cells were scored as a colony and counted all colonies on a plate under a microscope (Axiovert40CFL, CarlZeiss, Göttingen, Germany) (Osathanon, Nowwarote, Pavasant et al., 2011) . The cell morphology will be investigated by fluorescent microscope (Carl Zeiss™Apotome2 apparatus, Germany).

Reverse transcription polymerase chain reaction (RT-PCR)

The expressions of stem cells markers were evaluated using reverse transcriptase polymerase chain reaction (RT-PCR) (Govitvattana, Osathanon, Taebunpakul and Pavasant, 2012). Total cellular RNA was extracted with Trizol reagent (RocheDiagnostics, USA). RNA samples (1 µg) were converted to cDNA using the ImPromII Reverse Transcription System (Promega, Uk). A polymerase-chain reaction (PCR) was performed using Taq polymerase (Invitrogen, Brazil). The PCR reaction was performed in the DNA thermal cycler (Biometra GmH, Göttingen, Germany). The sequences of the primers and number of cycles were shown in Table 1. The amplified DNA was then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide fluorostaining (Bio- Rad, USA) The band density was measured using Scion Image-Release alpha 4.0.3.2. GAPDH was used as an internal control. The relative expression was calculated by normalizing the density of target gene expression against GAPDH.

Table 1 Primer sequences and cycles used in the experiment

Gene	Primer sequence	Base pairs	cycles
Rex-1	Forward: 5' AGAATTCGCTTGAGTATTCTGA3' Reverse: 5' GGCTTTCAGGTTATTTGACTGA 3'	470	45
Oct-4	Forward: 5' GCAACCTGGAGAATTTGTTCCT 3' Reverse: 5' AGACCCAGCAGCCTCAAAATC3'	182	30
GAPDH	Forward: 5' TGAAGGTCGGAGTCAACGGAT3' Reverse: 5' TCACACCCATGACGAACATGG'	396	22

Statistical analyses

All experiments were performed in triplicate. Data were reported as mean ± standard deviation and were statistically analyzed by Student *t*-test for two-group comparison. Differences at $P<0.05$ were considered to be statistically significant.

Results

Using MTT assay (Fig.1), cells from both passage 5 and 10 had increased in cell proliferation from day 1 to day 7. Interestingly, the proliferation rate was dramatically decreased in cells from passage 10 when compared to cells from passage 5 at the same day of culture. However, statistically significant difference ($p<0.05$) was not found at any time point between both passages (P5 VS P10) or in the cells from same passage (1d, 3d and 7d).

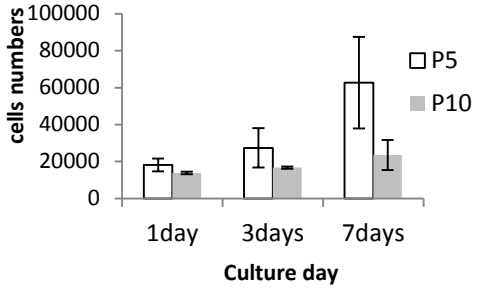

Fig. 1 Effect of increased passage on self-renewal ability of deciduous dental pulp cells. The deciduous dental pulp cells at passage 5 and passage 10 were measured for cell proliferation by MTT assay ($P<0.05$)

Colony formation unit of cells from passage 5 is obviously higher than cells from passage 10, but statistical significance was not observed (Fig. 2A and 2B). As for cell morphology, cells from both passages were identical with long, spindle, fibroblast-like shape (Fig. 2C).

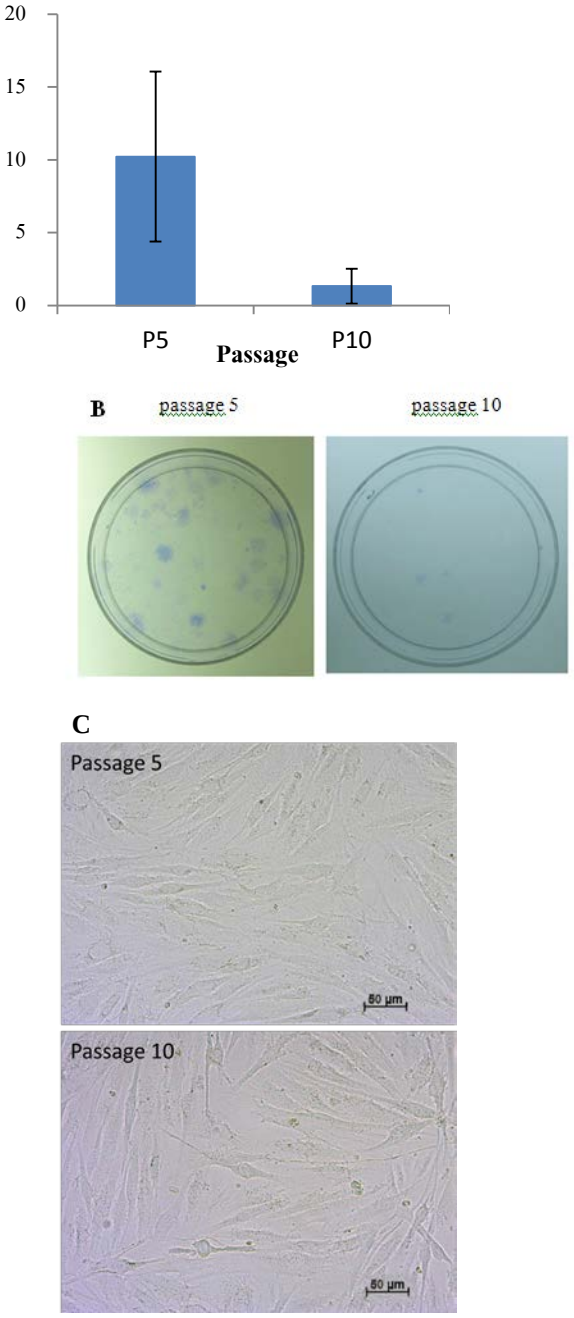

Fig. 2 Effect of increased passage on self-renewal ability of deciduous dental pulp cells. A: Graph illustrated the average number of colony per plate ($P<0.05$). B: Colonies of deciduous dental pulp stained with methylene blue. C: Cell morphology of deciduous dental pulp cells at passage 5 and passage 10

mRNA expressions of pluripotent markers, Rex-1 and Oct-4 were evaluated using RT-PCR. Rex-1 and Oct-4 mRNA were strongly detected in cells form passage 5 and the intensity of band were decreased in the cells from passage 10 (Fig. 3A), However, no statistical difference was found when the relative gene expression was calculated from band density ($p<0.05$) (Fig. 3B and C).

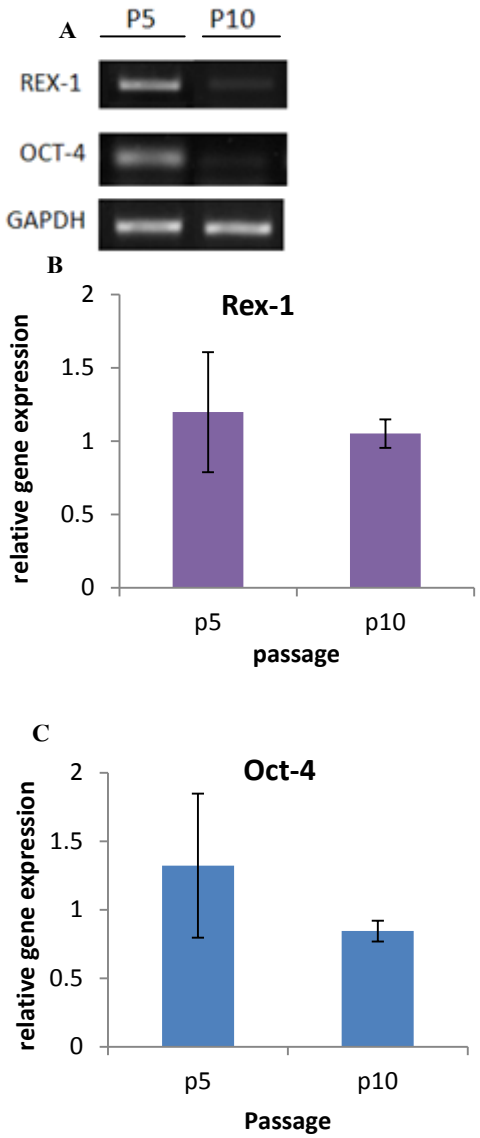

Fig. 3 Effect of increased passage on pluripotent markers mRNA expression of deciduous dental pulp cells at passage 5 and passage 10

A: mRNA expression of Rex-1 and Oct-4 were decreased in passage 10 when compared to passage 5. GAPDH was used as an internal control. B: Graph illustrated the relative expression levels of Rex-1 against GAPDH ($P<0.05$). C: Graph illustrated the relative expression levels of Oct-4 against GAPDH ($P<0.05$)

Discussion and Conclusions

SHEDs is an alternative source of postnatal stem cell which is highly accessible and exhibited several stem cells characteristics which are promising for stem cell therapy (Miura et al., 2003). In spite of the convenient in harvesting, the limited quantity of isolated stem cells from SHEDs is a major obstacle. Although the process of cell expansion might give rise to sufficient cell numbers, but the effects of long-term cultivation, cultured medium (Pradel, Mai, Gedrange, and Lauer, 2008) and the atmosphere (Sanden, Dhobb, Berger, and Didier, 2010) might influence stemness qualities of SHEDs. The ultimate goal of stem cell application is the hope of expanding a large number of cells while maintaining their stemness for prolonged passages. In our study, we demonstrated that increased passage numbers showed obvious effects on human deciduous dental pulp cells properties including self renewal and colony forming ability. The mRNA expression of well known stem cell markers such as Rex-1 and Oct-4 also decreased in the cells with higher passage number and these might affect the pluripotency of SHEDs. Although cell morphology of SHEDs was not altered in long-term culture and we found no statistical difference in every parameter measured, our results correspond to previous report that SHEDs proliferation was slower after cultured in

long term (Suchanek et al., 2010). Other studies also suggested that cell expansion process affected proliferative and differentiation capacity of mesenchymal stem cells (Stenderup, Justesen, Clausen, and Kassem, 2003; Baxter et al., 2004) and may increased the probability of genetic changes (Rubio D et al., 2005). Taken together, we suggested using SHEDs with caution. SHEDs in low passage number (less than P10) might be suitable for experiment and give more precise and reproducible results. Moreover, adding some growth factors responsible for maintaining stemness, such as bFGF, might improve the quality of SHEDs in long-term culture (Shimabukuro et al., 2009; Wu et al., 2012). However, the effect of bFGF on self-renewal and differentiation potential in SHEDs requires further study.

In conclusion, SHEDs can be expanded in vitro during long term culture. However, high passage number has some influence on self renewal and may attenuate the pluripotency of SHEDs. This effect should be of concern in SHEDs amplification for experiment and further therapeutic application.

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References

Baxter, MA., Wynn, RF., Jowitt, SN., Wraith, JE., Fairbairn, LJ., and Bellantuono, I. 2004. Study of telomere length reveals rapid aging of human marrow stromal cells following in vitro expansion. Stem cells. 22(5): 675-82.

Cordeiro, MM., Dong, Z., Kaneko, T., Zhang Z., Miyazawa, M., Shi, S., et al. 2008. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. J Endod. 34(8): 962-69.

Derubeis, AR., and Cancedda R. 2004. Bone marrow stromal cells (BMSCs) in bone engineering: limitations and recent advances. Ann Biomed Eng. 32:160-5.

Govindasamy, V., Abdullah AN., Ronald VS., Musa, S., Ab Aziz, ZA., Zain, RB., et al. 2010. Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth. J Endod. 36(9): 1504-15.

Govitvattana, N., Osathanon, T., Taebunpakul, S., and Pavasant, P. 2012. IL-6 regulated stress-induced Rex-1 expression in stem cells from human exfoliated deciduous teeth. Oral Dis. doi: 10.1111/odi.12052

Ishkitiev, N., Yaegaki, K., Calenic, B., Nakahara, T., Ishikawa, H., Mitiev, V., et al. 2010. Deciduous and permanent dental pulp mesenchymal cells acquire hepatic morphologic and functional features in vitro. J Endod. 36(3): 469-74.

Lizier. NF., Kerkis, A., Gomes, CM., Hebling, J., Oliveira, CF., Caplan, AI., et al. 2012. Scaling-up of dental pulp stem cells isolated from multiple niches. PLoS ONE 7(6): e39885.doi:10.1371/journal.pone.0039885.

Miura, M., Gronthos, S., Zhao, M., Lu, B., Fisher, LW., Robey, PG., et al. 2003. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A. 100(10): 5807-12.

Nakamura, S., Yamada, Y., Katagiri., Sugito, M., Ito, K., and Ueda, M. 2009. Stem cell proliferation pathways comparison between human exfoliated deciduous teeth and dental pulp stem cells by gene expression profile from promising dental pulp. J Endod. 35(11): 1536-42.

Nishino, Y., Ebisawa, K., Yamada, Y., Okabe, K., Kamei, Y., and Ueda, M. 2011. Human deciduous teeth dental pulp cells with basic fibroblast growth factor enhance wound healing of skin defect. J Craniofac Surg. 22(2): 438-42.

Nourbakhsh, N., Soleimani, M., Taghipour, Z., Karbalaie, K., Mousavi, SB., Talebi, A., et al. 2011. Induced in vitro differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells. Int J Dev Biol. 55: 189-95.

Pradel, W., Mai, R., Gedrange, T., and Lauer, G. 2008. Cell passage and composition medium effects proliferation and differentiation of human osteoblast-like cells from facial bone. J Physiol pharmacol. 59(suppl 5): 47-58.

Requicha, JF., Viegas, CA., Albuquerque, CM., Azevedo, JM., Reis, RL., and Gomes, ME. 2012. Effect of anatomical origin and cell passage number on the stemness and osteogenic differentiation potential of canine adipose-derived stem cells. Stem Cell Rev. 8(4): 1211-22.

Rubio, D., Garcia-Castro, J., Martin, MC., de la Fuente, R., Cigudosa, JC., Loyd, AC., et al. 2005. Spontaneous human adult stem cell transformation. Retrieved January 18, 2013, from http://cancerres.aacrjournals.org/content/65/11/4969.1.full.pdf.

Sakai, VT., Zhang, Z., Dong, Z., Neiva, KG, Machado, MA., Shi, S., et al. 2010. SHED differentiate into functional odontoblasts and endothelium. J Dent Res. 89(8): 791-96.

Sanden, BVD., Dhobb, M., Berger, F., and Didier, W. 2010. Optimizing stem cell cuture. J Cell Biochem. 111(4): 801-7.

Seo, BM., Sonoyama, W., Yamaza, T., Coppe, C., Kikuiri, T., Akiyama, K., et al. 2008. SHED repair critical-size calvarial defects in mice. Oral Dis. 14(5): 428-34.

Shimabukuro, Y., Ueda, M., Ozasa, M., Anzai, J., Takedachi, M., Ito. M., et al. 2009. Fibroblast growth factor-2 regulates the cell function of human dental pulp cells. J Endod. 35(11): 1529-35.

Stenderup, k., Justesen, J., Clausen, C., and Kassem, M. 2003. Aging is associated with decreased maximal life span and accelerated senescene of bone marrow stromal cells. Bone. 33(6): 919-26.

Suchanek, J., Visek, B., Soukup, T., El-Din Mohamed, SK., Ivancakova, R., Mokry, J., et al. 2010. Stem cells of human exfoliated deciduous teeth - isolation, long term cultivation and phenotypical analysis. Acta Medica. 53(2): 93-9.

Wang, X., Sha, XJ., Li, GH., Yang, Fs., Ji, K., Wen LY., et al. 2012. Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells. Arch Oral Biol. 57(9): 1231-40.

Osathanon, T., Nowwarote, N., and Pavasant, P. 2011. Basic fibroblast growth factor inhibits mineralization but induces neuronal differentiation by human dental pulp stem cells through a FGFR and PLCγ signaling pathway. J Cell Biochem. 112(7): 1807-16.

Wu, J., Huang, GT., He, W., Wang, P., Tong, Z., Jia, Q., et al. 2012. Basic fibroblast growth factor enhances stemness of human stem cells from the apical papilla. J Endod. 38(5): 614-22.

Yamaza, T., Kentaro, A., Chen, C., Liu, Y., Shi, Y., Gronthos, S., et al. 2010. Immunomodulatory properties of stem cells from human exfoliated deciduous teeth. Stem Cell Res Ther. 1(1): 5.