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STUDY OF ALU METHYLATION LEVELS IN VARIOUS PASSAGES OF HUMAN DENTAL PULP STEM CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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เซลล์ต้นกำเนิดจากเนื้อเยื่อในฟันของมนุษย์ซึ่งได้จากเนื้อเยื่อในของฟันเป็นแหล่งหนึ่งของเซลล์ต้นกำเนิดสำหรับเซลล์บำบัดและวิศวกรรมเนื้อเยื่อ การแก่ตัวของเซลล์ซึ่งส่งผลต่อศักยภาพในการกำเนิดใหม่และการเจริญเป็นอุปสรรคหนึ่งที่พบในระหว่างการเพาะเลี้ยงเซลล์ต้นกำเนิดเนื้อเยื่อในฟันในห้องปฏิบัติการ ถึงแม้ว่าทั้งกลไกทางพันธุกรรมและกลไกเหนือพันธุกรรมนั้นมีส่วนในกระบวนการแก่ แต่การศึกษาเกี่ยวกับกลไกเหนือพันธุกรรมที่เกี่ยวข้องนั้นยังมีค่อนข้างจำกัด มีรายงานว่าระดับเมทิเลชั่นของอะลูซึ่งเป็นการเปลี่ยนแปลงเหนือพันธุกรรมนั้นเกี่ยวข้องกับความชราของมนุษย์ งานวิจัยนี้ได้ทำการศึกษาระดับเมทิเลชั่นของอะลูในพาสเสจต่างๆของเซลล์ต้นกำเนิดเนื้อเยื่อในฟัน โดยใช้วิธีการวิเคราะห์คอมไบน์ไบซัลไฟต์เรสตริกชั่น รวมทั้งได้ทำการสังเกตสัณฐานและความสามารถในการเพิ่มจำนวนเซลล์ต้นกำเนิดเนื้อเยื่อในฟันมนุษย์ในทุกพาสเสจ พบว่า เซลล์ต้นกำเนิดเนื้อเยื่อในฟันในพาสเสจท้ายแสดงความแก่ของเซลล์ เปลี่ยนแปลงทางสัณฐาน และมีระดับเมทิเลชั่นของอะลูน้อยกว่าในพาสเสจต้นอย่างมีนัยสำคัญทางสถิติ ผลการศึกษาชี้ให้เห็นว่าระดับอะลูเมทิเลชั่นอาจเกี่ยวข้องกับการแก่ตัวของเซลล์ต้นกำเนิดเนื้อเยื่อในฟันมนุษย์ ควรมีการศึกษาต่อไปเพื่อให้เข้าใจถึงกระบวนการที่ควบคุมการแก่ตัวของเซลล์ต้นกำเนิดเนื้อเยื่อในฟัน เพื่อการจัดการที่เหมาะสมในการนำเซลล์ต้นกำเนิดเนื้อเยื่อในฟันไปใช้ในการรักษาต่อไป

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ATITAYA VONGPROMMOOL: STUDY OF ALU METHYLATION LEVELS IN VARIOUS PASSAGES OF HUMAN DENTAL PULP STEM CELLS. ADVISOR: ASST. PROF. KESKANYA SUBBALEKHA, Ph.D., CO-ADVISOR: PROF. APIWAT MUTIRANGURA, Ph.D., 71 pp.

Human dental pulp stem cells (DPSCs) which can be isolated from pulpal tissue of tooth are being recognized as an alternative source of stem cells for cell-based therapies and tissue engineering. The replicative senescence which results in the alteration of regeneration and differentiation potential is an obstacle encountered during DPSCs culture in vitro. Although both genetic and epigenetic mechanism was found participating in the aging processes, the study in epigenetic mechanisms that involved with these biological processes of DPSCs is still very limited. Methylation level of Alu, an epigenetic alteration, was reported to be associated with human aging. In this study, the methylation level of Alu in different passages of DPSCs was studied by combined bisulfite restriction analysis. Moreover, the morphology and proliferation ability of human DPSCs were observed in every passages. We found that DPSCs in late passage showed replicative senescence, morphological change and had significant lower Alu methylation level than early passage. Our results suggest that Alu methylation level may involve in replicative senescence of DPSCs. Further study should be performed to clarify the mechanism controlling senescence of DPSCs in order to properly manipulation of DPSCs in therapeutic use.

Department:	Oral and Maxillofacial Surgery	Student's Signature .....
		Advisor's Signature .....
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CHAPTER I

INTRODUCTION

Background and Rationale

Stem cell has been a powerful tool for therapy of various disorders. The stem cell-based therapies and the tissue engineering have their potential to repair injured tissue, regenerate missing tissue and restore organ functions (1, 2) since their particular characteristics of proliferation, differentiation and plasticity (3-5). Stem cells can be isolated from many tissues and organs, including dental tissue which become an alternative source of stem cells due to their easy access and isolation (6). Human dental pulp stem cells (DPSCs), can be non-invasively obtained with very low morbidity from pulpal tissue of the tooth that need to be extracted or discarded including wisdom tooth (7). DPSCs have the potential to differentiate into several types of cell, including neurons, adipocytes, chondrocytes, osteoblasts, keratocytes or even insulin-producing cells (8-14).They have high proliferation rate and show typical fibroblast-like morphology with high clonogenic activity similar to the human bone-marrow derived stem cells (15, 16). They also express STRO-1, a mesenchymal stem cell surface marker, which is also present in bone-marrow derived stem cells (15). The variety of practicable differentiation potential leads DPSCs to be a future promising tool that plays important role in regenerative medicine especially in stem cells- based therapy, for example, in corneal reproduction (17), myocardial infarction (18, 19), ischemia (20), muscular dystrophy (21), neural repair (22, 23), hepatocyte reproduction (24, 25), insulin-producing cell production (26), and bone regeneration (27, 28).

Unfortunately, after an extent passage of cultures in vitro, DPSCs display some degrees of replicative senescence, stop to replicate and lose their differentiation potential (29). Liu et al. reported that DPSCs at the 7^{th} passage changed in their morphology with enlarged cell size, irregular cell shape, increased nuclear/cytoplasm ration and decreased cell proliferation rate (30). Generally, proper cell differentiation and function are regulated by gene expression pattern (31-34)

which is not only mediated by genetic events such as DNA sequence, but epigenetic regulatory processes also participate (35, 36).

Epigenetics refers to heritable changes in the genetic expression without any changing in the DNA sequences. Major epigenetic mechanisms including histone modifications, DNA methylation, and DNA acetylation have been related to gene regulation and other cellular processes including division and survival (37). DNA methylation is known to be associated with the regulation of many biological processes including genomic stability, long-term gene silencing (36, 38-40), parental genomic imprinting (41, 42), proper development (43-45) and gene expression (46). Each cell types has the unique pattern of methylation (47).

More than one-third of DNA methylation occurs in repetitive elements (48, 49). Interspersed repetitive sequences (IRSs) are a major contributor to human genome size (approximately 45 percentages of human DNA) (50, 51). IRSs can be categorized by their size and the association of transposable elements. Short interspersed elements (SINEs), long interspersed elements (LINEs), long terminal repeat (LTR)-retrotransposons, and DNA transposons are the commonly known IRSs (52). Among these elements, Alu is the most successful and abundant mobile elements (52-54), which the copy number is over a million and contributes to almost 11% of the human genome (55). Regarding to its richness in CpG residues, Alu is responsible for about 25% of all the methylation in genome (56).

Methylation of Alu varies in different tissues and appears to decrease in some tumors including familial breast cancer (57), HER2 enriched subtype of breast cancer (58), gastric cancer (59, 60), chronic lymphocytic leukemia (61), and nasopharyngeal cancer (62). The lower methylation level of Alu was reported in post-menopausal women with osteoporosis comparing to individuals with the same age and normal bone mass index (63). The catch-up-growth offsprings had higher methylation level of Alu comparing to non catch-up growth offsprings (64). A significant age-related loss of methylation was found in Alu (65, 66). Since the individual aging process are thought to be linked with cellular and stem cell aging (67), we hypothesized that the decreased Alu methylation level that found in aged individual might be found in aged DPSCs.

The purpose of this study is to investigate DNA methylation levels of Alu in DPSCs during multiple passages of cell culture. Moreover, the morphological changes and doubling time of DPSCs will be recorded during the expansion in vitro. The findings from this study will help us understand more about epigenetics involving aging of stem cell and may lead to efficiently application of these cells by maintaining their pluripotency property. Furthermore, understanding the underlying mechanisms of age-related decline in capacity of stem cells and mechanisms of Alu will help us gain better understanding of aging.

Research Questions

Do the methylation levels of Alu in human dental pulp stem cells change during expansion passages?

Objectives

To investigate the Alu methylation levels in varying passages of human dental pulp stem cells.

Hypothesis

Ho : There is no difference of Alu methylation levels in varying passages of human dental pulp stem cells.

Ha : Alu methylation levels from varying passages of human dental pulp stem cells are different.

Research Design

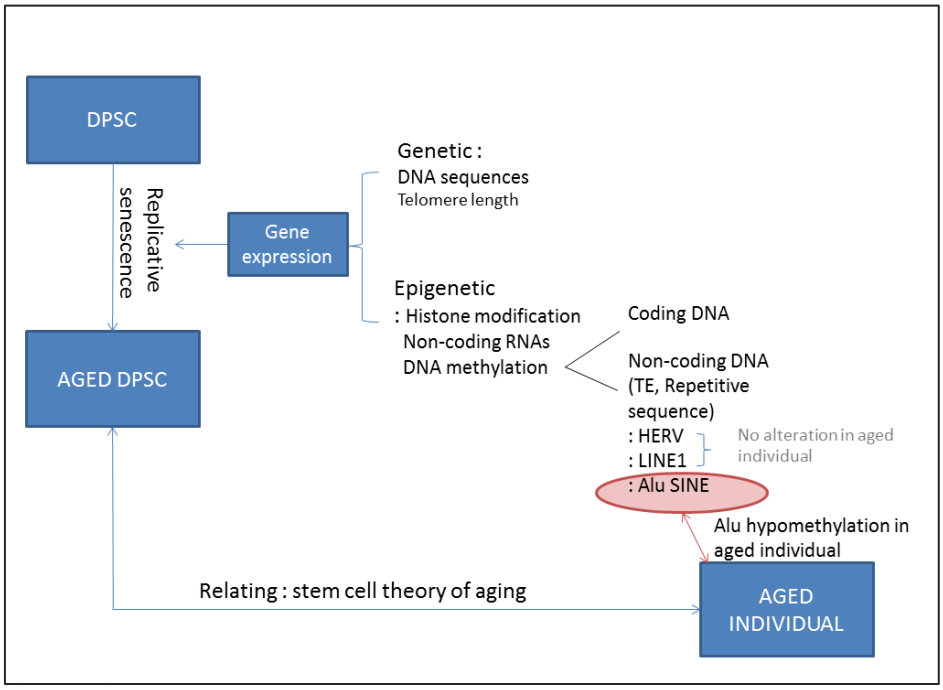
Descriptive analytical study

Expected Benefit

The finding will lead to a better understanding of biological process especially in epigenetic mechanisms that regulate stem cell behavior. The integration of knowledge from both epigenomics and genomics will help the researchers to appropriately manipulate, maintain proper characteristic of DPSCs in prolonged period of preservation in vitro, and precisely produce the cells they demand for the secure and effective application of these cells in therapeutic use.

Further studies may open the new therapeutic approaches and drug development combining genetic and epigenetic strategies. Moreover, the information from this study may imply the connection between changes of Alu methylation level in individual and cellular aging process. Besides the better understanding of stem cell aging, the findings will help us gain better understanding of aging mechanism in organisms.

Research Methodology Framework



CHAPTER II

REVIEWS AND RELATED LITERATURES

1. Human Stem Cells

Stem cells are defined as unspecialized cells that possess the ability for self-renewal without significant changes in their general properties and the ability for differentiation into at least one type of specialized descendant under certain condition (68).

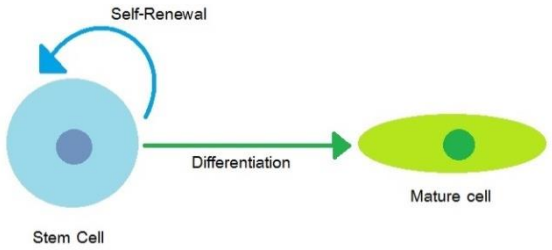


Figure 1 Stem Cell Capacity.

Stem cells have the capacity for self-renewal and differentiation into specialized cell lineage.

The pluripotent embryonic stem cells (ES cells) were successfully isolated from the inner cell mass of embryos. Although their ability to differentiate into almost all cell lineages made them the most promising cells for regenerative medicine, their isolation brought ethical concerns. The induced pluripotent stem (iPS) cells which possess several properties similar to ES cells without ethical concerns were developed instead. However, both ES and iPS cells occupy the potential for teratoma formation which seriously compromise their utility, adult and mesenchymal stem cells which are free of ethical concern and teratoma formation had been widely developed.

Post-natal tissues contain reservoirs of specific stem cells that contribute to tissue maintenance and regeneration. Stem cells that isolated from adult tissues e.g., bone marrow, skin, skeletal muscle and gastrointestinal tract are known as adult or somatic or postnatal stem cells (68, 69).

1.1 Human Mesenchymal Stem Cells

Mesenchymal stem cell (MSC) or mesenchymal stromal cell was first isolated and characterized in 1974 by Friedenstein et al. (70). MSC is a subset of non-hematopoietic adult stem cells that originate from the mesoderm. MSCs are defined as plastic adherent cells, expressing a variety of surface markers, e.g., CD44, CD63, CD105, CD146, with the ability for in vitro self-renewal and multilineage differentiation into mesoderm, endoderm and ectoderm lineages. MSCs exist in almost all tissues and can be isolated from different tissue e.g., umbilical cord, bone marrow, adipose tissue, muscle, lung, and liver (69, 71, 72). Several MSC sources have been identified in oral and maxillofacial region including bone marrow-derived mesenchymal stem cells (BMSCs), dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), apical papilla, dental follicle stem cells (DFSCs), tooth germ progenitor cells (TGPCs), stem cells from the apical papilla (SCAPs), oral epithelial progenitor/stem cells (OESCs), gingiva-derived mesenchymal stem cells (GMSCs), periosteum-derived stem cells (PSCs), salivary gland-derived stem cells(SCSCs) (73, 74). (figure 2)

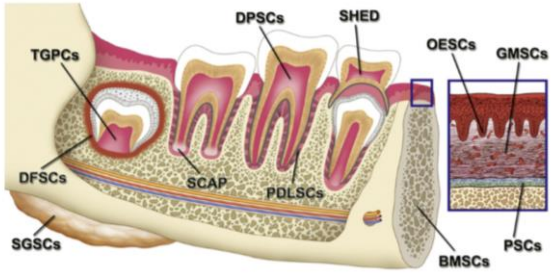


Figure 2 Sources of Mesenchymal Stem Cells (MSCs) in Oral and Maxillofacial Region. Sources of MSC in oral and maxillofacial region include bone marrow-derived mesenchymal stem cells (BMSCs), dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous tooth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle stemcells (DFSCs), tooth germ progenitor cells (TGPCs), stem cells from the apical papilla (SCAPs), oral epithelial progenitor/ stem cells (OESCs), gingiva-derived mesenchymal stem cells (GMSCs), periosteum-derived stem cells (PSCs), salivary gland-derived stem cells (SGSCs) (73).

1.2 Therapeutic uses of stem cells

Human tissues have different regenerative properties. For examples: epithelia can repair any defects after an injury and regenerate themselves all life long, bone or cartilage can regenerate limited defects and in particular conditions, myocardium or neural tissues do not show this ability. Therefore stem cells, which possess the extensive proliferation and differentiation plasticity, are theoretically a promising tool for tissue regeneration and repair of injured tissues. Stem cells-based therapy, a sub-type of regenerative medicine, is the process of introducing stem cells into tissue to treat a disease (3).

An obstacle of the therapeutic use of stem cells is the identification of accessible sites where an adequate amount of stem cells can be collected. The surgical access to the collection site remains a limiting point, due to the morbidity of the site itself. Nevertheless, the selected site must contain a high percentage of stem cells per total volume of the collected tissue to avoid an excessive weakening of the organ**.** At present, the use of an autologous stem cell is the golden standard of stem cell-based therapy because homologous stem cells transplantation need immunosuppression and can cause pathogen transmission.

MSCs in tissue repair

Several clinical and preclinical studies of MSCs on reparative effects strongly suggested that MSCs play a critical role in healing process and are effective in the treatment of tissue injury or degenerative diseases. They are thought to be responsible for wound healing, growth, and replacing the lost cells.

Autologous bone marrow mesenchymal stem cells improved the clinical indices of liver function in patients with liver cirrhosis and liver failure caused by hepatitis B (75, 76). They showed the effectiveness in regeneration of diabetic critical limb ischemia and burn-induced skin defects (77, 78). They are also effective in treating cornea damage(79) and myocardial infarction (80) in preclinical studies. Similar outcome has been reported for MSCs in treating various tissue injuries including brain, spinal cord and lung (81, 82).

MSCs in immune disorder therapy

MSCs are able to suppress inflammation and reduce kidneys and bowel damage in patients with systemic lupus erythematosus and Crohn's disease (83, 84). The success of MSCs application to treat graft-versus-host disease (GvHD) in patients that received bone marrow transplantation (85) especially in severe steroid resistant patients (86), multiple asystem atrophy (87), multiple sclerosis, amyotrophic lateral sclerosis (88), and stroke (89) has also been reported. Moreover, Osiris' Prochymal, the first stem cell-based drug approved by Food and Drug Administration, success in phase III clinical trials in treating GvHD and Crohn's disease (90).

1.3 Human dental pulp stem cells

Human dental pulp derived stem cells (DPSCs) were initially identified by Gronthos et al. in 2000 (91). The connective tissue residing in the center of tooth is found structurally resembles primitive connective tissues and contains special population of adult stem cells (91, 92). The dental pulp comprises of four layers. The most external layer is odontoblastic layer which produce dentin. The second layer, rich in extracellular matrix and poor in cells, is called cell free zone, the third layer, which contains progenitor cells that present pluripotential capabilities and plasticity, is called cell rich zone. The innermost layer contains the vascular and nervous plexus.

DPSCs are readily available from extracted teeth which are usually discarded as medical waste. Their accessibility and rapid proliferation provide a potential for expansion. Furthermore, it is also possible to bank DPSCs as it can be effectively cryopreserved with excellent viability and function (93, 94). The extraction, preservation and isolation of DPSCs can be advantageous for prospective regenerative medicine.

Under the specific stimuli, DPSCs have the potential to differentiate into several types of cell, including neurons, adipocytes, chondrocytes , osteoblasts, keratocytes and insulin producing cells (8-14). They have high proliferation rate. Their cultures show typical fibroblast-like morphology and high clonogenic activity similar to the human bone marrow colony forming units. They also expressed STRO-1, a

mesenchymal stem cell surface marker, which is also present in bone-marrow derived stem cells (15). The phenotypic characteristics of DPSCs were similar to the mesenchymal stem cells from bone marrow (16).

1.4 Roles of DPSCs in regenerative therapies

Role in corneal reproduction

The reconstruction of corneal epithelium with tissue engineered DPSC sheet was successful in animal study. In 2010, Gomes et al. performed a transplantation of a tissue engineered DPSC sheet on the corneal bed of rabbits and covered it with de-epithelialized human amniotic membrane. Its postoperative histological analysis showed the formation of a healthy uniform corneal epithelium (17).

Role in myocardial infarction

Cardiomyocytic differentiation of DPSCs has been studied. The tissue specific MSCs including DPSCs can differentiate into cardiomyocytes (18). Another study in animal model also demonstrated that human DPSCs secrete multiple pro-angiogenic apoptotic factors and suggested that human DPSCs have the therapeutic potential in repair of myocardial infarction (19). These studies support the potential use of DPSCs in the stem cell based cardiac therapies.

Role of DPSCs in Ischemia

A highly vasculogenic DPSCs which is similar to endothelial progenitor cells was successfully isolated from the dental pulp. These cells showed increased proliferative and migration activities including multi-lineage differentiation and vasculogenic potential. The engraftment of DPSCs in a rat model with hind limb ischemic was also successful and lead to an increase in the blood flow including high density of capillary formation (20).

Role of DPSCs in muscular dystrophy

The myogenic potential of DPSCs was evaluated. The therapeutic potential of DPSC in differentiation into dystrophin producing muscle cells was successfully utilized in muscular dystrophy which dystrophin cannot be produced by the body. A transplantation of human DPSCs by arterial or muscular injections in golden retriever dogs with muscular dystrophy showed a significant engraftment in dog muscles (21).

Role of DPSCs in neural repair

The neuro-protective effect of DPSCs in vitro models of Parkinson's and Alzheimer's disease has been studied (22). DPSCs can express a neuronal phenotype and produce several neurotropic factors including nerve growth factor (NGF), Glial cell-derived neurotrophic factor (GDNF), Brain-derived neurotrophic factor (BDNF) and Bone morphogenetic protein 2 (BMP2). An experiment on DPSC transplantation in rats with severed spinal cords demonstrated that DPSCs protected primary neurons and helped in the cell viability. They helped in regeneration of transacted axons by prevention of apoptosis of neurons, astrocytes and oligodendrocytes and by inhibiting axon growth inhibitors. They also differentiated into mature oligodendrocytes to replace the lost cells (23, 95).

DPSCs differentiation to Hepatocyte

The initial research in hypatocytic differentiation of DPCSs reported that DPSCs may possibly be differentiated into cells with phenotypic, morphological and functional characteristics similar to hepatocytes (24). A recent study in vitro showed that DPSCs have the capacity to differentiate into hepatic family (25).

DPSCs considerations for Type 1 diabetes

DPSCs can differentiate into insulin producing cells (IPCs) (14) and have the capacity to differentiate into islet-like aggregates in vitro (96).

Role of DPSCs in bone regeneration

In dentistry, the need for bone is increasing since inadequate bone foundation or bone loss is one of the major problems in dental treatment. The DPSCs are ectomesenchymal in origin and contain osteogenic markers that respond to the inductors of osteogenic and odontogenic differentiation. Since DPSCs have the differentiation profiles which are similar to those showed during bone differentiation, they become interesting as a model to study the osteogenesis and the relationship with scaffolds (27, 28).

Some studies have demonstrated that DPSCs possess the potential to express osteoblastic characteristics and form mineralized nodules in vitro (97-99). Osteogenic medium, a mixture of β-glycerophosphate (β-Gly), dexamethasone (Dex), and ascorbic acid, is now a standard procedure used for the osteogenic induction of

stem cells (100). Dental mesenchymal cells display osteogenic differentiation and form calcified mass when treated with osteogenic medium (101-103). Moreover, the studies in vivo on bone regeneration by DPSCs using with collagen scaffold demonstrated the complete bone regeneration in clinical and radiographic analysis (104, 105).

1.5 Clinical application of DPSCs

The clinical application of DPSCs has not been approved yet. The use of DPSCs is still limited to in vitro and in animal studies. More information and further study about DPSCs in vivo is still needed before their virtually clinical application.

2. Aging and Senescence

Aging (ageing) means becoming older. It refers to the biological ageing of organisms in narrow sense. The term can also refer to single cells within an organism in the broader sense but (cellular senescence). Similarly, the word “senescence” which means "to grow old” can refer either to the senescence of whole organism or to cellular senescence.

Humans aging or senescence represents the accumulation of changes over time in an individual, including change in physical, psychological and social. This complex process is gradual deterioration of physiological function involves entire cells and tissues in an organism. The functional decline is associated with a reduced capacity to maintain normal tissue homeostasis and a decreased regenerative capacity of tissues in response to injury in old organisms. It is commonly believed that cellular senescence underlies the senescence of organismal.

2.1 Theories in aging

The definite causes of aging are still unknown. Several theories have been proposed to explain the alterations related to aging in human bodies. The present theories are based on the damage concept where the accumulation of externally induced damage e.g., mutation, or the programmed aging concept where the internal processes drive aging e.g., telomere shortening. Harman in 1956 first introduced “the free radical theory of aging” to explain the molecular mechanisms of aging (106). Mitochondria dysfunctions, accumulation of DNA damage and telomere length are

also considered as causative factors of aging mechanisms. Moreover, “the stem cell theory of aging” which explained that stem cells are crucial for maintaining tissue homeostasis has also been proposed to explain aging mechanism. As stem cells continuously replace effector cells in the organ, loss of their number and function leads to a breakdown of organ function (67). Exhaustion of adult stem cell pools as an outcome of deranged metabolic signaling, premature cellular senescence as a response to oncogenic offense, and other factors contribute to age-related tissue degeneration. Oxidative stress, DNA damage and telomere length are also interrelated phenomena and affect adult stem cells (107, 108).

2.2 The hallmarks of aging

Lopez-Otin et al. in 2013 summarised nine tentative cellular and molecular hallmarks of aging in organisms. They are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and altered intercellular communication (100).

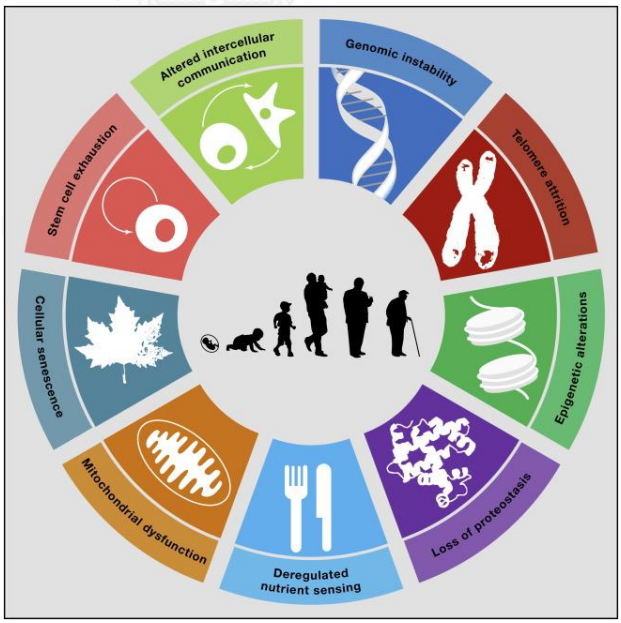


Figure 3 The Hallmarks of Aging.

The nine hallmarks described by Lopez-Otin et al. representing aging in organisms are genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction,

cellular senescence, stem cell exhaustion, and altered intercellular communication.

2.2.1 Genomic Instability

The accumulation of genetic damage throughout life is a common denominator of aging. Many premature aging diseases result from the increased DNA damage accumulation, such as, Werner syndrome and Bloom syndrome. The DNA stability is repeatedly challenged by internal threats including spontaneous hydrolytic reactions, reactive oxygen species, and DNA replication errors, as well as by external physical, chemical and biological threats. The genetic lesions from intrinsic or extrinsic damage are diverse and include point mutations, translocations, chromosomal gains and losses, telomere shortening, and gene disruption caused by the integration of viruses or transposons. DNA repair mechanisms have been evolved in organisms to minimize these genetic lesions. The genomic stability systems also include specific mechanisms for maintaining the appropriate length and functionality of telomeres, and for ensuring the integrity of mitochondrial DNA. Defects in nuclear architecture can also cause genome instability and result in premature aging syndromes. Genomic damage accompanies aging and its artificial induction can provoke the acceleration of aging.

2.2.2 Telomere Attrition

Telomere (terminal ends of DNA) shortening was found during normal aging. Telomere dysfunction can accelerate aging in mice and humans, and stimulation of telomerase can delay aging in mice. Somatic cells do not express telomerase, DNA polymerase that can replicate telomere, thus the progressive and cumulative loss of telomere-protective sequences from chromosome ends. Telomerase deficiency is associated with premature development of diseases in humans, such as dyskeratosis congenital, pulmonary fibrosis, and aplastic anemia.

2.2.3 Epigenetic Alterations

Many evidences suggested that aging is accompanied by epigenetic alterations. Epigenetic alterations including the alterations in chromatin remodeling, post-translational modification of histones, and DNA methylation patterns constitute

age-associated epigenetic marks. Alterations in epigenetic factors, epigenetic modifications in histones, and DNA-methylation determine changes in chromatin architecture, such as global heterochromatin loss and redistribution, which establish characteristic features of aging.

2.2.4 Loss of Proteostasis

Impaired proteostasis or protein homeostasis is associated with aging and some age-related diseases. The proteostasis participates the mechanisms for stabilization of the correctly folded proteins and mechanisms for proteins degradation by proteasome or the lysosome. Many studies have demonstrated that proteostasis is altered with aging.

2.2.5 Deregulated Nutrient-Sensing

Anabolic signaling accelerates aging, and decreased nutrient signaling extends longevity. Limited nutrient can extend longevity in mice.

2.2.6 Mitochondrial Dysfunction

Mitochondrial dysfunction can accelerate aging in mammals, but it is less clear that improving mitochondrial function can extend lifespan in mammals. The mitochondrial function has a profound impact on aging process. The efficacy of the respiratory chain decreases as cells and organisms age, and it bring about increasing electron leakage and reducing ATP generation. Mitochondrial dysfunction can conduce to aging independently of ROS. It may affect apoptotic signaling by increasing the propensity to permeabilize and trigger inflammatory reactions. Mitochondrial dysfunction may also directly impact on cellular signaling by affecting mitochondrial-associated membranes. The combination of increased damage and reduced turnover in mitochondria, due to lower biogenesis and reduced clearance, may contribute to the aging process.

2.2.7 Cellular Senescence

Cellular senescence is a compensatory response to damage that accelerates aging when tissues exhaust the regenerative capacity. The primary purpose of senescence is to prevent the propagation of damaged cell, and senescence is possibly a beneficial compensatory response that leads to rid tissues from potentially oncogenic and damaged cells. However, this cellular response requires a

competent cell-replacement process including clearance of senescent cells and collecting of progenitors to restore cell numbers. This turnover process in aged organisms may exhaust the regenerative capacity of progenitor cells and eventually contribute to the accumulation of senescent cells which may provoke damage and lead to aging. Not only telomere shortening but other aging-associated stimuli trigger senescence. The accumulation of senescent cells in aged tissues has been often inferred using surrogate markers such as DNA damage. However, cellular senescence is not a generalized property of all tissues in aged organisms. The accumulation of senescent cells with aging can conceivably reflect an increasing the generating rate of senescent cells or their decreasing clearance rate. It has been assumed that cellular senescence contributes to aging since the amount of senescent cells increases with aging.

2.2.8 Stem Cell Exhaustion

Stem cell exhaustion unfolds as the consequence of multiple types of aging associated damages. The decreasing in regenerative potential of tissues is one of the most obvious characteristics of aging. Functional decline of stem cells has been found in all adult stem cell compartments. It correlates with the accumulation of DNA damage and the overexpression of cell cycle-inhibitory proteins (eg. p16INK4a). Telomere attrition is also an important cause of stem cell decline with aging. Although deficient proliferation of stem and progenitor cells is obviously detrimental for the long-term maintenance of the organism, an excessive proliferation of stem cells and progenitor cells can also be deleterious by accelerating the exhaustion of stem cell niches.

2.2.9 Altered Intercellular Communication

Aging also involves changes at intercellular communication level. The neurohormonal signaling, such as renin-angiotensin, adrenergic and insulin-IGF1 signaling, tends to be deregulated in aging as inflammatory reactions increase, immunosurveillance declines, and composition of extracellular environment changes, therefore affecting the mechanical and functional properties of all tissues. Inflammaging, the prominent aging-associated alteration in intercellular communication, may arise from multiple causes such as the accumulation of pro-

inflammatory tissue damage, occurrence of a defective autophagy response, and the failure of dysfunctional immune system. The function of the adaptive immune system declines paralleling inflammaging. Moreover, aging-related alteration in one tissue can lead to aging-specific deterioration of other tissues, explaining the inter-organ coordination of the aging phenotype. Bystander effects which senescent cells induce senescence in neighboring cells via gap junction-mediated cell-cell contacts and processes involving ROS was also found. Aging was not a cell biological phenomenon, but also general alteration in intercellular communication, offering opportunities to modulate aging at this level.

3. Adult stem cells and aging

Although the definite mechanism in age-dependent decline of stem cell self-renewal is still unclear, it is fundamentally important to present understanding of aging.

Adult stem cells play a significant role in maintaining normal homeostasis and regenerative repair of tissues throughout the lifetime of an organism, owing to their special characteristic of self-renewal to preserve stem cell pools and differentiation into various cell types. The self-renewal capacity declines with advancing age and it eventually take to the accumulation of unrepaired, damaged tissues in old organisms (67).

Many studies have found the impairments of various tissue resident stem cells during aging process. For example, Nishimura et al. reported that melanocyte stem cells exhibit ectopic pigmentation or differentiation in aged mice and this event might link to graying hair which is a sign of aging in humans and some mammals (109, 110). Aged hematopoietic stem cells in humans and rodents were found decreasing in their engraftment potential and tending to differentiate into myeloid cells (111-113). Aged mice and humans had decreased number of muscle stem cells (114, 115).

4. Epigenetics

The definition of epigenetics is “The study of stable genetic modifications that results in changes in gene expression without a corresponding alteration in the DNA sequences” (116), or “Heritable changes in the genetic expression without any

changing in the DNA sequences" (117). Epigenetic marks integrate intrinsic and extrinsic or environmental stimuli and are considered as a link between genotype, environment, phenotype and disease (118). Epigenetic mechanisms include histone modifications, DNA methylation, post-translational and non-coding RNAs. Epigenetic mechanisms are widely implicated in cancer (119) and play an important role in developmental processes (120).

4.1 DNA Methylation

DNA methylation, the major epigenetic modification in human (121), is the covalent addition of methyl groups to cytosines which is catalyzed by DNA methyltransferases (DNMTs). A methyl group (CH_3) is transferred from the universal methyl donor S-adenosyl methionine to position 5-carbon of the cytosine ring resulting in 5-methylcytosine (5-mC) (122) (Fig. 3).

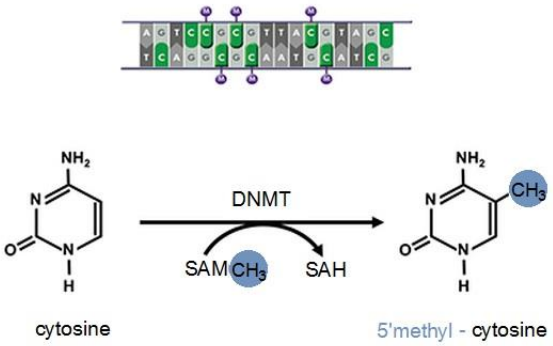


Figure 4 DNA Methylation Reaction Catalyzed by DNMT.

DNA methylation typically occurs at cytosines that are followed by a guanine. A methyl group is transferred from the universal methyl donor S-adenosyl methionine to 5-carbon of the cytosine ring resulting in 5-methylcytosine (5-mC) and S-adenosylhomocysteine (SAH) via the action of DNA methyltransferases (DNMTs).

DNA methylation is considered an efficient repressor of transcriptional activity since the methyl groups directly prevent the binding of essential transcription factors to their targets. New evidence support that the presence of methyl groups models the surrounding chromatin, inducing a DNA conformation less accessible to the transcription machinery (39). About 70 percents of CpGs are methylated, mainly in

repressive heterochromatin regions and in repetitive sequences such as retro transposable elements (123).

DNA methylation is known to be associated with the regulation of many biological processes, including genomic stability, parental imprinting and gene expression (43). DNA methylation also plays an important role in cell differentiation by silencing the expression of specific genes during the developmental processes (124-126).

It was also found to be related with many types of cancer. There are associations between aberrant DNA methylation patterns (hypermethylation and hypomethylation) and a large number of human malignancies (119, 127) . Global loss of DNA methylation has also been implicated in the development and progression of cancer through different mechanisms (128). Typically, there is hypermethylation of tumor suppressor genes and hypomethylation of oncogenes.

DNA methylations have been related with atherosclerosis. Vascular tissue and blood cells such as mononuclear blood cells displays global hypomethylation with gene-specific areas of hypermethylation. DNA methylation polymorphisms present before lesions are observed thus it may be used as an early biomarker of atherosclerosis and may provide an early tool for detection and risk prevention (129).

DNA methylations have also been related with aging. There is a global loss of DNA methylation during aging. However, some genes become hypermethylated with age (130).

More than one-third of DNA methylation is estimated to occur in repetitive elements (48, 49) which regard as a large portion of human genome (55). Alu and LINE-1, the most plentiful families (approximately 30% of human genome), have been used as the surrogate markers for estimating the global DNA methylation levels (131, 132).

5. Repetitive Sequences

Human genome contains large portion of DNA repeats which were once thought as junk DNA (Fig. 4).

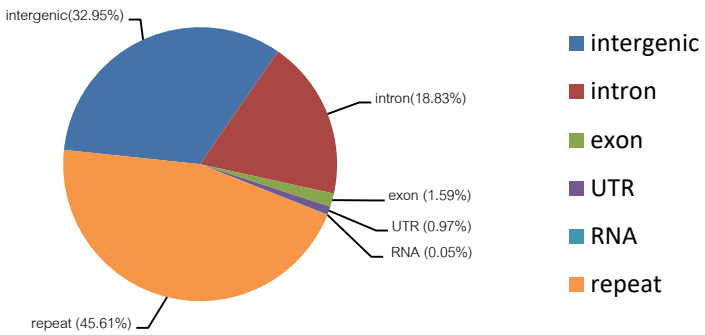


Figure 5 The Organization of Human Genome.

Repeats or repetitive sequences are the largest portion and contributed to about 45% of human genome (133).

"Repetitive sequences, repetitive DNA, repetitive elements, repeats or DNA repeats" are the terms refering to any DNA fragments which present in multiple copies in the whole genome. Repetitive sequences can be basically devided in to two types; tandemly arrayed and interspersed repeats (Fig. 5) (134).

1.) Tandem repeats: the DNA sequences which their copies lie immediately adjacent to each other in an array or in head-to-tail orientation, either directly or inverted
2.) Interspersed repeats: the DNA sequences which their copies are randomly scattered or dispersed throughout the genome

5.1 Interspersed Repetitive Sequences and Transposable Elements

Interspersed repetitive sequences are mostly inactive. They are the incomplete copies of transposable elements inserted into genomic DNA.

Transposable elements (TE or transposon), sometimes called jumping genes or mobile elements, were discovered more than 50 years ago and are now known as the most abundant constituent in eukaryotic genomes and account for at least about 45% of human genome. TEs are the segments of DNA sequences which can change their position within the genome by being duplicated and inserted anywhere in the host genome. Almost half of human genome is derived from the ancient transposable elements (134).

5.2 Types of Transposable Elements

TEs can be categorized by several criteria. On the basis of self-sufficiency, all types of TEs present two variants; autonomous and nonautonomous. The autonomous variants (e.g., HERV, LINE) encode the necessary enzymes and are self-suffcient for transposition, such as transcriptase and endonuclease/integrase. The nonautonomous variants (e.g., Alu, SVA) change their positions by borrowing the protein machinery encoded by its autonomous relatives.

Despite their diversity, human transposable elements can also be divided into two basic types depending on how they transpose or the requirement of reverse-transcription; DNA transposons and retrotransposons (134, 135).

5.2.1 DNA transposons (Type II TEs)

DNA transposons do not require any reverse-transcription process in their transposition. They move their DNA copies from one location to another by a cut-and-paste mechanism, without an RNA intermediate. A transposase which is the transposon-encoded protein recognizes the terminal inverted repeats that flank the TE, then cuts the TE out from the donor position, and integrates the transposon into a new site. The gap occurred at the donor site can then be repaired either with or without the replacement of the TE.

5.2.2 Retrotransposons (Type I TEs)

Retrotrasposons are the TEs that need a reverse-transcription. They move by a process, liken to a copy-and-paste mechanism, called retrotranposition through an RNA intermediated which is expressed in the host cell. After reverse transcription using mRNA, the resulting complementary DNA (cDNA) copy is integrated back into the host genome. The enzymes used in reverse transcription and integration, reverse transcriptase and endonuclease/integrase (EN/INT), are encoded by autonomous elements.

Retrotransposons can be subdivided into long terminal repeat (LTR) retrotransposon and non-long terminal repeat (non-LTR) retrotransposon, on the basis of the presence of long terminal repeats (LTRs) which are direct repeats at the ends of the elements (136) .

- Long terminal repeat (LTR) retrotransposon

Proteins pol and gag found in LTR retrotransposon are very closely related to retroviral proteins, however these elements lack the envelope (env) protein which is required to exit the host cell. LTR retrotransposons are human endogenous retroviruses (HERVs) that have an intracellular existence as a result of a nonfunctional envelope gene.

- Human endogenous retroviruses (HERVs)

- Non-long terminal repeat (Non-LTR) retrotransposon

Non-LTR retrotransposon can be classified on the basis of size into long interspersed elements and short interspersed elements .

- Short interspersed elements (SINEs) is the repetitive interspersed DNA sequences which are less than 500 base pair long[53,189,194,195]
- Long interspersed elements (LINEs)

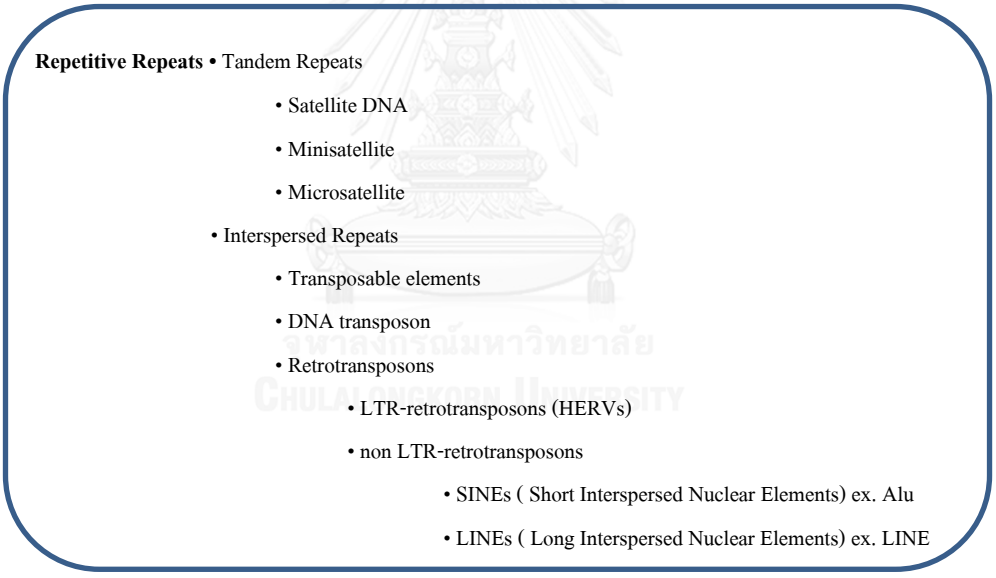


Figure 6 The Overall Classification of The Repetitive Sequences.

The flow chart shows the overall classification of the repetitive sequences.

5.3 Alu elements

An Alu element, being about 300 basepairs long, is classified as a non-autonomous short interspersed nuclear element and was identified originally 30 years ago as a component in human, characterized by the action of the Alu (Arthrobacter luteus) restriction endonuclease. Alu elements are unique to primates.

The name ‘Alu elements’ was given to these repeated sequences as members of this family of repeats contain a recognition site for the restriction enzyme AluI. Alu is one of the most successful mobile elements. It is the most abundant mobile elements which the copy number excesses one million, contributing to almost 11% of the human genome (49, 137).

The elements are non-autonomous and acquire trans-acting factors in their amplifying process from the only active family of autonomous human retroelements: LINE-1 (138). Alus insert in genome using a process termed target primed reverse transcription. Because these elements insert through an RNA intermediate, each new insertion effectively increases the copy number of them. However, the vast majority of the Alu copies are not transcriptionally active (54). Most Alus in the genome are repressed transcription by methylation or chromatin context (139, 140). Alu is formed from two diverged dimers, which are ancestrally derived from the 7SL RNA gene, and separated by a short A-rich region. Having two similar dimeric structures, distinct monomers and joined by an A-rich linker, it is believed that modern Alu elements emerged from a head-to-tail fusion of two distinct fossil antique monomers over 100 million years ago (141).

The general structure of an Alu, presenting in Fig. 6, is (5' Part 1 - A5TACA6 - Part 2 – Poly A Tail - 3'). Typical Alu structure is composed of 2 part, with the 3’ half (right arm) containing additional 31 basepairs insertion relative to the 5’ half (left arm) Total length of each Alu is approximately 300 basepairs, depending on the length of poly A tail which varies between Alu families, while the body of Alu is about 280 basepairs long. Two dimers are sererated by a central A-rich region. The entire Alu element is flanked by short direct repeats of variable length which are derived from the site of insertion, called target site duplication or TSDs (black arrow in the figure). The 3’ end of an Alu element has a longer A-rich region that plays a critical role in its amplification mechanism. The elements have an internal RNA polymerase III promoter in the 5’half (Box A and B) that potentially initiates transcription at the beginning of the Alu and produces RNAs that are responsible for their amplification. However, Alu elements have no terminator for transcription and

the transcripts terminate at nearby genomic locations using a TTTT terminator sequence (53, 142).

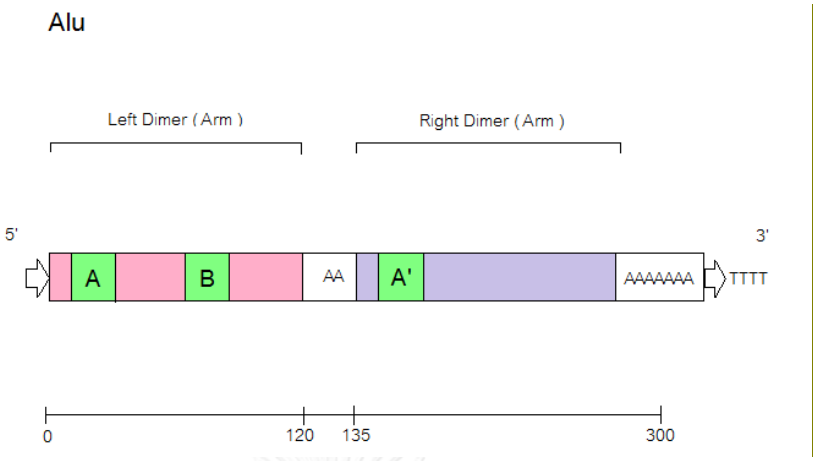


Figure 7 The Illustration of The Basic Structure of Alu Element.

Alu element is approximately 300 bp long. A dimeric structure is composed of two related but not equivalent monomers (left and right arms). Both arms are linked by an A-rich region. At the end of the right arm lies the poly-A tail, which is a long stretch of adenine characteristic to both SINEs and LINEs. Box A, B are the control sequences, the RNA polymerase III promoter and box A' is the complement of box A. The element is flanked by target site duplication (TSD), presenting by black arrows.

Alus are rich in CpG residues which are widely subject to methylation, thus responsible for about 25% of all of the methylation in genome (56). Methylation of Alu varies in different tissues and appears to decrease in some tumors (58, 59, 62). Demethylation of an Alu may increase the expression from that Alu locus. Recent study also reported that decreased methylation level of Alu was associated with aging (65, 66), post-menopausal women with osteoporosis (63).

5.4 Association of Alu methylation levels

DNA methylation also results in transposable element silencing. It was found that the repression of LINE (143) and SINE (144) promoters in human genome occurs when DNA methylation is reduced. The actual biological significance of TEs repression is unproven. There are two explanations discussing; the repression is essential for preventing DNA damage from unconstrained transposition (145), or the

repression is required to reduce transcriptional noise, as the transcription of a large excessive irrelevant promoters could bring an unacceptable level of transcriptional noise which would interfere gene expression programs (146). Increasing in transcription of the elements human yet has not been found related with increasing transposition.

Alteration of Alu methylation level was observed relating with various conditions. Decrease in methylation level of Alu was found in many types of cancer such as familial breast cancer (57), HER2 enriched subtype of breast cancer (58), gastric cancer (59, 60) , chronic lymphocytic leukemia (61), and nasopharyngeal cancer (62). Hypomethylation of Alu was also associated with developmental process, it has been reported in menopausal women who has lower bone mass and osteoporosis (63), and in blood cells of aging people (66). On the contrary, higher Alu methylation has been observed in various conditions, such as systemic lupus erythematosus (147), insulin resistance (148) colorectal cancer (149), and cardiovascular risk, including hypertension and diabetes (150).

6. Stem cell behavior and epigenetic regulations

Stem cell behavior is not only mediated by genetic events such as DNA sequence, multiple levels of regulation including posttranscriptional, translational, posttranslational, but epigenetic regulatory processes also take part (35). Recently, the epigenetic regulation has emerged as an important modulator of stem cell differentiation (151). Epigenetic modifications are largely responsible for the activation and repression of specific genes at specific time points during the lifespan of the cells, allowing for their terminally differentiated phenotype. The epigenetic modification is heritable, the changes in DNA remain as cells divide and last for multiple generations. Major epigenetic mechanisms include histone modifications and DNA methylation which have been related to gene regulation and other cellular processes including division and survival (37). DNA methylation, histone modification and noncoding RNA regulation collaborate in controlling chromatin plasticity.

Gene expression potential in stem cell renewal and differentiation is regulated by epigenetic mechanisms that alter the transcriptional permissiveness of

chromatin, of which DNA methylation is the best characterized compartment (152). It is generally accepted that DNA methylation silences gene expression.

The maintenance of the pluripotency state in embryonic stem cells (ESCs) is given by development associated transcription factors, including OCT4, NANOG, and SOX2, which activate genes of self-renewal at their unmethylated promoters (153). Epigenetic profiles of mesenchymal stem cells (MSCs) reflect a more limited differentiation potential as compared to ESCs, but numerous epigenetic modifications concomitantly happen during both osteogenic and adipogenic differentiation (35).

In bone marrow derived mesenchymal stem cell, OCT4 is silenced by hypermethylation of its promoter, whereas SOX2 and NANOG are unmethylated despite the repressed state of the genes (154), indicating the implications of other epigenetic mechanisms.

Each cell type has the unique pattern of methylation. MSCs isolated from human adipose tissue, bone marrow, and muscle show similar DNA methylation profiles (155). It has been reported that human embryonic stem cells contain a unique epigenetic signature based on methylation profiles (156).

A study on MSC undergone replicative senescence reported that the overall DNA methylation remained constant after long-term culture of MSC but highly significant differences were observed at specific CpG sites, homeobox genes and genes involved in cell differentiation. The study inevitably shows that the long-termed culture leads to significant methylation changes at specific CpG sites and seems to affect genes involved in development. It also supports the notion that replicative senescence represents developmental processes that are regulated by specific epigenetic modifications (157).

CHAPTER III

MATERIALS AND METHODS

This study was an observational laboratory investigation. DPSCs were expanded until reaching the passage that their proliferation rate was extremely retarded. DNA of DPSCs from each passage were collected and submitted to methylation study. Combined bisulfite restriction analysis (COBRA) technique was used for studying methylation levels of Alu. The methylation levels of Alu were compared among various passages of DPSCs. Light microscope was used for examining the morphological change. Population doubling time was recorded during cell passage. The senescence-associated ß-Galactosidase assay was performed to detect cellular senescence.

Sample

Sample Selection

The non-pathological third molars removed from healthy adult patients for treatment of impacted third molars at the Department of Oral and Maxillofacial Surgery, Faculty of Dentistry, Chulalongkorn University were collected with the consent of patients. Dental pulp tissue were then isolated for DPSC extraction.

Sample size

Calculation of the sample size for hypothesis testing was performed using the data from pilot study which the percentage of Alu methylation in early passages and late passages of 3 DPSCs samples were cultured and investigated. Independent t-test was used for comparing means of two populations under the condition of normal distribution data. The Alu methylation data from preliminary study was shown in the table 1.

Table 1 The Alu methylation data from preliminary study.

	% Alu Methylation of DPSC	
Sample	early passages	late passage
1	39.94	36.72
2	37.59	32.72
3	36.86	36.84
Mean (μ)	38.13	35.43
Standard deviation (s)	1.31	1.91
s^2	1.72	3.65

Sample size is calculated with the following formula.

$$n = \frac{2\sigma^2(Z_{1-\alpha/2} + Z_{1-\beta})^2}{(\mu_1 - \mu_2)^2}$$ If $n_1 = n_2$, $\sigma^2 = \frac{(S_1^2 + S_2^2)}{2}$

n = sample size by group

σ = standard deviation

α = 0.05 (at 95% confidence interval)

β = 0.1 (power of test 90%)

μ_1 = mean of Alu methylation percentage in osteogenic induced DPSCs

μ_2 = mean of Alu methylation percentage in original DPSCs

μ_1	38.13	μ_2	35.43
S_1	1.31	S_2	1.91
S_1^2	1.72	S_2^2	3.65
$Z_{1-\propto/2}$	1.96	$Z_{1-\beta}$	1.28
σ^2	2.69	$(Z_{1-\alpha/2} + Z_{1-\beta})^2$	10.50
$(\mu_1 - \mu_2)^2$	7.29		

From the formula $n = \frac{2\sigma^2(Z_{1-\alpha/2} + Z_{1-\beta})^2}{(\mu_1 - \mu_2)^2}$, n = 7.75

Therefore, at least 8 samples should be included in each group.

Dental Pulp Stem Cells Isolation and Culture

The Isolated dental pulp tissues were washed with sterile phosphate buffered saline (PBS) and plated onto 60 mm culture plates and maintained in 3 ml. of Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) containing 10%fetal bovine serum (FBS; Gibco, NY, USA), 2mM L-glutamine (Gibco, NY, USA), 100U/ml penicillin, 100µg/ml streptomycin, and 5µg/ml amphotericin B (Gibco, NY, USA) in 100% humidity, 37°C and 5% carbon dioxide for 24 hours, under sterile condition. Medium was changed every 48 hours. After reaching confluence, the cells were subcultured to 3 plates at 1:3 ratios.

Investigation of Morphological Changes

The morphology of DPSC in every passage was observed under light microscope, and their images were recorded.

MTT Cell Proliferation Assays

Doubling time of each passage of DPSC was analyzed by MTT proliferation assay used for assessing cell metabolic activity. The yellow tetrazolium MTT salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was reduced by NADH or NADPH which generated by dehydrogenase enzymes in the mitochondria of the metabolically active and viable cells to yield purple insoluble formazan product.

In this study, 20,000 cells of DPSC form each passage were plated in the 24-well plates in triplicate. MTT assay was performed at day 1, 2 and 3 of the culture. At the time of MTT assay, plates were rinsed with PBS twice and added with 0.5 mg/ml MTT solution, prepared with Thiazolyl blue (Sigma, MA, USA) in serum free media without phenol red. After incubated for 30 minutes and purple formazan was visible, MTT solution was removed and 1ml detergent solution (1 Glycine buffer: 9 Dimethylsulfoxide) was added to solubilize the colored crystal.

The Senescence-Associated β-Galactosidase Assay

The cellular senescence was investigated by the detection of senescent-associated beta galactosidase activity (SA-β-Gal), a biomarker for cellular senescence, using Senescence β-Galactosidase Staining Kit (Cell Signaling Technology, MA, USA) according to the manufacturer's instruction. In brief, DPSCs from each passage were plated in 6-well plate and incubated overnight. After removing growth medium and

rinsing the plate with 2 ml PBS, 1ml of Fixative Solution was added and incubated at room temperature for 15 minutes. The plate was then rinsed twice with PBS, added with ß-Galactosidase Staining Solution, sealed with parafilm and incubated at 37°C overnight in a dry incubator without CO_2. The development of blue color representing the ß-Galactosidase activity in senescent cell was investigated under a microscope (200X magnification).

DNA Extraction

DPSCs from each passage were collected to extract the DNA for the analysis of Alu methylation. The cells are harvested into 1ml tube by trypisinization and centrifuged at 5,000 rpm for 5 minutes to pellet cells. After removal of all medium and washing with PBS, 20 µL of Proteinase K and 500 µL of lysis buffer were added to the sample. They were mixed well by vortexing and incubated at 50°C for 48 hours for cell lysis. Genomic DNA was then isolated by using standard phenol-chloroform extraction, ethanol precipitation, washing and elution. The final DNA concentration is measured using nanodrop. The purified DNA is stored at –20°C until use.

Modified Combined Bisulfite Restriction analysis (COBRA)

The process consists of 4 steps as follow (Fig. 7)

1. DNA denaturation and sodium bisulfite treatment
2. Polymerase chain reaction (PCR) for DNA amplification
3. Restriction digestion of the amplified DNA
4. Quantification (gel electrophoresis) and measuring the intensity of interesting bands

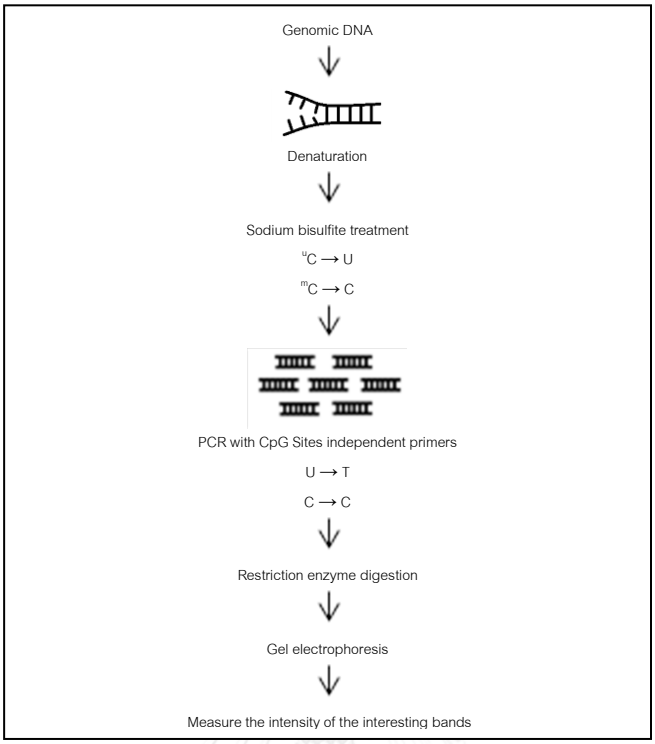


Figure 8 Steps in COBRA.

1. DNA denaturation and Sodium Bisulfite Treatment

Principle

Sodium bisulfite treatment of DNA converses all “unmethylated cytosine” (^{u}C) to “uracil” (U) residues. “Methylated cytosine” (^{m}C) are unaffected and remain as “cytosine” residue (C). Bisulfite conversion results in a mixed population of DNA which reflects the difference in a methylation pattern.

The unmethylated cytosine is changed to uracil in bisulfite treatment through the deamination of cytosine. The reaction involves in several steps (Fig 8). Deamination of cytosine begins when the bisulfite are added to 5-6 double bond of cytosine, then a hydrolytic deamination of the resulting cytosine-bisulfite derivative takes place to form a Uracil-bisulfite ,and the sulfonate group is then removed by subsequent alkaline treatment. However, the reactivity of 5-methylcytosine is greatly lower thus the methylated cytosine remains unchanged.

The reaction requires the DNA to stay in a single-stranded form because sulfonation of cytosine can only occur on single-stranded DNA. Therefore, the

genomic DNA is needed to be fully denatured and remains denatured until the sulfonation is complete.

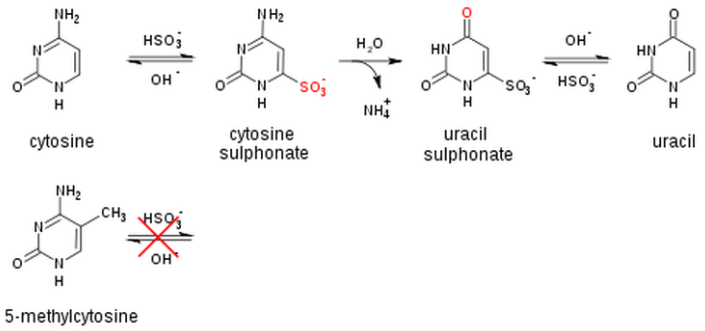


Figure 9 Reaction in Bisulfite Treatment.

Bisulfite treatment changes unmethylated cytosine to uracil but 5-methylcytosine remains unaffected.

Technique

Bisulfite modification of the DNA samples was performed using an EZ DNA methylation kit (Zymo Research, Orange, CA, USA) according to the manufacturer's protocols. Modified DNA was ready for analysis or was strored at -20°C for later use.

2. Polymerase Chain Reaction (PCR)

Principle

PCR is one of revolutionary method for DNA amplification. This method is ground on using DNA polymerase to synthesize new strand of DNA that complementary to the offered DNA template strand. Since DNA polymerase can only add a nucleotide on a preexisting 3'-OH group, a DNA primer is need to initiate the first nucleotide addition. The PCR components are DNA template, DNA polymerase, primers and nucleltides. DNA template is the sample DNA which contains target DNA sequences. DNA polymerase is the heat resistant enzyme used in synthesizing new strands of DNA which complementary to the target sequences. The most common enzyme is *Taq* DNA polymerase acquired from *Thermis aquaticus*. DNA polymerase can generate new strands of DNA by using a DNA template and primers. Primers are short segment of single-stranded DNA that bind in complementary direction to the

target DNA strand. Nucleotides (deoxynucleotide triphosphates or dNTPs) are single units of bases A, T, C, and G, which are the essential substances for new DNA strands.

One cycle of PCR consist of three steps; denaturation, annealing and extension, which are conveniently triggered by temperature. Each cycle of PCR generates double amount of target DNA sequence, the copies of target sequence will be exponentially increased at the end of the reaction. The first step at the beginning of reaction is “Denaturation”. High temperature (approximately 94°F) is applied to separate the strands of DNA’s double helix. The heat breaks the hydrogen bonds between amino acids on each strands, and the helix then unwinds and separates into two strands. In “annealing” step, the temperature is decreased to approximately 58°F which enable the primers to attach to the single-strands of target DNA by reforming the hydrogen bonds. The DNA polymerase continues the process in “extension” step. To activate the polymerase, the mixture in extension is warmed. After initially attaching to the primer, the polymerase begins to synthesize new DNA strand from the end of the primer. The polymerase generates new copying strand along the DNA template by attaching to base pairs in a 3’ to 5’ direction. The polymerase reforms the original sequence following the primer as it attaches nucleotides to the new strand complementary to the mother strand. To continue the cycle, the mixture can be reheated as long as the nucleotides and primers are enough for synthesizing new strands.

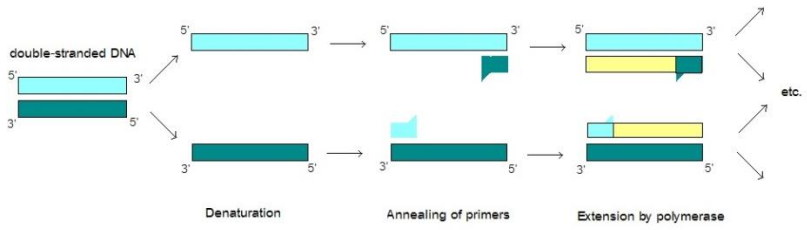


Figure 10 Polymerase Chain Reaction Cycle.

One cycle of PCR consist of three steps; denaturation, annealing and extension.

Technique

After DNA is treated with sodium bisulfite, PCR is performed to amplify ALU sequences, using the primer sequences that correspond to the nucleotides in the regulatory region of Alu elements.

Technique For Alu PCR

One microlitre of bisulfite DNA is subjected to initial denaturation at 95°C for 15min, followed by 45 cycles of 95°C for 45 seconds, annealing at 63°C for 45 seconds, extension at 72°C for 45s and final extension at and 72°C for 7 min, using the following primer sets Alu (F), 5=-GG(T/C) G(C/T)G GTG GTT TA(C/T) GTT TGT AA-3= (R), 5=- CTA ACT TTT TAT ATT TTT AAT AAA AAC RAA ATT TCA CCA-3= Alu amplicon after PCR is 133 bp.

3. Specific Restriction Enzyme Digestion

Principle

Specific restriction digestion is the technique using enzymes called Restriction Endonucleases (Restriction Enzymes or RE's) to cleave DNA molecules at specific sites. The enzymes recognize particular sequence (for example GGATAT) and cut the DNA molecules where that sequence exist. A buffer specific for enzymes is also added to optimize the condition for reaction. All ingredients for a restriction digestion are usually stored in a freezer until they are used to prevent proteins denaturation.

The resctriction enzyme used in this experiment is *Taq*I restriction enzyme which recognize at 5' TCGA, 3' AGCT and makes the cut as 5'---T / CGA---3' and 3'---AGC/ T---5'.

Technique

Resulting from PCR, the COBRA Alu amplicon is 133 bp. The PCR products of Alu are digested with 2 units of *TaqI* (Fermentas International, ON, Canada) at 65°C overnight. The products are then identified and quantified.

4. Quantification

Principle

The digested fragments from restriction enzyme digestion are separated by polyacrylamide gel electrophoresis. Quantitative amount of DNA in the bands appearing from electrophoresis can be determined with a phosphoimager. The

Methylation levels (percentage of methylation) can be calculated as the intensity of methylated bands divided by the sum of the methylated and unmethylated bands, showing in the following equation:

$$\% \text{ Methylation} = 100 \text{ x } \frac{\text{Digested Fragments}}{\text{Undigested Fragments} + \text{Digested Fragments}}$$

Technique

After the restriction digestion, the DNA fragments were separated on 8% non-denaturing polyacrylamide gels. The gel was then stained with SYBR green nucleic-acid stain (Invitrogen, Carlsbad, CA, USA). The intensities of the DNA fragments were measured with a PhosphoImager, using ImageQuant software (Molecular Dynamics, GE Healthcare, Slough, UK).

The PCR product of Alu was 133 bp and the amplicons after digestion were 133, 90, 75, 58, 43 bp (Figure10). The number of CpG dinucleotides of each COBRAAlu band was also normalized as follows: %133/133 = A; %58/58 = B; %75/75 = C; %90/90 = D; %43/43 = E; and %32/32 = F. In addition to overall methylation level, we also classified Alu loci based on the methylation statuses of 2 CpG dinucleotides as hypermethylated loci (mCmC), hypomethylated loci (uCuC), and 2 types of partially methylated loci (mCuC and uCmC) (Fig. 10).

Alu methylation levels are calculated with the following formula: (49,174,181)

Overall Alu methylation level percentage (%mC) = 100 x (E+B)/(2A+E+B+C+D)

percentage of mCmC loci (%mCmC) = 100 xF/(A+C+D+F)

percentage of uCmC loci (%uCmC) = 100 xC/(A+C+D+F)

percentage of mCuC loci (%mCuC) = 100 xD/(A+C+D+F)

percentage of uCuC loci (%uCuC) = 100 xA/(A+C+D+F)

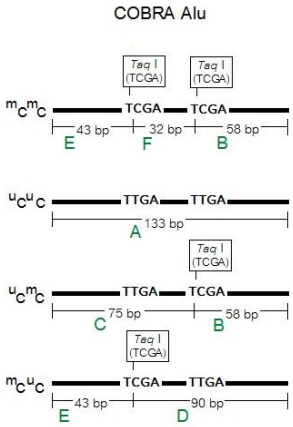


Figure 11 COBRA Alu Amplicons

Alu loci were classified based on the methylation statuses of 2 CpG dinucleotides as hypermethylated loci (mCmC), hypomethylated loci (uCuC), and 2 types of partially methylated loci (mCuC and uCmC).

Statistical Analysis

Statistical analyses were performed using SPSS version 17.0 (SPSS Inc., Chicago, IL). All *p*-values less than 0.05 were considered significant. Data were reported as mean and standard deviation. Total passage numbers of each sample were different, so we classified DPSCs into three groups depending on passage number; early, middle, and late passage, to perform the proper statistical analysis. Independent sample t-test was used for the comparison between two passage groups.

CHAPTER IV

RESULTS

Long-term Culture of DPSCs

Twelve samples of DPSCs were explanted from 12 third molars of t12 healthy subjects who underwent the surgical extraction, age 18-35.

However, 3 samples were excluded from the study; including 2 bacterial contaminated samples, and 1 sample explanted from microdontia showing an unusually slow proliferation rate. Therefore, 9 samples of dental pulp stem cells were expanded in culture medium with fetal bovine serum (FBS) until they obviously lose their proliferation property or enter replicative senescence. Overall, the proliferation rate remained relatively high during the first two months before it declined. All DPSCs sample entered growth arrest after 140±15 days.

After primary explant culture, DPSCs grew out from dental pulp tissue. Under light microscope, DPSCs showed a typical fusiform fibroblast-like morphology, single monolayer with a well-spread morphology attached to the culture plate. The morphological alteration was demonstrated in figure 12.

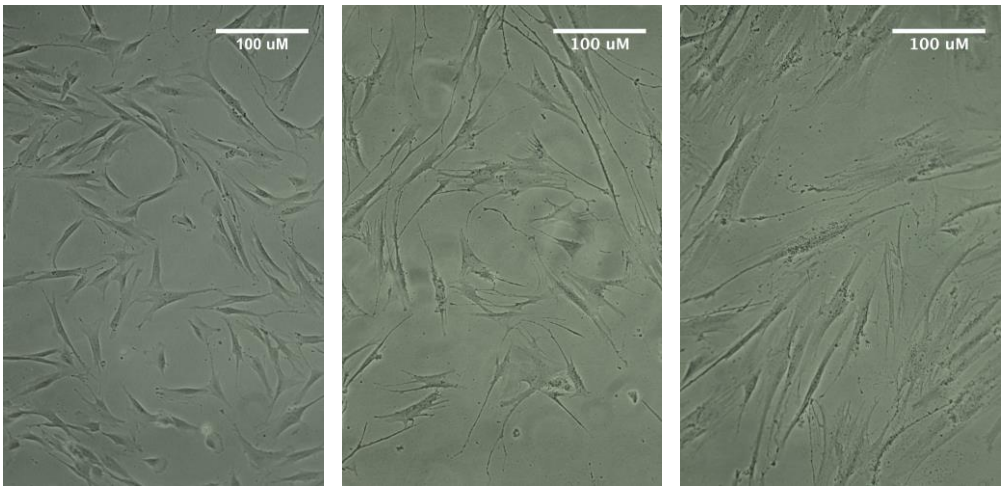


Figure 12 The Morphological Alteration of Long-term Cultured DPSCs.

Long-term culture of DPSCs showed the morphological alteration. DPSCs in passage 2 showed fibroblast-like morphology with spindle shape. DPSCs in

passage 9 showed larger cell size and more cytoplasmic processes compared to DPSCs in passage 2. DPSCs in passage 15 also showed larger cell size and more cytoplasmic processes compared to DPSC in passage 9.

Cell Proliferation and Doubling Time

MTT cell proliferation assay was performed in 3 samples of DPSCs, in triplicate. Doubling time of DPSCs in each passage was calculated, plotted and shown in table 2 and figure 13. Spearman's Correlation was used to analyze the relationship between passage number and doubling time. There was a statistically significant positive correlation between passage number and doubling time ($r = .774$, $p < .001$).

Table 2 Doubling time of DPSCs in each passage.

Passage Number	Average Doubling Time (hour)	Passage Number	Average Doubling Time (hour)
3	30.39278	13	90.15596
4	67.23596	14	99.37973
5	69.78801	15	106.7616
6	63.15861	16	107.385
7	55.09325	17	93.52593
8	76.86075	18	106.5855
9	114.3359	19	99.78152
10	94.49278	20	111.4373
11	93.36996	21	128.0004
12	107.531	23	124.2271

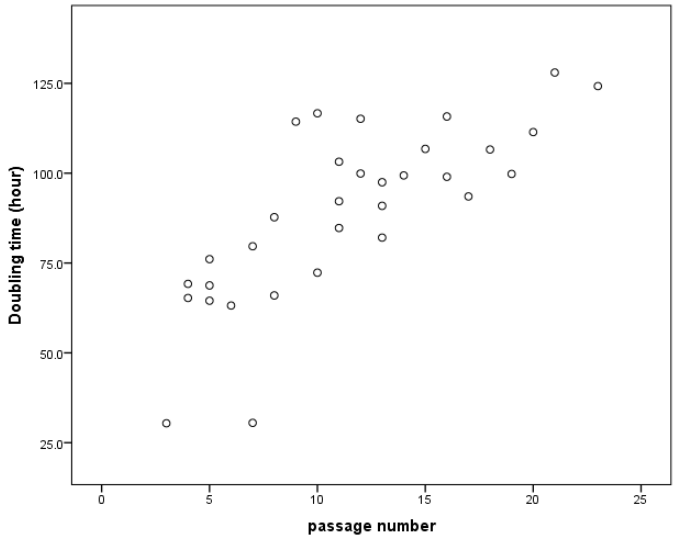


Figure 13 Correlation of Passage Number and Doubling Time.

Passage number was positively correlated with doubling time with statistical significance.

Methylation level of Alu

Percentage of overall Alu methylatioin level (%mC) of each DPSC sample were demonstrated in figure 14. The levels of mCmC, uCuC, uCmC and mCuC were also demonstrated in figure 15, 16, 17 and 18, repectively. In each passage, the DNA methylation level was measured in tripicate. The linear regression lines were also created to show the trend of methylation level.

Figure 14, all trendlines from each sample showed the declination of %mC during DPSCs culture. All DPSCs samples tended to lose Alu methylation level during cell culture in vitro. Figure 15, DPSCs showed either the declination or escalation of %mCmC during DPSCs culture. 5 out of 11 samples of DPSCs showed the declination of trendline, 2 samples showed the escalation of trendline, and 1 sample showed the constancy of trendline. Figure 16, trendlines of %uCuC from 10 samples showed the escalation of %uCuC during DPSCs culture, whereas 1 sample showed the constancy of %uCuC. Figure 17, DPSCs showed the different trendlines of %uCmC. Figure 18, Most of all DPSCs tend to lost %mCuC. 9 samples of DPSCs showed the declination while 1 sample showed the escalation of trendline.

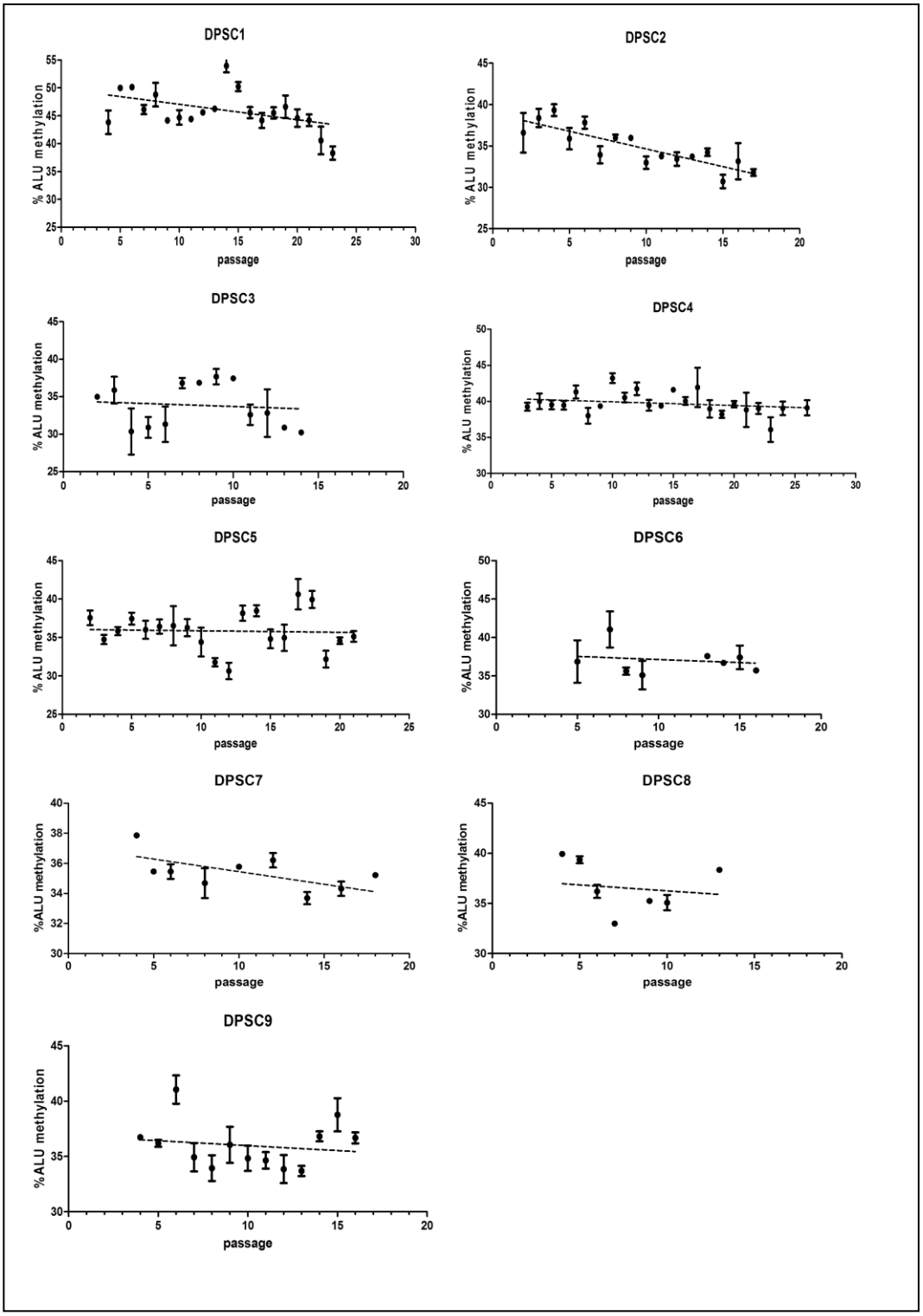


Figure 14 Percentages of Overall Alu Methylation (%mC).

Each passage, the methylation level was measured in tripicate. The verical line indicates the variance of %mC. Dashed lines represents trendlines, and all trendlines showed the declination of %mC during DPSCs culture.

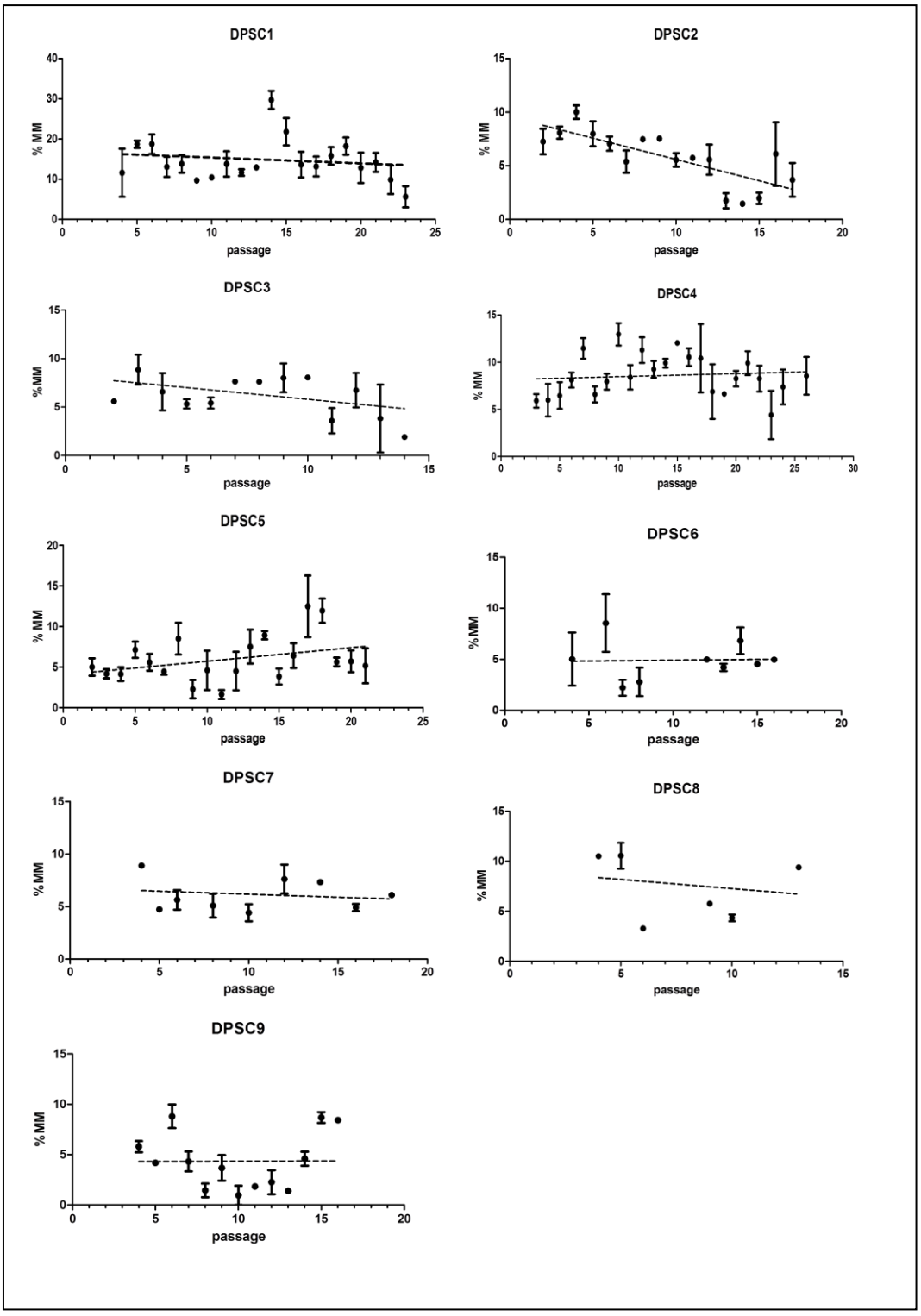


Figure 15 Percentage of Hypermethylation Alu (%mCmC).

The verical line indicates the variance of %mCmC. Dashed lines represents trendlines. DPSCs showed either the declination or escalation of %mCmC during DPSCs culture.

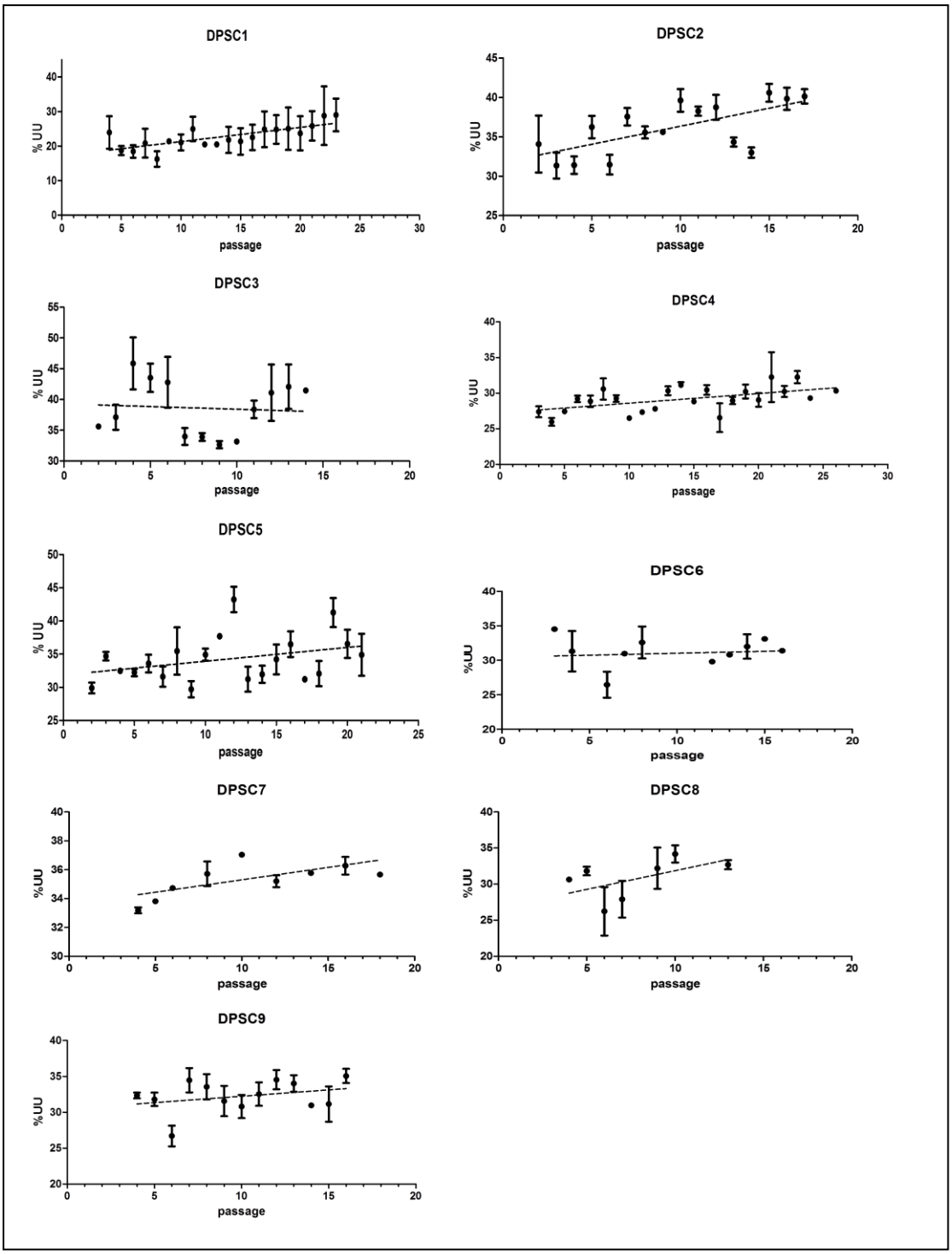


Figure 16 Percentage of Hypomethylation Alu (%uCuC).

The verical line indicates the variance of %uCuC. Dashed lines represents trendlines. Most samples of DPSCs showed the escalation of %uCuC during DPSCs culture

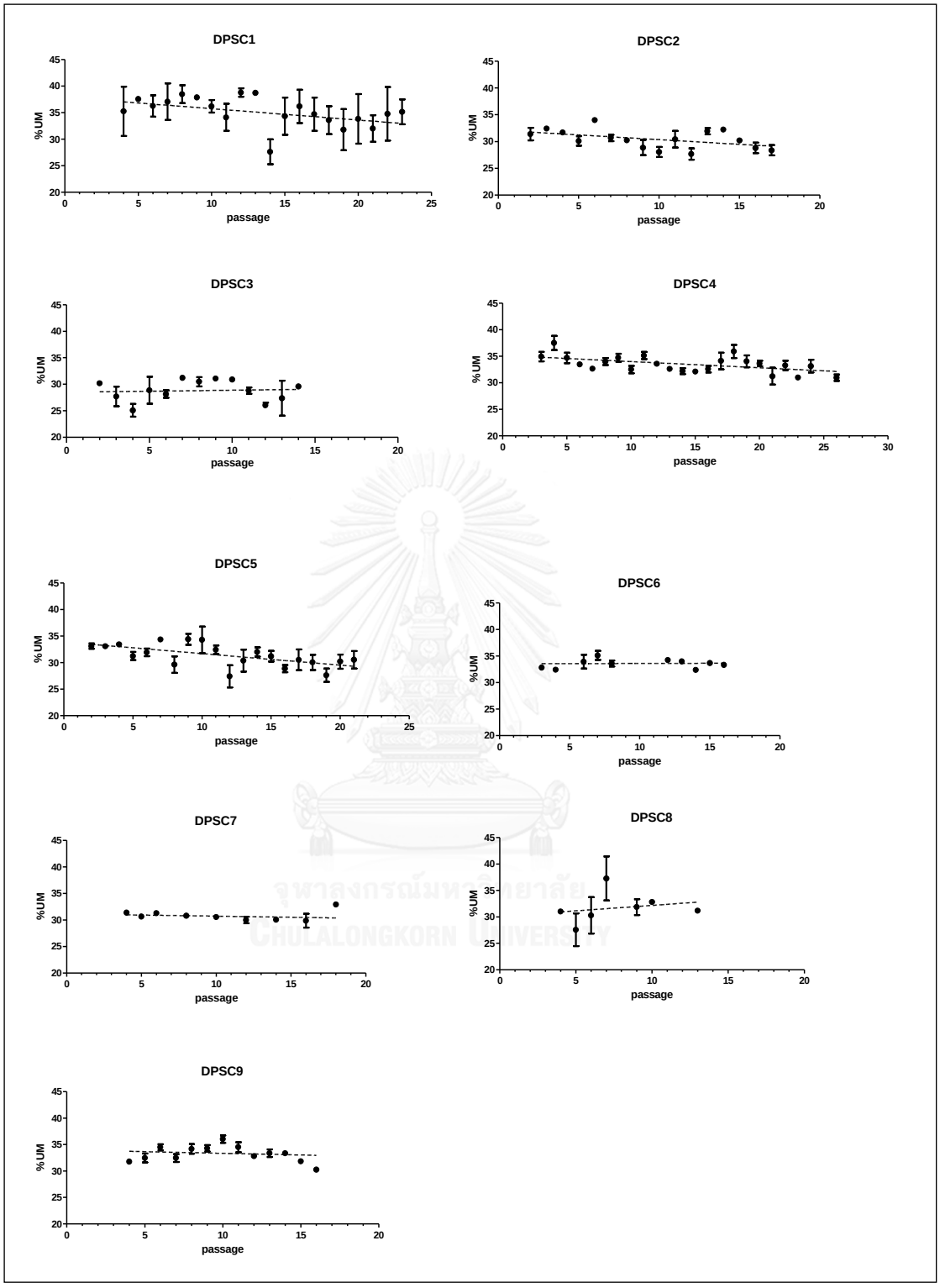


Figure 17 Percentage of Partial Methylated Alu (%uCmC).

The verical line indicates the variance of %uCmC. Dashed lines represents trendlines. DPSCs showed either the declination or escalation of %uCmC during DPSCs culture.

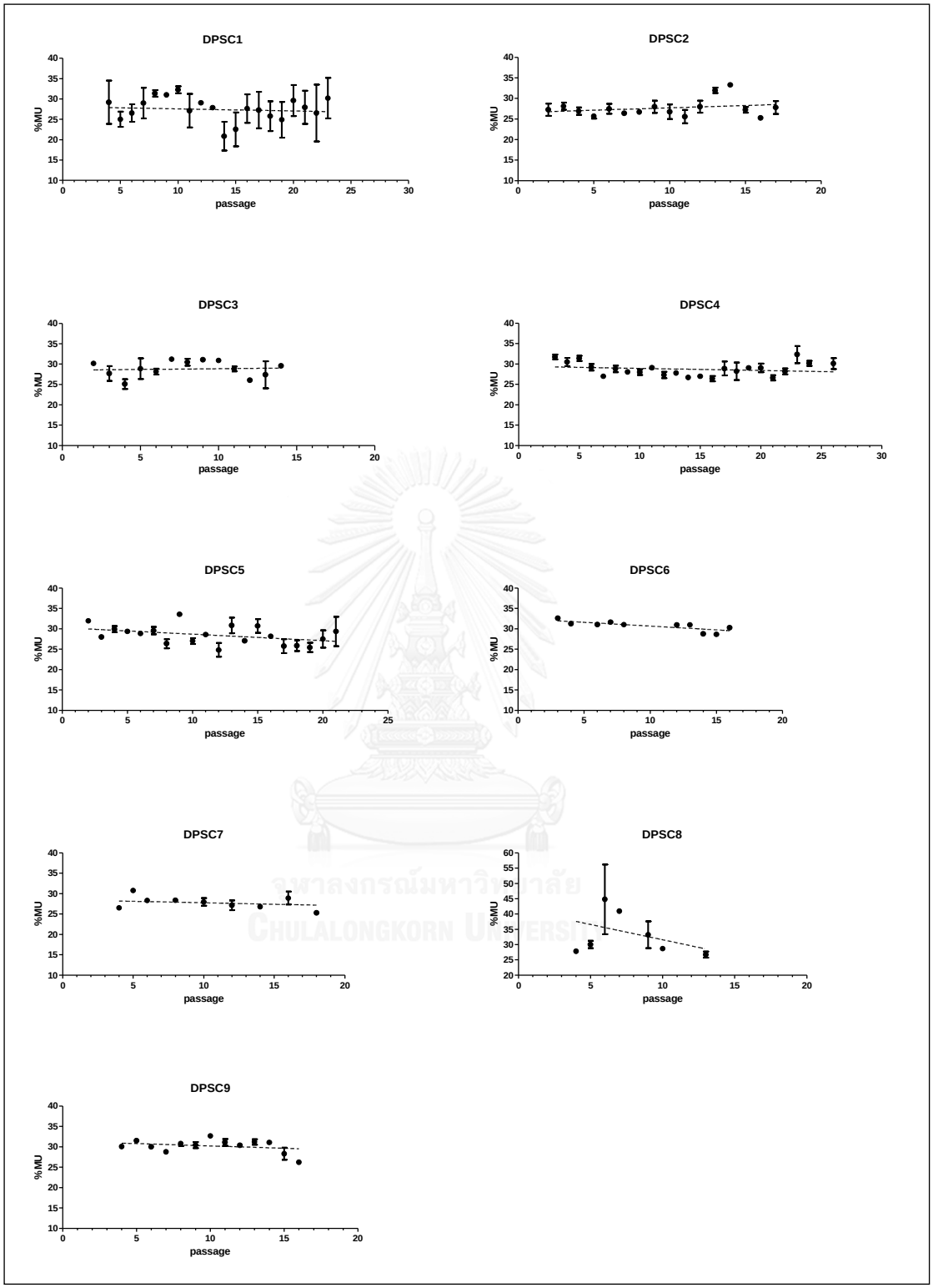


Figure 18 Percentage of Partial Methylated Alu (%mCuC).

The verical line indicates the variance of %mCuC. Dashed lines represents trendlines. Most samples of DPSCs showed the slight declination of %mCuC during DPSCs culture.

The mean of overall Alu methylation level in each passage of DPSCs were described in Table 3. A linear trendline was also created to see the trend of the average %mC in each passage during cell culture (Fig.17). Eventhough each sample of DPSCs showed the declination of %mC during DPSCs culture (Fig.12), the average of %mC showed the escalation of %mC (Fig.17). This resulted from the different level of %mC and the unequal passage number among samples.

Table 3 Mean of overall Alu methylation level (%mC) in each passage of DPSCs.

passage	% Alu methylation		passage	% Alu methylation	
	Mean	S.D.		Mean	S.D.
2	36.7787	1.03364	14	31.213820	1.3182128
3	36.9925	.72260	15	31.595687	2.3399897
4	38.3516	.94303	16	32.787958	1.7822080
5	39.1143	1.36098	17	30.691849	1.9201973
6	39.1086	1.25634	18	28.695845	1.7298963
7	38.6640	1.05333	19	32.181187	3.0558134
8	38.3771	1.20603	20	29.768135	2.4412537
9	37.7847	.67889	21	30.842524	2.3973397
10	38.0139	1.13413	22	29.752424	2.2656386
11	37.1134	1.53149	23	30.310265	2.7205845
12	33.835446	2.0713771	24	29.307444	.0388163
13	32.460061	1.3153781	26	30.342838	.0827342

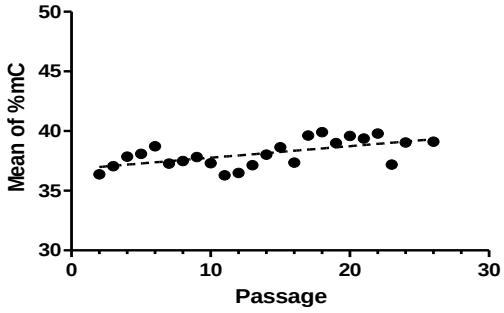


Figure 19 Mean of Overall Methylation Level (%mc).

The average overall methylation level in each passage of DPSCs showed the increasing trend line which resulted from the unequal passage number and unequal initiated methylation level of each sample.

Due to the late passages of DPSC subculture could not be reached equally in all samples, DPSCs were categorized in to three groups; early, middle, and late passage group for the proper performance of statistically analysis. The categorization of samples was shown in Table 4.

Table 4 Total passage numbers of each samples and the categorization.

Sample	Total passage no.	Category		
		Early Passage	Middle Passage	Late Passage
DPSC1	23	1-8	9-16	17-23
DPSC2	17	1-6	7-12	13-17
DPSC3	14	1-5	6-10	11-14
DPSC4	26	1-9	10-18	19-26
DPSC5	21	1-7	8-14	15-21
DPSC6	16	1-5	6-11	12-16
DPSC7	18	1-6	7-12	13-18
DPSC8	13	1-4	5-9	10-13
DPSC9	16	1-5	6-11	12-16

The overall Alu methylation level (%mC) of each group was demonstrated in table. The average of Alu methylation levels of DPSCs in early, middle and late passage were 38.9686 ± 4.72261, 38.2052 ± 5.13613 and 37.1838 ± 4.46660 respectively. (Table 5)

Each pattern of Alu methylation was also analysed. The average %mCmC of DPSCs in early, middle and late passage were 7.9868 ± 4.42643, 8.0140 ± 5.79163 and 7.2535 ± 4.79511 respectively. The average %uCuC of DPSCs in early, middle and late passage were 30.0496 ± 6.33365, 31.2287 ± 6.01295 and 32.8858 ± 6.11275 respectively. The average %uCmC of DPSCs in early, middle and late passage were 28.6396 ± 3.05794, 28.6568 ± 4.38776 and 28.3307± 3.75756 respectively. The average %mCuC of DPSCs in early, middle and late passage were 33.3240 ± 3.26219, 32.4754 ± 3.43170 and 31.5300 ± 3.21139 respectively. (Table 5)

Table 5 The average Alu methylation level of each DPSCs group.

	DPSCs		
	Early Passage	Middle Passage	Late Passage
%mC	38.9686 ± 4.72261	38.2052 ± 5.13613	37.1838 ± 4.46660
%mCmC	7.9868 ± 4.42643	8.0140 ± 5.79163	7.2535 ± 4.79511
%uCuC	30.0496 ± 6.33365	31.2287 ± 6.01295	32.8858 ± 6.11275
%uCmC	28.6396 ± 3.05794	28.6568 ± 4.38776	28.3307± 3.75756
%mCuC	33.3240 ± 3.26219	32.4754 ± 3.43170	31.5300 ± 3.21139

For statistical analysis we used one-way ANOVA to compare the Alu methylation levels of DPSCs among groups. There was a significant difference of Alu methylation level (%mC) among DPSCs groups (p=.030). DPSCs in late passage showed lower level of Alu methylation than DPSCs in middle passage with no statistical significance (p=.103). DPSCs in middle passage also showed lower level of Alu methylation than DPSCs in early passaged with no statistical significance (p=.233). However, DPSCs in late passage showed lower level of Alu methylation with statistical significance when compared with the early passage (p=.006). (Fig.20)

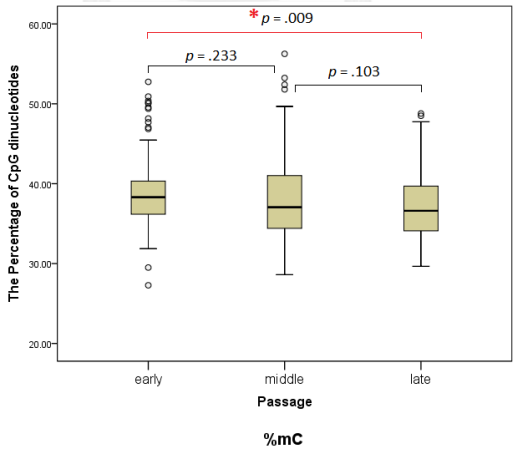


Figure 20 Comparison of Overall Alu Methylation Level (%mC) among Groups.

%mC represents the overall methylation level of Alu. The horizontal line within each box indicates mean of the percentage. Star indicates statistical significance at p< 0.05. The results demonstrated that DPSCs in late passage had a significantly lower level of Alu methylation than DPSCs in early passage.

One-way ANOVA was also used to compare all patterns of Alu methylation levels of DPSCs among passage groups. There was no statistically significant difference of %mCmC among groups (p=.325). DPSCs in late passage showed lower level of %mCmC than DPSCs in middle and early passage with no statistical significance (p=.144, p=.302). There was a significant difference of %uCuC among DPSCs groups (p=.004). DPSCs in late passage had statistically significant higher %uCuC than DPSCs in middle passage (p=.038) and DPSCs in early passage (p=.001). There was no statistically significant difference of %uCmC among groups (p=.778). DPSCs in late passage had lower %uCmC than DPSCs in middle and early passage with no statistical significance (p=.567, p=.514). Finally, There was a significant difference of %mCuC among DPSCs groups (p=.001). DPSCs in late passage had significant lower %mCuC than DPSCs in middle and early passage (p=.029, p<.001). (Fig.21)

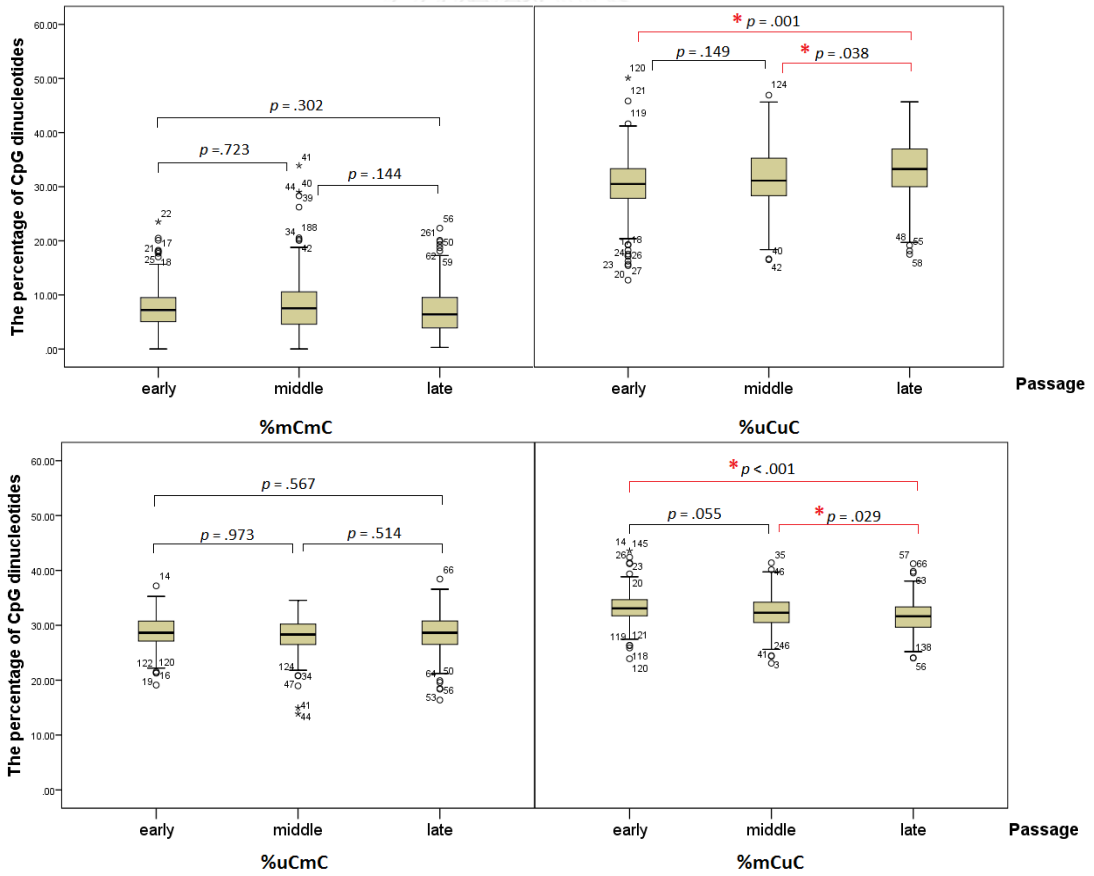


Figure 21 Comparison of each Alu Methylation Pattern Among Groups.

The horizontal line within each box indicates mean of the percentage. Stars indicate statistical significance at $p < 0.05$. The results demonstrated that DPSCs in late passage had a significantly higher level of uCuC than DPSCs in middle and early passage. DPSCs in late passage had a significantly lower level of mCuC than DPSCs in middle and early passage.

Morphological Observation and The Senescence-Associated β-Galactosidase Staining

After primary explant culture, DPSCs grew out from dental pulp tissue. Under light microscope, DPSCs showed a typical fusiform fibroblast-like morphology, single monolayer with a well-spread morphology attached to the culture plate. The senescence-associated (SA) β-Galactosidase staining assay was performed in one sample of DPSCs (DPSC1). The morphology and the result of β-Galactosidase staining were shown in figure 22, 23 and 24.

Figure 22 demonstrated the morphology and SA β-Galactosidase staining of DPSCs in early passage (passage 4-8 of DPSC1). All DPSCs in early passage showed a typical fibroblast-like morphology without expression of SA β-Galactosidase.

The morphology of DPSCs has slightly changed over time. DPSCs in the middle passage (passage 9-17 of DPSC 1) showed larger cell size, comparing to the early passage, and increased nuclear/cytoplasm ratio. However, DPSCs in the middle passage did no present the blue color of SA β-Galactosidase activity (Fig. 23).

DPSCs in late passage (passage 18-23 of DPSC 1) showed more alteration of morphology, enlarge cellular size, increased in cell debris, more cellular processes. They displayed the typical large and flat cellular morphology and expressed senescence-associated beta galactosidase (Figure 24).

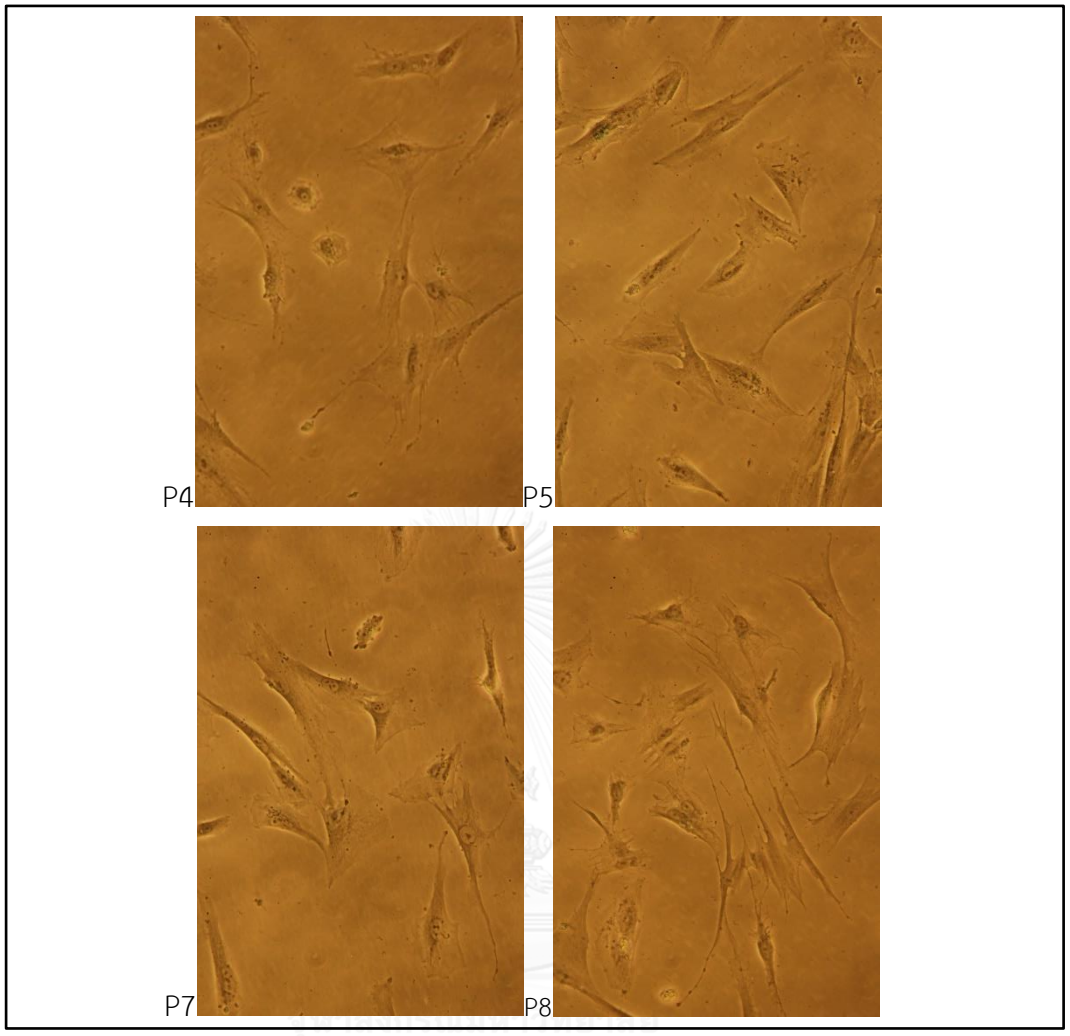


Figure 22 Illustration of ß-Galactosidase Staining on DPSCs in “Early Passage” Group. DPSCs in early passage showed a typical fibroblast-like morphology. Blue color which represents ß-Galactosidase activity was not present in these passages.

Figure 23 Illustration of ß-Galactosidase Staining on DPSCs in“Middle passage” Group. DPSCs in middle passage showed morphological changes comparing to DPSCs in early passage. Blue color which represents ß-Galactosidase activity was not present.

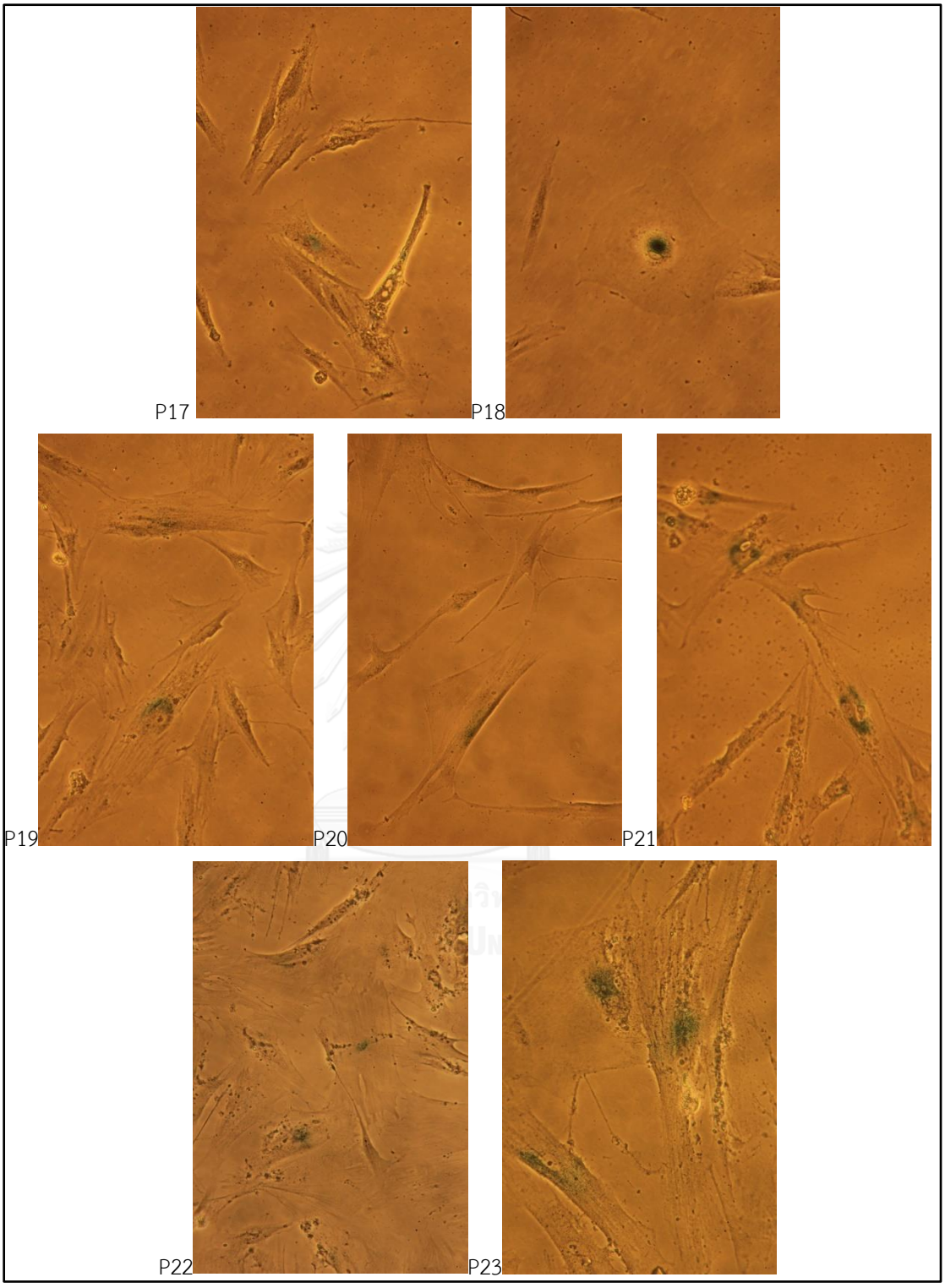


Figure 24 Illustration of ß-Galactosidase Staining on DPSCs in “Late Passage” Group. Blue color represents ß-Galactosidase activity which indicates cellular senescence. Every passage of DPSCs in late passage showed ß-Galactosidase activity.

CHAPTER V
DISCUSSION

The result from this study demonstrated that DPSCs in late passage had a significantly lower level of overall Alu methylation (%mC) than DPSCs in early passage. In addition, DPSCs in late passage had statistically significant higher %uCuC and lower %mCuC than DPSCs in middle and early passage. In other words, the lower level of Alu methylation in aged DPSCs arised from the increased %uCuC and decreased %mCuC.

We provided the DPSCs grown in an optimal condition. Our results demonstrated that under this in vitro condition, DPSCs were capable of long-term culture without changing their viability but losing their proliferation rate and changing in their morphology. After some passages, the morphology of DPSCs displayed some sign of replicative aging during in vitro expansion, such as, alteration in cell shape, enlarged cell size, increased cell secretion and nuclear/cytoplasm ratio. These findings are in agreement with observation of Liu et al. (30) who demonstrated the morphological alteration of DPCs at passage 7 in long-term cultivation of dental pulp cells. The declination of the reprogramming markers expression Oct-4, Sox2, and c-Myc in nucleus which were detectable in the early passages were also reported during the long-term in vitro cultivation.

DPSCs with increasing passage number were associated with rising expression of senescence-associated beta galactosidase, a biomarker for cellular senescence. This finding is in agreement with the study of Li et al. which reported the higher SA-ß-Gal staining in senescent dental pulp cells than in young cells (158). Furthermore, this morphological alteration and higher expression of SA-ß-Gal staining in senescent cells were also found in mesenchymal stem cells (159). Although DPSCs in late passage showed blue color in SA-ß-Gal staining in this study, not every DPSC presented ß-Gal activity. It reflects the heterogeneous population of DPSCs. These findings are in agreement with Alraies et al. who reported the proliferative and regenerative heterogeneity between DPSC populations. They found that different

DPSC population reached different population doubling before senescence. DPSCs with delay-onset senescence correlated with high proliferative capacities, possessed longer telomeres, exhibited prolonged stem cell marker expression, but lacked CD271. They also identified that proliferative and regenerative heterogeneity is related to contrasting telomere lengths and CD271 expression between DPSC populations (160).

Our study demonstrated that DPSCs lost their proliferation ability during expansion in vitro. This finding is in agreement with many evidences (30, 92, 161). Decreasing in regeneration potential in human DPSCs over time they replicate in vitro was found associated with progressive telomere shortening, the major genetic event linked to cellular aging. The relative telomere length of DPSC was inversely correlated with cumulative doubling time. DPSC lines were able to grow beyond Hayflick's limit, which the doubling time of cultured DPSCs increased when number of passages increased. The excessive ex vivo expansion of adult stem cells was also indicated to be reduced at minimum to avoid detrimental effects on telomere maintenance and measurement of telomere length should become a standard when certificating the status and replicative age of stem cells prior therapeutic applications (92, 161).

Our study also demonstrated that Alu methylation level of DPSCs in late passage was significant lower than DPSCs in early passage, and Alu methylation level decreased in the higher number of passages of DPSCs. However, there is no prior study about Alu methylation level in multiple passages of DPSCs. In the study of epigenetic regulation, global hypomethylation or the decreasing in genome-wide DNA methylation is an epigenetic change usually found in senescent cells (162). Jintaridth et al. (66) evaluated the methylation levels of peripheral blood mononuclear cells of interspersed repetitive sequences and found that human ageing was negatively correlated with methylation levels of Alu. According to our result which also indicated that aged DPSCs was negatively significant correlated with methylation levels of Alu, it implied the linkage between cellular aging and human aging as both of them are associated with the decreasing in Alu methylation level. In addition,

global hypomethylation and hypomethylation of Alu were associated with the increased genomic instability (163) which is one hallmark of aging.

The exact cause of aging is still unknown, however, aging mechanisms involve with the accumulation of DNA damage, mitochondria dysfunctions and telomere length shortening. The stem cell theory of aging was proposed to explain the aging mechanisms; the stem cells are important in the maintenance of tissue homeostasis by replacing effector cells in the organs, loss of their number and function leads to a breakdown of organ function.(67) The impairments of various tissue resident stem cells during aging process have found in many studies (110, 112, 113, 115).

The limitation of this study was an unequal number of passages among samples, so we had to categorize the DPSCs in to 3 groups to statistically calculate the difference of Alu methylation level. We reported the declination of Alu methylation level in the higher number of passages of DPSCs in this study; however, the passage number is not the exact cellular age. It simply refers to the number of times the cells in the culture have been subcultured. The consideration of inoculation densities and recoveries should be involved. The population doubling should be calculated. Some experiments cannot be performed in some passages due to the inadequacy of cells, and it lead to the incomplete of data set of each sample. In addition, even in the proper condition for DPSCs, not every explanted dental pulp provided a satisfactory primary culture which presented the optimum growth rate. The microbial contamination was also a hindrance of long-term cell cultivation.

The finding from this study reflects the association between changes of Alu methylation levels in cellular and human aging process. Besides, the better understanding of stem cell aging, the more we concern in aging mechanism of human. Further studies may open the new therapeutic approaches and drug development combining genetic and epigenetic strategies.

CHAPTER VI

CONCLUSION

In conclusion, Alu methylation level of dental pulp stem cells in late passage was statistically significant lower than early passage, as %uCuC significantly increased and %mCuC significantly decreased. Dental pulp stem cells underwent replicative senescence and lost their methylation level of Alu during cultured passage in vitro. It implied that replicative senescence of DPSCs was associated with hypomethylation of Alu. Other epigenomic regulations of DPSCs and aging process should be further investigated.

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