# Evaluation of pluripotency gene in Thai human dental pulp stem cells

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**Abstract**

The introduction of stem cells in regenerative medicine have given us valuable opportunities to repair the disfunctional tissues and organs of human. The more variety of cell types stem cells can give rise to the more diseases and disability we can overcome. 'Pluripotency' refers to the ability of stem cells to differentiate into all three germ layers. The aim of this study was to evaluate the expression of pluripotency markers in Thai human dental pulp stem cells. Stem cells was isolated and cultured from dental pulp tissue of 3 permanent teeth (DPSC 1-3) and 3 human exfoliated deciduous teeth (SHED1-3). The total RNA was extracted from the dental pulp stem cells in passage 3-5. The cDNA of DPSCs and SHEDs was measured the gene expression level of OCT-4, NANOG, SOX-2, and HNF-3b by RT-qPCR. The statistical difference was determined by Mann-Whitney U test ($P\text{-value}<0.05$). The result showed variation in level of pluripotency gene expression among both DPSC 1-3 and SHED 1-3. Comparatively, the expression of OCT-4, NANOG, SOX-2 gene appeared to demonstrate higher trend in SHEDs. Whereas, higher trend of HNF 3b gene expression was detected in DPSCs. The genetic investigation of stem cell can reveal many useful data for the future of stem cell research and regenerative medicine. This study was the first to provide comparative pluripotency markers information between SHED and DPSC of selected Thai patients.

**Keywords**: Pluripotency markers, Dental pulp stem cells, Thai, Primary teeth, Permanent teeth

## Introduction

Stem cells are a class of undifferentiated cells capable of self-renewal and differentiation. The pluripotency of stem cells is the potential of stem cells to differentiate into any of the three germ layers: ectoderm, mesoderm or endoderm. Pluripotent stem cells can develop into any fetal or adult cell types. With such ability, pluripotent stem cells are considered an important key to the future of regenerative medicine. Regenerative medicine may lessen the invasiveness of dental procedure such as pulp vascularization lowering the likeliness of tooth extraction, or maximize the treatment success such as localized bone regeneration extending the longevity of prosthetic implants. These stem cells can be found in dental pulp tissue inside both permanent (Gronthos, 2000) and exfoliated deciduous teeth (Miura, 2003). Stem cells found in dental pulp tissue have demonstrated promising potential beyond the quality of other adult stem cells. Several studies have shown that dental pulp stem cells can be cultivated into many types of cells from the same germ layer such as osteocytes, odontocytes (Kitagawa, 2007), chondrocytes (Iohara, 2006; Yu, 2010), adipocytes (Lee, 2014), and myocytes (Nakatsuka, 2010), and also have shown potentials to differentiation into cells from different germ layers such as ectoderm (Srisawasdi, 2007; Arthur, 2008; Govindasamy, 2010) and endoderm (Govindasamy, 2011; Ishkitiev, 2012). This indicates that pluripotency are no longer restricted only within

embryonic stem cells, but also can be found in adult stem cells such as DPSC and SHED. Not all pluripotent stem cells are exactly the same. There may be differences in morphology, ability to contribute to chimeras, and gene expression profiles (Rossant, 2008). Describing the difference in gene expression profiles will be helpful in the process of investigating the ability of stem cells. Several genetic studies have identified many pluripotency marker genes (Calloni, 2013; Miang-Lon, 2011). The well-recognized marker genes are OCT-4, NANOG, and SOX-2. They are the core of a transcription factor network regulating the cell pluripotency in early embryos (Pesce, 1999; Niwa, 2007). These three transcriptional factors co-occupy putative enhancer elements of other pluripotency promoting genes, and repress differentiation (Ralston, 2010). The study of Huang et al. in 2014 suggested that the signaling of OCT4-NANOG is a regulatory switch to maintain properties such as cell proliferation, osteogenic/chondrogenic/adipogenic differentiation of DPSCs (Huang, 2014). HNF-3b (FOXA2) is a member of the HNF-3/fork head family of transcription factors. One of the same family members is also an important pluripotency marker, FOXD3. HNF-3b was previously found to be essential in visceral endoderm for normal primitive streak morphogenesis (Dufort, 1998). Later in the year 2002, a study have found that not only HNF-3b definitely exists at early stage of embryos but HNF-3b also plays an integral role in the formation of axial mesendoderm (Hallonet, 2002). Moreover, the profiling of transcriptional and epigenetic changes during directed endothelial differentiation of human embryonic stem cells astonishingly identified HNF-3b as a marker of early mesoderm commitment (Howard, 2013). Stem cells from dental pulp has shown great potentials to give rise to various cell types of mesoderm and ectoderm, but few have demonstrated the ability to differentiate into endodermal cells. The molecular investigation on pluripotency marker expression in Thai human dental pulp stem cells has not been clearly established. A comparative study of dental pulp stem cells in Thai population has been done in 2010, but no investigation was done molecularly (Guan, 2011). The genetic investigation of pluripotency markers in DPSC and SHED will clarify the pluripotency level in DPSC and SHED. Unpublished studies have been conducted at the faculty of Dentistry, Mahidol University, prior to this proposal research. Those studies have found characteristics of stem cell such as proliferation rate, surface markers and differentiation in both the dental pulp cells isolated from permanent and primary teeth. The results had confirmed to characteristics of stem cells. The present study was focusing on evaluating the pluripotency gene markers (OCT-4, NANOG, SOX-2, and HNF-3b) in those dental pulp stem cells isolated from permanent and primary teeth using RT-qPCR technique.

## Materials and Methods

*Tooth samples collection*

Six of extracted caries-free permanent (n=3, patient's age = 18-25 years) and exfoliated deciduous teeth (n=3, patient's age = 5-12 years) were collected from selected Thai subjects at faculty of Dentistry, Mahidol University. Teeth with previous restorations, history of trauma, and/or signs of pulpal pathology, were not a part of this study. The experiment was approved by the Ethics Committee on Human rights Related to Human Experimentation of Faculty of dentistry of Mahidol University (COE. No. MU-DT/PY-IRB 2014/036.0310).

*Isolation and culturing of dental pulp stem cells*

Within 24 hours of tooth extraction, fragments of dental pulp tissues were extracted and submerged in culture medium using 25 $mm^2$ culture flasks (Jet Biofil). The culture media consisted of Dulbecco's Modified Eagle Medium (DMEM)/ high-glucose (Hyclone), 10% Fetal bovine serum (Biochrome) and 1% Penicillin streptomycin (Gibco). The dental pulp stem cells were obtained using the outgrowth methods and incubating condition of 37°C, 5%$CO^2$ and 95% humidity. The culture medium was changed every 2 days. The obtained stem cells were continually cultured in a 75 $mm^2$ culture flask (Jet Biofil). When primary culture reached 80% confluent, cells were collected by trysinization using 0.25% Trypsin solution (Gibco) and processed for subsequent passages. Three lines of DPSCs and three lines of SHEDs in Passage 3-5 were collected to investigate the genetic expression of pluripotency gene markers using RT-qPCR technique.

*The evaluation of plutipotency gene expression using RT-qPCR*

The total RNA was extracted from the three lines of DPSCs and three lines of SHEDs in passage 3-5 using RNeasy®Mini Kit (Qiagen). The RNA was reversed-transcribed into cDNA using iScript™ Select cDNA Synthesis Kit (BioRad) according to the manufacturer's instruction. For the real-time PCRs, cDNA amplification was performed in the C1000™ thermal cycler of CFX96 real time system (Bio-Rad) by using KAPA SYBR® Fast qPCR Kit (Kapa Biosystem). Using 25 ng of DNA per capillary, 40 PCR cycles were monitored with SYBR green I at the annealing temperatures (50-61°C). After the PCR cycles, melting curve analysis was performed by heating to 95°C for 10 seconds, followed by cooling to 65°C for 5 seconds and gradual heating to 95°C at 0.2°C/second. All PCRs were done in triplicates. All measurements were normalized by the expression level of the housekeeping genes, GAPDH. Primers of OCT-4, NANOG, SOX-2, HNF-3b, and GAPDH were referenced from other previous human stem cell researches (Atari, 2011; Kim, 2013). The relative quantification of gene expression was assessed by $\Delta\Delta$Cq method.

**Table 1** Primers used in this study

| Forward primer (F)<br>Reverse primer (R) | | Base pairs | Annealing temperature |
|---|---|---|---|
| OCT-4 | F: GACAGGGGGAGGGGAGGAGCTAGG<br>R: CTTCCCTCCAACCAGTTGCCCCAAAC | 144 | 61.0°C |
| NANOG | F: AAAGAATCTTCACCTATGCC<br>R: GAAGGAAGAGGAGAGACAGT | 110 | 50.0°C |
| SOX-2 | F: GGGAAATGGGAGGGGTGCAAAAGAGG<br>R: TTGCGTGAGTGTGGATGGGATTGGTG | 151 | 50.0°C |
| HNF-3b | F: CTACGCCAACATGAACTCCA<br>R: GAGGTCCATGATCCACTGGT | 199 | 55.1°C |
| GAPDH | F: GTCAGTGGTGGACCTGACCT<br>R: AGGGGAGATTCAGTGTGGTG | 395 | 50-61°C |

*Data and statistical analysis*

Statistical analyses were performed by using PASW Statistics 18 (SPSS, New York, U.S.A.). The data of relative normalized expression was presented by descriptive analysis. Difference among the two groups was analyzed using Mann-Whitney U test. Significant differences was set at 95% confidence.

## Result

*Isolation and culturing of dental pulp stem cells*

Fragments of dental pulps were extracted and immersed in culture medium for 7-14 days before the spindle shape cells were seen migrating from the fragments. This occurred both in dental pulps extracted from permanent teeth and from exfoliated deciduous teeth (Figure 1-2). The morphology of the cells have remained the same as fibroblastic-like appearance throughout the passage 0-5 (P0-P5). The stem cells in P3-P5 were collected as pellets and continued with the RT-qPCR.

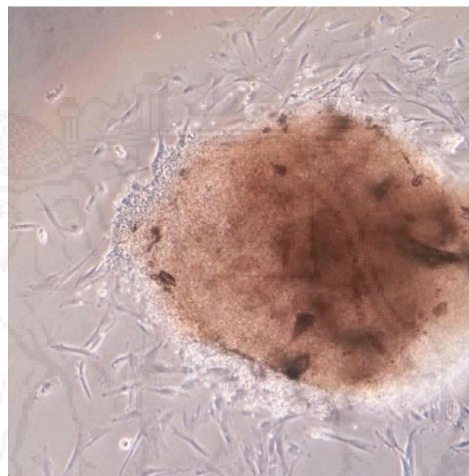

**Figure 1** Primary cells outgrowth from dental pulp fragment of a exfoliated deciduous tooth on day 7-14

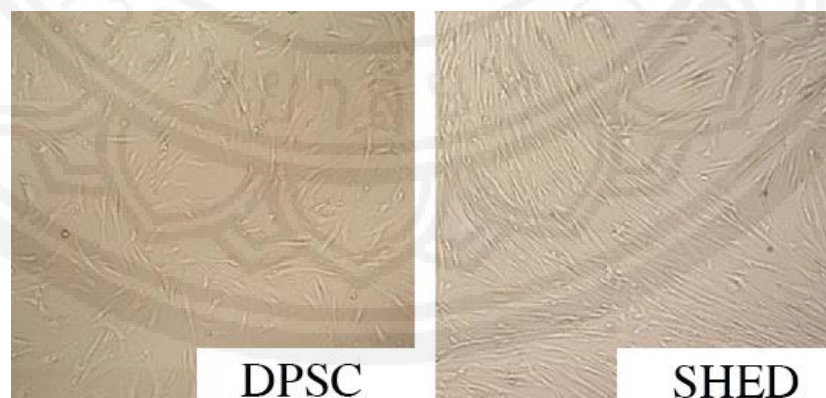


**Figure 2** Morphology of the dental pulp cells from permanent and primary teeth

*Expression of pluripotency markers in denta lpulp stem cells*

Total RNA was extracted from the stem cell pellets which was later reverse-transcribed into cDNA. The cDNA amplification and quantification was performed using qPCR. Each pluripotency gene marker of dental pulp stem cells was evaluated relatively to GAPDH, and all PCRs were performed in triplicates.

*DPSC:* The gene expression of OCT-4, NANOG, SOX-2, HNF-3b have been detected in all DPSCs cell lines (Figure3). Comparison within the group of DPSCs, DPSC 3 had the highest expression level of NANOG and SOX-2. The second highest was DPSC 2 and the lowest was DPSC 1, respectively. For the expression of OCT-4, the highest expression was found in DPSC 2, DPSC 1, and DPSC 3 accordingly. For the expression of HNF-3b, DPSC 1 showed a very high level of expression, 3 folds of the expression level of DPSC 2 and DPSC 3.

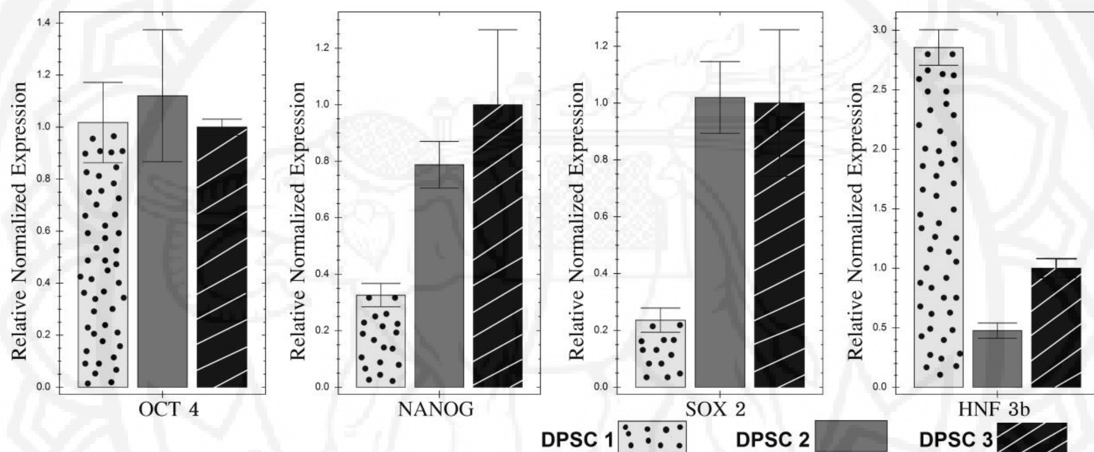


**Figure 3** The relative normalized expression of pluripotency markers in dental pulp stem cells from permanent teeth (DPSC 1-3).

*SHED:* The gene expression of OCT-4, NANOG, SOX-2, HNF-3b have been detected in all SHED cell lines (Figure 4). Comparison within the SHED group, SHED 3 had the highest expression level of NANOG and SOX-2. The second highest was SHED 2 and the lowest was SHED 1. For the expression of OCT-4, the highest expression was found in SHED 2, SHED 3, and SHED 1 respectively. For the expression of HNF-3b, SHED 1-3 demonstrated similar level of gene expression with the highest from SHED 1 to SHED 2, and SHED 3 respectively.

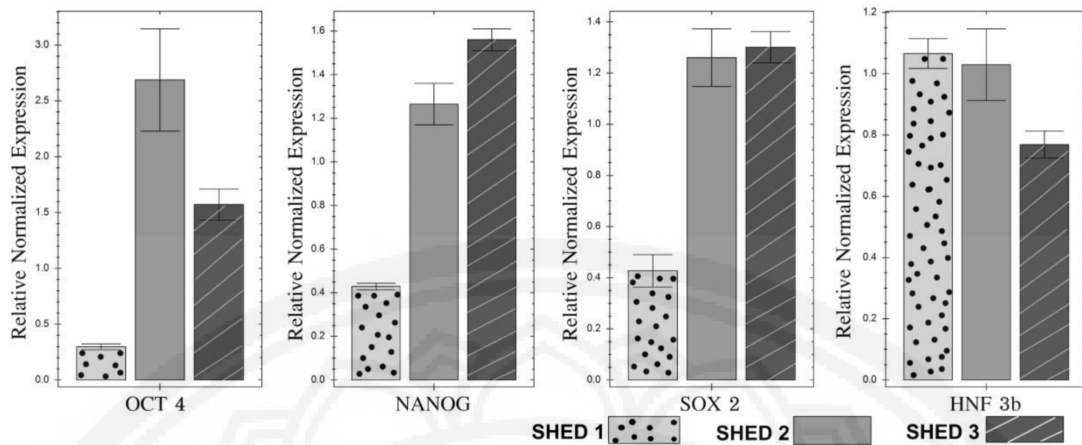


**Figure 4** The relative normalized expression of pluripotency markers in dental pulp stem cells from primary teeth (SHED 1-3).

*Comparison of pluripotency gene expression between DPSCs and SHED*

The study of gene expression in-between groups of DPSCs and SHEDs had indicated the higher trend of OCT-4, NANOG, and SOX-2 expression in SHEDs than in DPSCs. On the other hand, DPSCs had shown the higher expression trend of HNF-3b gene when compared to SHEDs. The overall data of comparative gene expression was exhibited in Figure 5. However, no statistically difference was found between the two groups in the expression of OCT-4, NANOG, SOX-2 and HNF-3b.

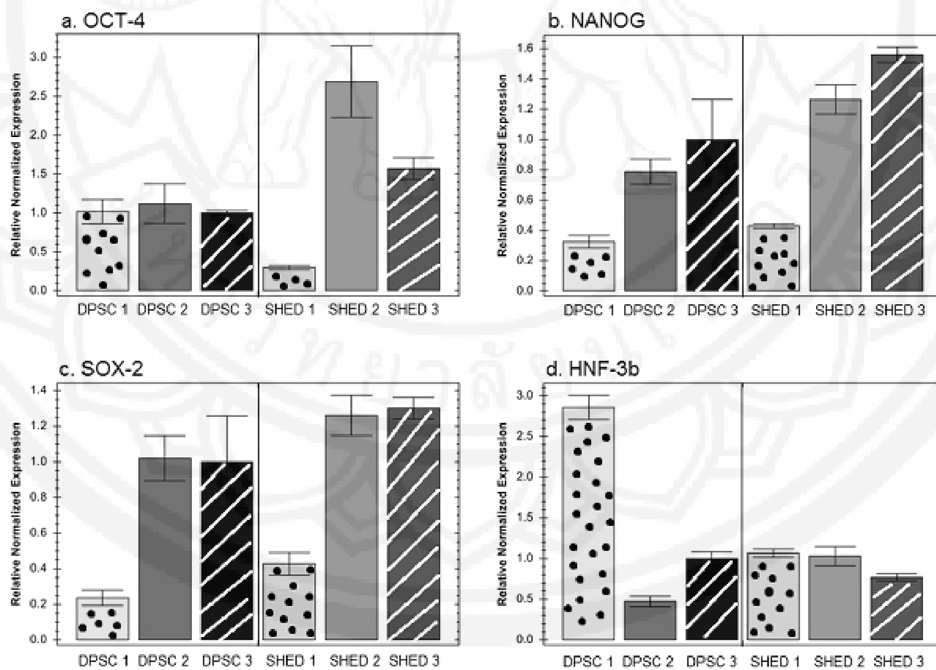


**Figure 5** (a-d): The overall data of comparative gene expression in DPSC 1-3 and SHED 1-3

## Discussion

Our research colleague at faculty of Dentistry, Mahidol University have investigated the mesenchymal stem cell property in the isolated dental pulp cells. We have found that the primary cells from the outgrowth methods have demonstrated all the required characteristics of mesenchymal stem cell (Lanza, 2014, pp.255-256), including plastic-adhesion when maintained in standard culture conditions, formation of CFU-Fs, and expression of CD105, CD73, CD44 surface molecules, lack expression of CD34 molecules, and the ability to differentiate *in vitro* into committed mesoderms such as osteoblasts.

The significant correlation between our previous studies of proliferation rate by Dr. Aiyared (unpublished data) (Figure 6-7) and the level of NANOG and SOX-2 expression was found in this study. This can be explained by the main function of NANOG in mesenchymal dental pulp stem cells (Huang, 2014). In 2014, Huang and his colleagues had investigated the physiological functions of OCT-4 and NANOG expression in DPSCs. They have found that down-regulation of OCT-4 and NANOG co-expression significantly reduced the cell proliferation of DPSC. Contrastingly, the co-overexpression of OCT-4 and NANOG have enhanced the proliferation rate of DPSC.

SOX-2 is also one of the essential pluripotency markers that together with OCT-4 and NANOG plays an important role in maintenance the pluripotency, controlling the expression of a numbers of genes involved in cell proliferation. The study of SOX-2 effects on proliferation of human dental pulp stem cells was done by Liu et al. in October of 2015 (Liu, 2015). In the study, they have found that the overexpression of SOX-2 resulted in the obvious increase in cell proliferation, migration, and adhesion of DPSCs. The same data correlates to our results from this study. The DPSC 3 and SHED 3 displayed high proliferation rate as they have expressed high NANOG and SOX-2 gene expression.

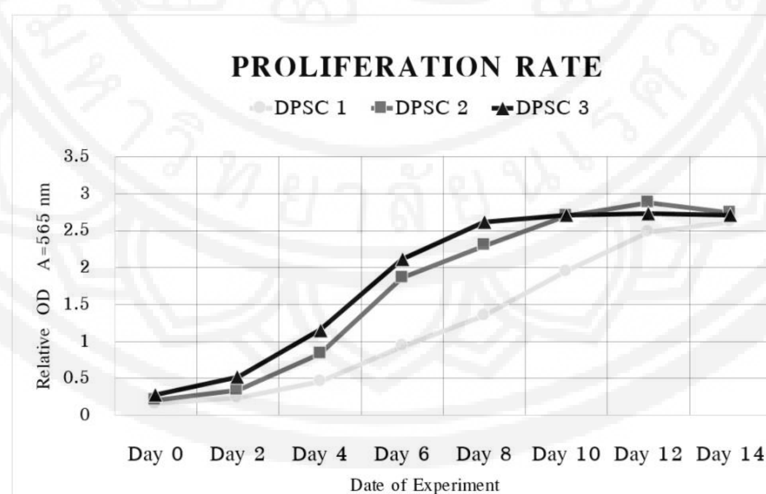


**Figure 6** The proliferation rate of dental pulp stem cells from permanent teeth (DPSC 1-3).

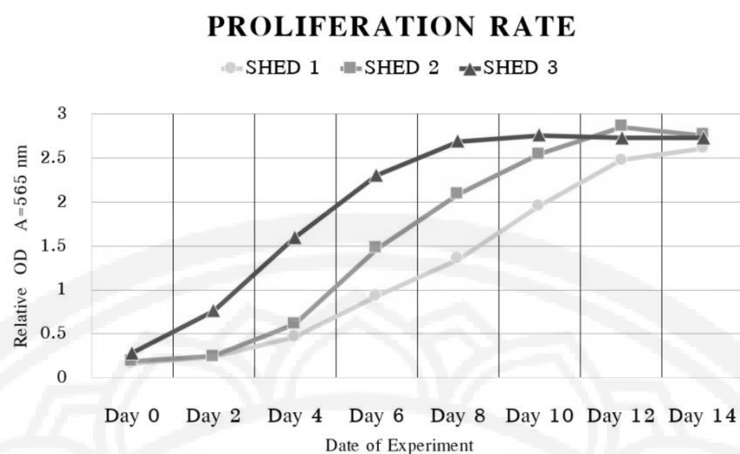


**Figure 7** The proliferation rate of dental pulp stem cells from primary teeth (SHED 1-3).

A study on isolated dental pulp stem cells has reported that even though the age of patients at the time of stem cell harvesting was not an important factor regarding the maximal cell division potential, it showed an impact on the doubling time (Kellner, 2014). Another study also indicated higher activities of dental pulp stem cells during the physiologic root resorption (Zhu, 2012). The teeth used in this study were collected from healthy Thai donors of different ages and at the different root stages. Consequently, the different levels of gene expression within each sample were observed in this study. A further investigation should be conducted with limitation criteria of age and the stage of root resorption in order to find the true relation of the age or the degree of root resorption to the level of pluripotency gene expression.

The relevance of HNF-3b to the endodermal commitment of human embryos may suggest the likelihood of DPSC 1 toward endodermal differentiation, as a research conducted in Chulalongkorn have been able to differentiate DPSCs into insulin-producing cells in 2014 (Sawangmake, 2014).

The result from this study in Thai patients resembles the result studied in Malaysian population. The study from University of Malaya reported OCT-4, NANOG, and SOX-2 to be higher in SHED as compared with DPSCs (Govindasamy, 2010). However, the study was done using pooled samples which was different from our method of analysis using pulp cells from different individual pulps without pooling. As a result, we also found different expression levels of pluriotency markers among each individual sample. Further studies should continue to focus on individual pulp cells and more samples should be collected in order to represent the major trend of Thai population.

## Conclusions

The genetic investigation of stem cell can reveal many useful data for the future of stem cell research and regenerative medicine. The comparable gene expression of pluripotency markers provides basic information on proliferation and differentiation of dental pulp cells in Thai population. The result from this study indicates that pulp cells from both permanent and deciduous teeth may also be beneficial for the future of regenerative dentistry.

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