The Methylation Level of Alu in Human Dental Pulp Stem Cell-derived Osteoblasts: Preliminary Report

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ABSTRACT

Introduction Dental pulp stem cells (DPSCs) have the potential to differentiate into osteoblasts and to be used in tissue engineering for bone regeneration. However, mechanism of differentiation is still unknown. Methylation is one of the key mechanisms that associated with cells differentiation. One of the major targets of methylation resides in Alu elements, which is an intersperse repetitive sequence (IRS). Many studies reported that the decreasing of Alu methylation level was associated with both pathological change such as cancer and physiological change such as ageing. Nevertheless, the methylation level of Alu in DPSCs and the relation with their differentiation have not been reported yet. The result of this study will gain a better understanding about mechanism of differentiation and may lead to new development of stem cell-based therapies, which can be a safe treatment for patients.

Objective To compare the levels of Alu between DPSC-derived osteoblasts (DPSC-DOSs) and DPSCs.

Materials and Methods DPSCs from 5 impacted third molars were extracted and induced into osteoblasts. Alu levels of DPSC-DOSs and DPSCs were measured by quantitative combined bisulfite restriction analysis-Alu (qCOBRA-Alu) technique.

Results The Alu methylation level of DPSC-DOs from four samples were higher than DPSCs with no statistically significant difference. While one sample had slightly lower Alu methylation level than DPSCs.

Conclusion DPSC-DOs has higher tendency of Alu methylation level than DPSCs. However, the mechanisms of differentiation are complicated and compose of varying patterns of DNA, so further studies are needed to confirm the result.

Keywords: Epigenetic, DNA methylation, Alu, Dental pulp stem cell, Osteoblast

1. Introduction

In dentistry, inadequate bone support is one of the major problems. Pathological or physiological bone loss result in tooth mobility and eventually tooth loss, poor retention of dental prosthesis, failure of dental implants, and facial disfiguration. Bone graft is one of the most common procedures for gaining more bone support. However, bone graft materials such as autologous bone, allograft and xenograft still have disadvantage. Autologous bone is associated with second site surgery which increase more pain and operating time. Allograft and xenograft materials are lack of osteogenesis ability and bone-forming cells. Stem cells which are now successful in many kinds of regenerative treatment may be an interesting alternative tool for bone generation.

Stem cells have special characteristics including self-renewal ability and capability to differentiate into multiple lineages that make them to be a promising tool for tissue engineering and regenerative medicine (Caplan, 2007; Watt & Hogan, 2000). Adult stem cells can be extracted from many human organs including dental pulp. DPSCs have been reported the ability to regenerate many tissue including bone (d'Aquino et al., 2007; d'Aquino et al., 2009; Takeda et al., 2008; Yamada et al., 2010; Yu et al., 2010). However, since DPSCs are multipotency, the cell type specific differentiation needs to be well controlled.

Generally, proper cell differentiation and function are regulated by gene expressions which are controlled by genetic and epigenetic mechanisms. Epigenetic mechanism regulates gene expression by modified the structure of chromatin (Kim, Samaranayake, & Pradhan, 2009). DNA methylation, one of the major epigenetic mechanism, is an addition of a methyl group to cytosine-phosphate-guanine (CpG) dinucleotides (Smith & Meissner, 2013). This mechanism was shown to have important roles in cellular process (Hellman & Chess, 2007; Hoffman & Hu, 2006; Smith & Meissner, 2013). A considerable portion of methylation sites are found in Interspersed repetitive sequences (IRSs). The most of IRSs are Alu element which comprise 13.7% of the human genome and corresponds to over 23% of all CpGs (Luo, Lu, & Xie, 2014). Several studies reported that methylation of Alu element was associated with cells activity during development, aging and tumor genesis.

The methylation levels of Alu elements have been associated with tumor malignancy and aggressiveness of many cancers (Bae et al., 2012; Goel, Boland, & Hur, 2012; Park et al., 2014; Sirivanichsuntorn et al., 2013). The lower level of Alu methylation was found when activate the naive B cells to differentiate into plasma cells and memory B cells (Lai et al., 2013). Decreased Alu methylation levels were reported in post-menopausal women with osteoporosis comparing to individuals with the same age and normal bone mass index (Jintaridth, Tungtrongchitr, Preutthipan, & Mutirangura, 2013) and also found in patients with Alzheimer's disease (Bollati et al., 2011). Yet, the methylation levels of Alu in dental pulp stem cells and the relation with their differentiation has not been reported. This study is investigating the DNA methylation levels of Alu in human DPSC-DOs compared with that of undifferentiated human DPSCs by using the combined bisulfite restriction analysis (COBRA) method.

2. Objectives of the study

To investigate the Alu Methylation levels of DPSC-DOs compared with undifferentiated DPSCs.

3. Materials and methods

Dental pulp cell isolation and culture

The impacted non-pathological third molars were removed from healthy adult subjects (N=5). The protocol was approved by the Human Research Ethics Committee, Faculty of Dentistry, Chulalongkorn University. Written consents were obtained from all participants. The dental pulp were minced and placed on 35 mm tissue culture dishes. The isolated cells were cultured in Dulbecco's Modifed Eagle Medium (Gibco BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B at 37 °C in a humidified 5% carbon dioxide atmosphere. The medium was changed every 48 hours. After reaching confluence, the cells were undergone standard passaging procedure until reached the 4th passage.

Osteogenic differentiation

The 4th passage of DPSCs were cultured in osteogenic medium, which consisted of growth medium supplemented with 50 μg/mL ascorbic acid (Sigma-Aldrich Chemical, St. Louis, MO, USA), 250 nM dexamethasone (Sigma-Aldrich Chemical), and 5 mM β-glycerophosphate (Sigma-Aldrich Chemical), for 2 weeks. The medium was changed every 48 hours.

Matrix Mineralization

For confirmation of osteogenic differentiation, the induced DPSCs were assessed for matrix mineralization by Alizarin red and Von Kossa staining. For both staining, cells were fixed with ice cold methanol for 10 min, washed with deionized water. For Alizarin red staining, cells were incubated with 1% Alizarin Red S solution (Sigma) for 3 min at room temperature. Then washed extensively with deionized water to remove unbound stain. For Von Kossa staining, cell were fixed by 4% Formalin in PBS, then added 3% $AgNO_3$ solution and incubated under 100 W UV light for 60 min in order to develop color.

DNA preparation for COBRA

2-week-cultured 4th passage DPSCs and DPSC-DOs were harvested by trypsinization and extracted DNA by using Phenol-chloroform extraction. Bisulfite conversion of the DNA was performed using the EZ DNA methylation Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocol.

Detection of COBRA-Alu by using the COBRA technique

The combined bisulfite restriction analysis-Alu (COBRA-Alu) technique was used to detect 2 CpG dinucleotide sites. Bisulfited-treated DNA was amplified by using primers with the following sequences: Alu forward 5'-GGRGRGGTGGTTTARGTTTGTAA-3 and Alu reverse 5'-CTAACTTTTTATATTTTTAATAAAAA CRAAATT TCACCA-3'. The PCR amplification was then performed the cycling: initial denaturation at 95 °C for

15 min followed by 45 cycles of denaturation at 95 °C for another 45s, annealing at 63 °C for 45s, extension at 72 °C for 45s and ending with the final extension at 72 °C for 7 min. After amplifcation, the Alu PCR products (133 bp in length) were digested with 2 U of the Tag1 restriction enzyme (Fermentas International Inc., Burlington, Canada) and incubated overnight at 65°C. The DNA fragments were stained with the SYBR green (Gelstar, Lonza, Rockland, ME, USA) and separated on 8% polyacrylamide gels, resulting in separated 5 bands of DNA. The intensity of the DNA fragments was measured using a Phosphoimager with Image Quant software (Molecular Dynamics, GE Healthcare, Slough, UK). We used DNA templates from HeLa cell lines as control for normalization of the inter-assay variation between each experiment.

Alu methylation analysis

Amplicons were cut by Taq1. Different sizes of fragments resulted from different methylation of 2 CpG dinucleotides, which were: 133, 90, 75, 58, 43 and 32 bp (Figure1). The number of CpG dinucleotides was calculated by dividing the intensity of each band by the number of double-stranded bp of DNA sequence as follows: A=intensity of the 133-bp fragment divided by 133; B=intensity of the 58-bp fragment divided by 58; C=intensity of the 75-bp fragment divided by 75; D=intensity of the 90-bp fragment divided by 90; E=intensity of the 43-bp fragment divided by 43; and F=intensity of the 32-bp fragment divided by 32. Alu methylation level percentage was calculated as follows: (%mC) =100× (E+B)/ (2A+E+B+C+D).

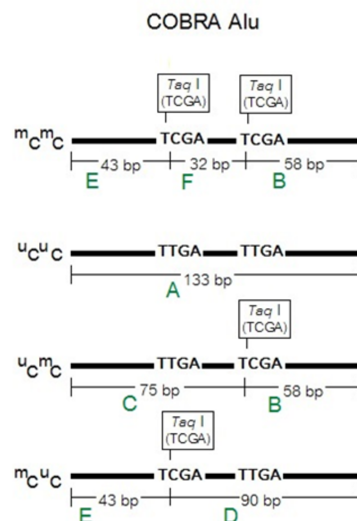

Figure1.The illustration of the COBRA-Alu technique

Statistical analysis

The statistical analysis was performed using SPSS software for Windows version 22.0 (SPSS Inc., Chicago, IL). Wilcoxon Signed-Rank test was performed to test the difference between Alu methylation levels of DPSC-derived osteoblasts and undifferentiated DPSCs. A P value<0.05 was considered statistically significant.

4. Results

Morphological Observation

After 2 weeks, undifferentiated DPSCs showed fibroblastic, spindle shape appearance which had narrow cytoplasm due to high level of confluence (Fig. 2A). DPSC-DOs also had fibroblast-like spindle shape morphology but larger nucleus. The formation of mineral nodules was observed in DPSC-DOs (Fig. 2B).

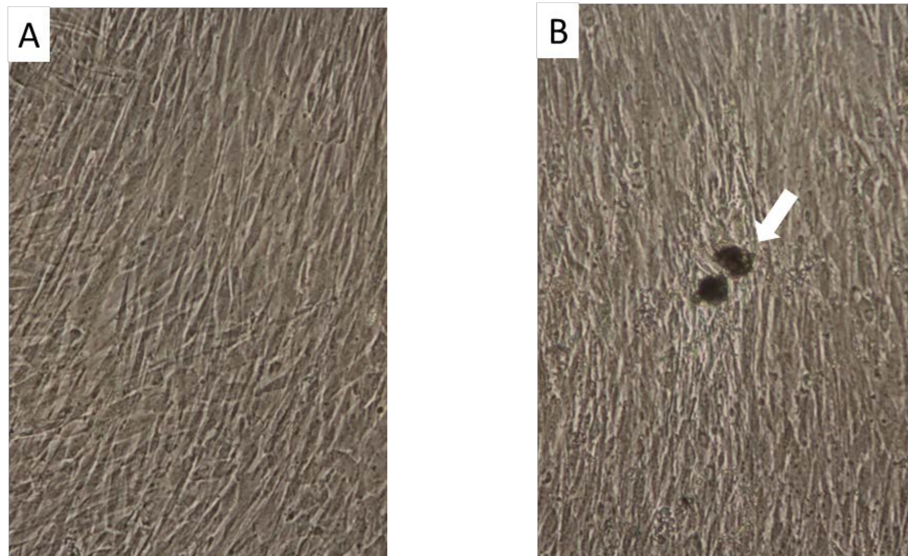

Figure 2. The morphology at 2 weeks of 4th passage DPSCs, observing under light microscope (original magnification, x100). (A) Undifferentiated DPSCs, (B) DPSC-DOs. White arrow showed the mineral nodule that was found scatter on the culture dish.

Matrix Mineralization

At 2 weeks, Alizarin red and Von Kossa staining were positive in DPSC-DOs. The mineral deposition of osteoblasts observed by Alizarin red staining was visualized as deep red color and Von Kossa staining was metallic silver. No positive staining was observed in undifferentiated DPSCs (Fig 3.).

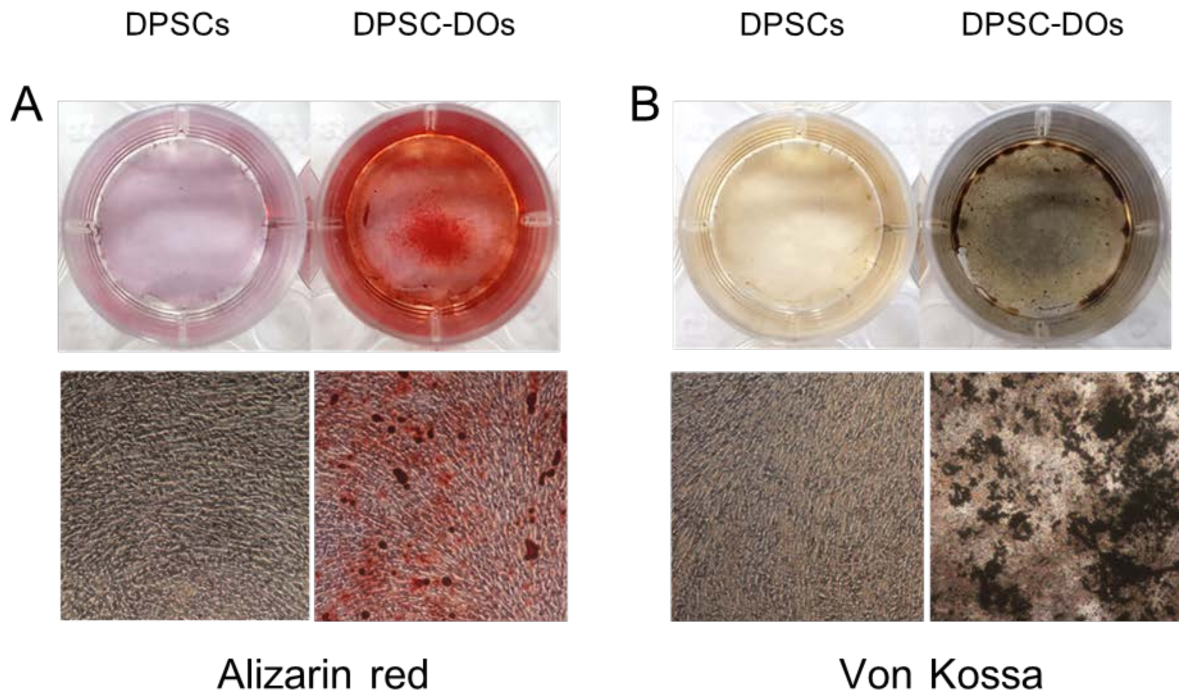

Figure 3. The mineral deposition after 2 weeks observed by (A) Alizarin red and (B) Von Kossa staining. Lower panel showed microscopic images (original magnification, x40).

<u>Methylation Level of Alu</u>

The Alu methylation level of DPSCs in sample 1, 2, 3, 4, and 5 were 35.43%, 37.73%, 36.16%, 36.77% and 46.50% while DPSC-DOs were 36.86%, 45.20%, 41.17%, 42.05% and 45.00% respectively (Fig 4). The Alu methylation level of DPSC-DOs from four samples were higher than DPSCs**,** while one sample had slightly lower than DPSCs. The average Alu methylation level of DPSCs was 38.52± 4.54 and DPSC-DOS was 42.06± 3.40. However, there was no statistically significant difference in Alu methylation level between DPSCs and DPSC-DOs ($p = .138$).

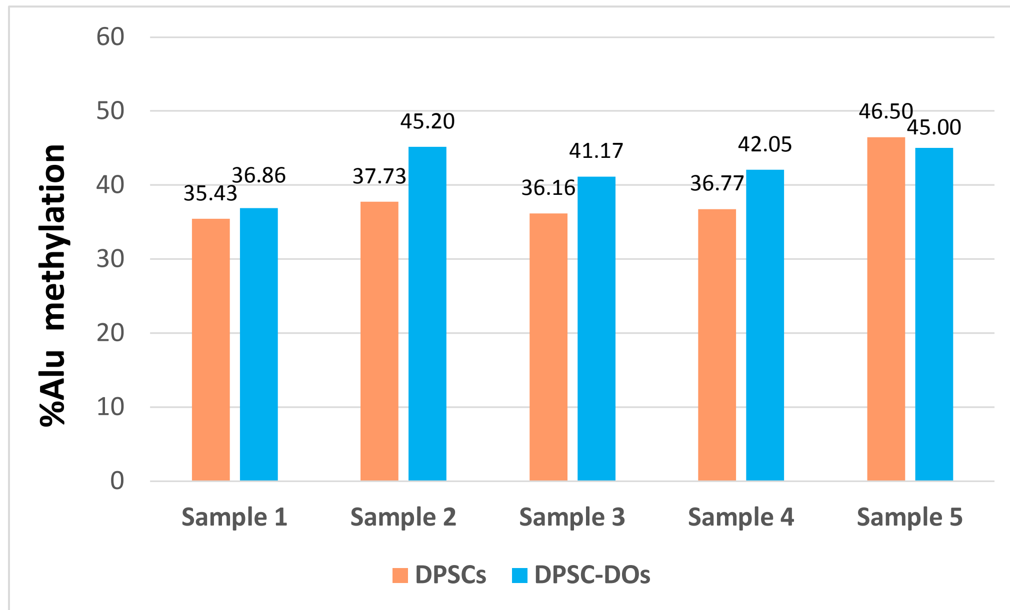

Figure 4. The Alu methylation levels of DPSCs and DPSC-DOs. DPSC-DOs in sample 1, 2, 3 and 4 had higher Alu methylation levels than DPSCs. However, there was no statistically significant difference.

5. Discussion

The average of Alu methylation level had no statistical difference between DPSC-DOs and DPSCs. However, DPSC-DOs had higher tended to have higher Alu methylation level than DPSCs. Because the sample size of this study was small, so larger sample size is needed in further study to clarify the result.

Alu methylation level has been reported in various conditions. Lower of Alu methylation levels were found associated with both pathological change such as cancer and physiological change such as aging, including disease phenotype due to aging such as osteoporosis (Jintaridth & Mutirangura, 2010; Jintaridth et al., 2013; Park et al., 2014; Sirivanichsuntorn et al., 2013). However higher levels of Alu methylation were found in catch-up growth in twenty-year-old offspring (Rerkasem et al., 2015). In this study, Alu methylation levels of DPSC-DOs were slightly higher than DPSCs. The possible explanation was the loss of Alu methylation may lead to genomic instability. On the other hand, cells of individuals with catch-up growth and differentiated cells may require higher levels of genome stability. In contrast, Lai et al. found that Alu methylation levels decreased when activated the naive B cells to differentiate into plasma cells and memory B cells (Lai et al., 2013). This distinction might be due to different cell types.

According to all of studies mentioned above, Alu methylation was associated in many conditions. However, each condition had specific mechanism and the knowledge about it is still unclear. Differentiation is a complex process involving many genes and mechanisms, further investigation should be performed to acquire more information about molecular basis before applying in therapeutic use.

6. Conclusion

The Alu methylation level of DPSC-DOs is slightly higher than DPSCs but no statistically significant difference. The larger sample size and methylation level of other IRSs should be recruited in further study.

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