Original Article

Preliminary Study of LINE-1 Methylation Level in Long-term Cultivation of Human Dental Pulp Stem cells

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Abstract

Human dental pulp stem cells become an alternative source of stem cells that play an important role in tissue engineering and cell-based therapies that will help the regeneration of impaired tissue in elderly, regarding to the easy access and the potential to renew and differentiate into many cell types. Cell expansion *in vitro* is necessary to amplify the small amount of cells that can be isolated before therapeutic use of DPSC. However, long-term cultivation leads to the alteration in morphology, proliferation and differentiation potential of DPSCs. Moreover, the accumulation of mutation during cell expansion can bring the risk of malignant transformation. Epigenetic mechanism including DNA methylation was found taking part in the regulatory processes in development. The global loss of methylation level relates with many events including tumor progression. Hypomethylation of LINE-1 is associated with many types of cancer and occurs during early event in carcinogenesis. In this study, we examined and compared the methylation levels of LINE-1 between the early and late passages of DPSCs using combined bisulfite restriction analysis. DPSCs showed the morphological change and lost methylation level of LINE-1 during expansion. DPSCs in late passage have lower level of LINE-1 methylation than the early passage but with no statistical significant difference. Further study about epigenetic and malignant transformation of DPSCs is still recommended and required for the secure application of these cells.

Keywords: LINE-1, Methylation, Dental pulp stem cell, Passages

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Introduction

Mesenchymal stem cells play an important part in tissue engineering and stem cell-based therapies for repairing and regenerating damaged tissue due to their potential in self-renewal and differentiation into many cell type.[1-3] Human dental pulp stem cell (DPSC), isolated from pulpal tissue inside the tooth chamber, is a practical source of stem cells in because of its easy access and low morbidity.[4] They can be harvested from discarded tooth including wisdom tooth. DPSCs have a high proliferation rate and display typical fibroblast-like morphology with high clonogenic activity similar to the mesenchymal stem cells from human bone-marrow.[5] They can differentiate into several cell types, such as neurons, adipocytes, osteoblasts, chondrocytes, keratocytes, and insulin-producing cells;[6-12] thus, they become a future promising tool in the large field of regenerative medicine.

A crucial problem for the application of DPSC is the small amount of cells that can be isolated; therefore, cell expansion *in vitro* is required. However, long-term culture leads to the changes in morphological, proliferation potential and differentiation potential of DPSCs.[13,14] Moreover, the possible accumulation of mutation that can bring the malignant transformation should be concerned.

Global loss of methylation level is an evident process that occur during tumor progression.[15] The DNA methylation is a major epigenetic regulatory process which methyl groups are covalently added to base cytosine in the DNA strand.[16,17] It is an efficient repressor of transcriptional activity as the methyl groups obviate the binding of transcription factors and found related with many regulation of biological processes including proper development,[18-20] parental genomic imprinting,[21,22] genomic stability, long-term gene silencing,[23,24] and gene expression that regulate cell function and differentiatio.[25] More than one-third of DNA methylation occurs in the repetitive elements.[26,27] Interspersed repetitive sequences are the major contributor to human genome, accounting for 45 percentages of human DNA,[28,29] and can be classified by size and the association of transposable elements. LINE-1 is the most abundant long interspersed elements (LINEs) and comprise approximately17 percentages of human genome. In addition, LINE-1 hypomethylation occur as an early event in carcinogenesis.[15] Decreasing in methylation level of LINE-1 is found relating with many types cancer, for example head and neck cancer, lung cancer, colon cancer, hepatic cancer, prostate cancer and bladder cancer.

In this study, we propose to preliminarily investigate and compare the LINE-1 methylation levels in different passage of DPSCs. The findings from this study will help us to initially understand more about epigenetic event of dental pulp stem cell and will lead to the appropriate further study. All aspects of biological mechanisms involving DPSCs culture should be clearly understood for the efficiently and securely application of DPSCs in the future.

Objectives

The objective of this pilot study is to preliminarily examine and compare the methylation levels of LINE-1 between the early and late passages of human dental pulp stem cells.

Materials and Methods

1. Sample and DNA Extraction

The protocol for the isolation of dental pulp stem cells was approved by the Ethical Committee, Faculty of Dentistry, Chulalongkorn University. Five non-pathological third molars from healthy adult subjects who underwent surgical extraction at Department of

Oral and Maxillofacial surgery, Faculty of Dentistry, were used for the isolation of DPSCs in this pilot study. Dental pulp tissue was explanted and plated in 60 mm. culture dish and maintained in 3 ml. of Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10 % fetal bovine serum (FBS, Gibco), 2 mM L-glutamine 100 (Gibco), 100 U/ml penicillin, 100 ug/ml streptomycin and 5 ug/ml amphotericin B (Gibco) in 100 % humidity, 37°C and 5 % carbon dioxide under sterile condition. Medium was changed every 48 hours. The cells were subcultured to 3 plates at a 1:3 ratio after reaching 80 % confluence.

DPSCs underwent standard passaging procedure until the cell proliferation rate was extremely decreased. DPSCs were observed their morphology under light microscope and collected for DNA extraction in order to analyze the methylation level of LINE-1. Collected cells were subject to lysis using proteinase-K lysis buffer at 50°C for 48 h. The DNA was then isolated by standard phenol-chloroform extraction.

2. Bisufite Modification

After DNA concentrations were determined with a spectrophotometer, bisulfite modification of the DNA samples was performed using an EZ DNA methylation kit (Zymo Research, Orange, CA, USA). Briefly, bisulfite treatment converts unmethylated cytosine to uracil, whereas the methylated cytosine remain unchanged.

3. The quantitative combined bisulfite restriction analysis-LINE1 (qCOBRA-LINE1)

Briefly, one microliters of bisulfite DNA was annealed with primers for COBRA LINE-1, 5-GTTAAAGAAAGGGGTGAYGGT -3 and 5-AATACRCCRTTTCTTAAACRA TCTA-3 at 55°C and amplified for 30 cycles.

The LINE-1 amplicons after amplification (92 bp length) were digested in 10 µL volumes with 2 U of TasI in 1x TaqI buffer (MBI Fermentas, Burlington, Canada) and incubated overnight at 65°C. The DNA fragments were then electrophoresed in 8 % polyacrylamide gel and stained with the SYBR green (Gelstar, Lonza, Rockland, ME, USA), resulting in separated five bands of DNA. The intensity of the DNA fragments was measured using a Phosphoimager with Image Quant software (Molecular Dynamics, GE Healthcare, Slough, UK). DNA templates from HeLa cell lines were used as controls for normalization of the inter-assay variation between each experiment. The 5 bands on the electrophoresis of LINE-1 are 92 bp, 60 bp, 50 bp, 42 bp, and 32 bp (Fig. 1).

4. LINE-1 Methylation Analysis

An illustration of the qCOBRA-LINE1 technique and an example of gel electrophoresis are shown in Figure 1. The COBRA LINE-1 loci were categorized into four groups: two unmethylated CpGs (uCuC); two methylated CpGs (mCmC); 5'-methylated and 3'-unmethylated CpGs (mCuC); and 5'-unmethylated and 3'-methylated CpGs (uCmC), based on the methylation status of 2 CpG dinucleotides in the 5' and 3' regions of the LINE-1 sequence. The DNA fragments derived from enzymatic digestion of the COBRA-LINE1 products were separated into 5 fragments of 92, 60, 50, 42, and 32 bp, which represented different methylation states. 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 92-bp fragment divided by 92; B=intensity of the 60-bp fragment divided by 56; C=intensity of the 50-bp fragment divided by 50; D=intensity of the 42-bp fragment divided by 40; E=intensity of the 32-bp fragment divided by 32; and F=((D+E)-(B+C))/2. The LINE-1 methylation levels were calculated with the following formula: LINE-1 methylation level percentage (% mC) = 100×(2C+A+F) / (2C+2A+2F+2B).

The statistical analysis was performed using SPSS software for Windows version 22.0 (SPSS Inc., Chicago, IL). A signed rank test was performed to test the difference between methylation levels of DPSCs in early and late passage. A P value<0.05 was considered statistically significant.

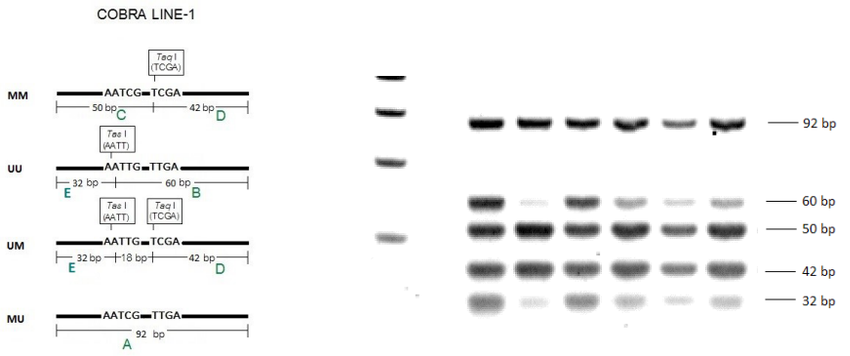

Figure 1 *The illustration of the qCOBRA-LINE-1 technique and the example of gel electrophoresis.*

Results

1. Morphological Observation

In the early passage, 4th passage of DPSCs showed a fibroblast-like morphology, single monolayer with a well-spread morphology attached to the culture dish (Fig. 2A). The morphology of DPSCs has gradually changed over time that we subculture *in vitro.* In the late passage, DPSCs at 15th passage showed enlarged cell size, increased cell secretion, increased nuclear/ cytoplasm ratio and more cytoplasmic processes (Fig. 2B).

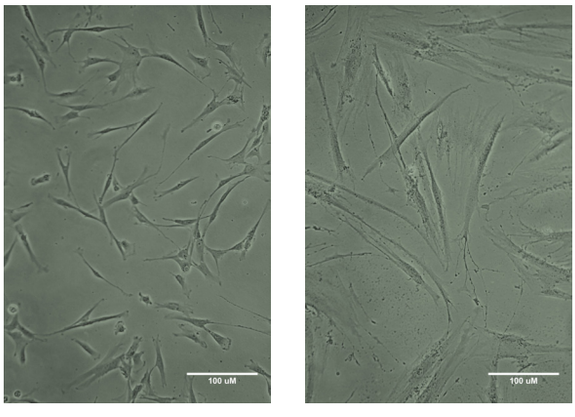

Figure 2 *The illustrations of dental pulp stem cells morphology in early and late passages. (A); DPSCs at 4th passage, (B); DPSCs at 15th passage under light microscope (original magnification, x100). DPSCs in late passage showed enlarged cell size and more cytoplasmic processes. White arrow showed the scattered cell secretion that was obviously seen on the culture dish background in late passage.*

2. Methylation levels of LINE-1

Figure 3 showed the methylation levels (% mC) of DPSCs in 4th (early) and 15th (late) passages. The LINE-1 methylation levels of sample 1, 2, 3, 4, and 5 are 57.229, 54.245, 55.604, 73.819 and 69.276 in 4th passage, while 57.509, 52.437, 53.119, 67.126, and 67.880 repectively in 15th passage. Four out of five DPSCs samples had lower level of LINE-1 methylation at 15th passage than the 4th passage, whereas one sample showed a very slightly higher level of LINE-1 methylation in 5th passage. The average LINE-1 methylation level is 62.035 ± 8.895 in 4th passage of DPSCs and 59.614 ± 7.465 in 15th passage of DPSCs. However, there was no statistically significant difference in LINE-1 methylation level between the early and late passage of DPSCs (p = .08).

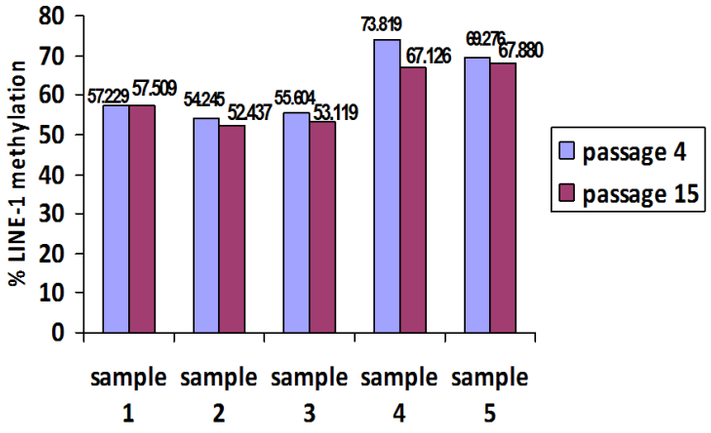

Figure 3 *The methylation levels (% mC) of DPSCs at 4th and 15th passages. Most of DPSCs samples had lower level of LINE-1 methylation at 15th passage than the 4th passage.*

Discussion

The limitations of this study are the sample size and the number of passages. More passage number is needed in the further study. The microbial contamination was one of the major hindrances in long-term cell cultivation that could limit the number of passage. Moreover, longer time was required for the more number of passages. DPSCs in early passage took 3-5 days to reach the confluence, while it took more than 2 weeks to reach the confluence in the late passage. In this study, two samples of DPSCs can reach more than passage 18 but we assorted the DPSCs at 15th passage as the late passage DPSCs since all samples showed the obvious alteration in morphology and could reach this passage number. However, passage number is not the exact cellular age. It simply refers to the number of times the cells have been subcultured. The inoculation densities and recoveries should be concerned.

The declination of LINE-1 methylation level of DPSCs at 15th passage, compared to 4th passage was rather small to detect the statistical significant difference, so the larger sample size is needed.

Our study reveals that DPSCs were capable of long-term cultivation under the *in vitro* conditions we provided without changing their viability but losing their proliferation rate. The morphologic alteration of DPSCs are found during *in vitro* expansion after some passages, such as, alteration in cell shape, enlarged cell size, increased cell secretion and nuclear/cytoplasm ratio. These findings are in agreement with the study of Liu *et al.*[14] who also reported the sequential loss of reprogramming markers Oct-4, Sox2, and c-Myc in the nucleus during dental pulp cell cultures.

LINE-1 (Long interspersed nuclear element-1) retrotransposons are the mobile elements or jumping genes that comprise about 17 percent of human DNA. They multiply themselves throughout the genome. Their methylation statuses are associated with cancer initiation and progression. Decreasing in methylation level of LINE-1 is found relating with many types cancer[30-35] and other pathologic conditions.[36,37] In this study, although we demonstrated that DPSCs at 15th passage lost LINE-1 methylation, we cannot consider their tumorigenesis because they also lost their proliferation rate. The further study about epigenetic mechanisms and malignant transformation of DPSCs is still recommended and required for the secure application of these cells.

Conclusion

Dental pulp stem cells slightly lost their methylation level of LINE-1 during cultured passage *in vitro* but with no statistically significant difference. The larger sample size and more passage number should be recruited in further study.

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