TECHNOLOGICAL APPROACHES FOR IMMORTALIZATION OF HUMAN DERMAL FIBROBLASTS THROUGH SIRT1 AND THE INVOLVEMENT OF MICRO-RNA

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การประยุกต์ใช้เทคนิคความเป็นอมตะของเซลล์ผิวหนังมนุษย์ผ่านกลไกของเซอทูอีนวันและศึกษาถึงความเกี่ยวเนื่องของไมโครอาร์เอ็นเอ

นางสาววิลาสินี พรมจันทึก

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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เซลล์ผิวหนังมนุษย์ทำหน้าที่สำคัญในการสร้างคอลลาเจน และอิลาสติน เพื่อใช้สำหรับการซ่อมแซมและรักษาบาดแผลในเนื้อเยื่อเกี่ยวพัน โดยเซลล์ผิวหนังมนุษย์ถูกนำมาใช้เป็นแบบจำลองในการศึกษาสรีรวิทยาของเซลล์ อีกทั้งยังถูกนำมาใช้ประโยชน์ทั้งในทางการแพทย์ และอุตสาหกรรมความงาม อย่างไรก็ตามเซลล์ผิวหนังมนุษย์มีข้อจำกัดในเรื่องของการเพิ่มจำนวนและการเข้าสู่ภาวะชราของเซลล์นำไปสู่การตายของเซลล์อย่างรวดเร็ว การเพิ่มปริมาณของเซลล์ผิวหนังมนุษย์ได้อย่างไม่จำกัดจึงมีความสำคัญต่อภาวะอมตะของเซลล์ หรือการหลีกหนีความชราของเซลล์ เทโลเมียร์ และเอนไซม์เทโลเมอเรส ซึ่งเป็นหนึ่งในแนวทางการเป็นอมตะของเซลล์ เนื่องจากเทโลเมียร์เป็นดีเอ็นเอที่อยู่บริเวณปลายสุดของโครโมโซม มีกลไกสำคัญในการควบคุมความชราของเซลล์ เปรียบเสมือนนาฬิกาชีวิตของเซลล์ นอกจากนี้ Simian virus 40 (SV40) large T antigen ในเซลล์ สามารถช่วยเพิ่มอัตราการรอดชีวิตของเซลล์ได้โดยการไปยับยั้ง pRB และ p53 ดังนั้น การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาภาวะอมตะของเซลล์ผิวหนังมนุษย์โดยการส่งถ่ายพลาสมิด hTERT, SV40 large T antigen และการทำงานร่วมกันของทั้งสองพลาสมิดเข้าสู่เซลล์เพื่อชักนำให้เกิดภาวะอมตะของเซลล์ และเพิ่มปริมาณการเพิ่มจำนวนเซลล์ จากการศึกษาแสดงให้เห็นว่า เซลล์ผิวหนังมนุษย์ที่ถูกชักนำให้เกิดภาวะอมตะ สามารถเพิ่มจำนวนเซลล์ต่อการเลี้ยงได้มากกว่า 60 ครั้งของการเลี้ยง และมีการทำงานของเทโลเมียร์เพิ่มขึ้น นอกจากนี้ยังไม่มีการแสดงออกของลักษณะการกลายเป็นเซลล์มะเร็งโดยการทดสอบโคโลนีฟอร์เมชั่น การศึกษาคุณสมบัติของเซลล์ผิวหนังมนุษย์ในเซลล์ผิวหนังมนุษย์ที่ถูกชักนำให้เกิดภาวะอมตะถูกตรวจสอบด้วยการแสดงออกของยีน ได้แก่ คอลลาเจน1ชนิด1 คอลลาเจน3ชนิด1 อีลาสติน ไวเมนติน และ p53 พบว่า มีการแสดงออกของยีนเพิ่มขึ้นจากเซลล์ปกติ สอดคล้องกับการแสดงออกของเซอทูอีนที่เพิ่มขึ้นเมื่อเทียบกับเซลล์ปกติ ผลที่ได้จากการแสดงออกของยีนถูกนำมาศึกษาต่อในระดับโปรตีนด้วยวิธีการย้อมสีโปรตีนด้วยแอนติบอดี้หรือที่เรียกว่าเทคนิคอิมมูโนฟลูออเรสเซนต์ การเพิ่มขึ้นของโปรตีนเซอทูอีนถูกตรวจสอบกลไกการทำงานด้วยการส่งถ่าย shRNA เพื่อลดการแสดงออกของเซอทูอีน เรสเวราทอลถูกใช้เพื่อกระตุ้นการแสดงออกของเซอทูอีน และเซอทีนอลซึ่งเป็นตัวยับยั้งการแสดงของเซอทูอีน จากผลการทดสอบพบว่า เซลล์ที่ถูกลดการแสดงออก

ของเซอทูอีน มีการแสดงออกของภาวะชรา โดยไซโทพลาซึมมีลักษณะแบนนิวเคลียสมีขนาดใหญ่ขึ้น และการแบ่งตัวของเซลล์ลดลงอย่างเห็นได้ชัด ดังนั้นกลไกการทำงานของเซอทูอินจึงถูกนำมาศึกษาต่อ มีรายงานการนำไมโครอาร์เอ็นเอมาศึกษาความเกี่ยวข้องในการยับยั้งการถอดรหัสของโปรตีน และการควบคุมกลไกต่างๆ ซึ่งอาจเกี่ยวข้องกับความชรา และภาวะอมตะของเซลล์ ดังนั้นเซลล์ผิวหนังมนุษย์ที่ถูกกระตุ้นให้เกิดภาวะอมตะจึงถูกทดสอบความเกี่ยวข้องกับไมโครอาร์เอ็นเอและเซอทูอีนด้วยเทคนิคเรียลไทม์พีซีอาร์ พบว่า มีไมโครอาร์เอ็นเอที่มีความเกี่ยวข้องกับเซอทูอีนและภาวะอมตะของเซลล์ ได้แก่ ไมโครอาร์เอ็นเอ 22 34เอ 93 217 และ449 นอกจากนี้ยังไม่มีการแสดงออกของไมโครอาร์เอ็นเอ 217 ในเซลล์ แตกต่างจากไมโครอาร์เอ 22 และ 449 ที่มีการแสดงออกสูงในเซลล์ที่ถูกส่งถ่ายด้วย SV40 large T antigen และไมโครอาร์เอ็นเอ 93 มีการแสดงออกสูงในเซลล์ที่ถูกถ่ายโอนโดยเทริตพลาสมิด เป็นที่น่าสนใจว่ามีการแสดงออกของไมโครอาร์เอ็นเอ 34เอ ลดลงในเซลล์ผิวหนังมนุษย์ที่กระตุ้นให้เกิดภาวะอมตะ ซึ่งการลดลงของไมโครอาร์เอ็นเอ 34เอ อาจจะส่งผลต่อการควบคุมการแบ่งตัวของเซลล์ที่เพิ่มมากขึ้น ถึงแม้ว่าผลลัพธ์จะสอดคล้องกันแต่กลไกที่แน่ชัดระหว่างไมโครอาร์เอ็น และเซลล์ผิวหนังมนุษย์ที่กระตุ้นให้เกิดภาวะอมตะที่ถูกส่งถ่ายด้วยพลาสมิดทั้งสองชนิดยังคงต้องได้รับการศึกษาเพิ่มเติมเพื่อการพัฒนาสู่แบบจำลองเซลล์ผิวหนังสามมิติที่สมบูรณ์แบบ

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา Wilasinee P.

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WILASINEE PROMJANTUEK: TECHNOLOGICAL APPROACHES FOR IMMORTALIZATION OF HUMAN DERMAL FIBROBLASTS THROUGH SIRT1 AND THE INVOLVEMENT OF MICRO-RNA. THESIS ADVISOR : ASST. PROF. PARINYA NOISA, Ph.D., 110 PP.

HUMAN DERMAL FIBROBLAST/IMMORTALIZATION/SIRT1/MIRNA

Human dermal fibroblasts are some of the skin cells that act as a collagen and elastin production source in connective tissue for wound healing. Fibroblasts can be used as a model in the study of skin physiology. The advantages of fibroblasts have been exploited continuously in cosmetic and medical therapeutics. However, fibroblasts have limitations of cell proliferation and entering cellular senescence and leads to cell death. Immortalization or escape senescence can help to increase unlimited cell proliferation. The telomere length and telomerase enzyme are the components maintaining the integrative structure at the end of chromosome. Telomere is an important mechanism that controls the aging of cells or also known as a biological clock, in which telomere and telomerase can increase cell proliferation. In addition, the Simian virus 40 large T antigen can improve cell viability by suppressing *pRB* and *p53*. Therefore, the primary aim of these studies was to discover the mechanism of immortalized fibroblasts. Plasmids were transfected into fibroblasts to induce immortalization and increase cell proliferation by overexpression of plasmid hTERT, SV40 large T antigen and co-transfection. The results showed that immortalized cells can be sub-cultured over 60 passages and increase telomere activity and behave as non-tumor cells by colony formation. Further, immortalized cells investigated the

efficiency of cells by the fibroblasts markers such as *COL1A1*, *COL3A1*, *ELASTIN* and *VIMENTIN*. In addition, the *p53* expression was increased in the immortalized cells in accordance with *SIRT1*. The relationship of these genes was demonstrated in the protein levels by immunofluorescence technique. The role of increasing the SIRT1 level was investigated via shRNA-knockdown transfection then treated the activator SIRT1 or resveratrol and the sirtinol inhibitor SIRT1. These results suggested that knockdown SIRT cells showed the characteristics of cellular senescence such as the flattened of cytoplasm, large nucleus and distinct decreased passage. Therefore, the SIRT1 mechanism should be further studied. In the present, several roles of miRNA have been reported in protein translated inhibition which are relevant in the control of ageing and immortalization. Immortalized cells were found to involve in miRNA-SIRT1 by the prediction and analysis with qPCR including miR-22, miR-34a, miR-93, miR-217 and miR-449a. The results showed that miR-217 was not involved in the immortalized cells. Nevertheless, miR-22 and miR-449a were highly up-regulated in SV40 large T antigen-transfected cells. MiR-93 was extremely expressed in hTERT-transfected cells. Interestingly, miR-34a was down-regulated in the immortalized cells, in which the suppression of miR-34a could increase cell proliferation. This connection between SIRT1 and miRNAs will need further exploration. The successful immortalized fibroblasts could be further developed for cosmeceutical screening, such as the development of 3D-skin models.

School of Biotechnology Student's Signature Wlasinee P.

Academic Year 2017 Advisor's Signature

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Wilasinee Promjantuek

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LIST OF ABBREVIATIONS

%	=	Percent
°C	=	Degree celsius
µg	=	Microgrum
µm	=	Micromate
µM	=	Micromolar
3D-skin	=	Three dimensional skin
3'UTR	=	3'untranslated regions
5'UTR	=	5' untranslated regions
ADP	=	Adenosine diphosphate
AMPK	=	5' adenosine monophosphate-activated protein kinase
ART	=	Mono-ADP-ribosyl transferase
aTL	=	Absolute telomere length
ATP	=	Adenosine triphosphate
BRCA	=	Breast cancer susceptibility gene
BrdU	=	5-bromodeoxyridine
CDK	=	Cyclin-dependent kinase
Chr	=	Chromosome
CNS	=	Central nervous system
CO_2	=	Carbon dioxide
COL1A1	=	Collagen 1 type I

LIST OF ABBREVIATIONS (Continued)

COL3A1	=	Collagen 3 type I
COUPTF	=	Chicken ovalbumin upstream promoter transcription factor
CoTF	=	Co-transfected cells
Ctrl	=	Control
DAC	=	NAD-dependent deacetylases
DAPI	=	4′,6-diamidino-2-phenylindole
DGCR8	=	Digeorge critical region 8
DMEM	=	Dulbecco's Modified Eagle Medium
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
EC	=	Esophageal cancer
ECM	=	Extracellular metrix
EVs	=	Extracellular vesicles
FOXO	=	Forkhead box factors regulated
g	=	Gram
GAGs	=	Glycosaminoglycans
GAPDH	=	Glyceraldehyde 3-phosphate dehydrogenase
gDNA	=	Guide DNA
GMP	=	Good manufacturing process
h	=	Hours
HA	=	Hyaluronan
HDACs	=	Mammalian histone deacetylases

LIST OF ABBREVIATIONS (Continued)

HDFs	=	Human dermal fibroblasts
HES1	=	Hairy and enhancer of split 1
HEY2	=	Hairy/enhancer-of-split related with YRPW motif 2
HMECs	=	Human mammary epithelial cells
HPV	=	Human papilloma virus
hTERT	=	Human telomerase reverse transcriptase
H-T-H-T	=	Helix-turn-helix-turn
IF	=	Intermediate filament
Kb	=	Kilobase
KRT18	=	Keratin 18
LB	=	Lysogeny broth
M0	=	Mortality stage 0
M1	=	Mortality stage
M2	=	Mortality stage 2
MDM2	=	Mouse double minute 2 homolog
MEF	=	Mouse embryo fibroblast
min	=	Minute
miRNA	=	MicroRNA
ml	=	Milliliter
MMPs	=	Matrix-metalloproteinases
mRNA	=	messengerRNA
MTT	=	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

LIST OF ABBREVIATIONS (Continued)

NAD	=	Nicotinamide adenine dinucleotide
NFĶB	=	Nuclear factor kappa B
nm	=	Nanometer
OIS	=	Oncogene-induced senescence
PBS	=	Phosphate buffered saline
PcG	=	Polycomb-group protein
PCR	=	Polymerase chain reaction
PGC1-α	=	peroxisome proliferator-activated receptor c co-activator 1- α
PPARγ	=	Peroxisome proliferator-activated receptor gamma
pRB	=	Retinoblastoma protein
qPCR	=	Quantitative realtime polymerase chain reaction
rDNA	=	Recombinant DNA
RISC	=	RNA-induced silencing complex
RNA	=	Ribonucleic acid
rpm	=	Revolutions per minute
RT	=	Room temperature
sec	=	Second
shRNA	=	Short-hairpin loop RNA
Sir2	=	Silent information regulator 2 gene
SIRT	=	Sirtuin
SV40	=	Simian virus 40
T2DM	=	Type2 diabetes

LIST OF ABBREVIATIONS (Continued)

TDNA	=	Transfer DNA
TRF	=	Terminal restriction fragment
U	=	Unit
UBF	=	Upstream binding factor
UV	=	Ultraviolet
v/v	=	Volume by volume

CHAPTER I

INTRODUCTION

1.1 Significance of the research

The expansion of somatic cells *in vitro* for research and medical therapy applications is limited by their scanty proliferative lifespans. Cellular senescence is a state of irreversible arrest of cell proliferation, exhibiting enlarge morphological changes and senescence-associated heterochromatin foci formation. Senescence, originating from the Latin word 'senax', refers to a physiological program towards permanent cell cycle arrest (Wang *et al*., 2010). Cellular senescence can be stimulated via a variety of factors such as tumor suppressor protein, activation of oncogene, telomere shortening, miRNA, cell cycle, genomic DNA damage and activation of p53/p21 pathways (Petkov *et al.*, 2018). Oncogene-induced senescence is a cellular response, found in premalignant tumors. Senescent cells exert a pleotropic effect on development, tissue aging and regeneration, inflammation, wound healing and tumor suppression (Audrey and Oliver, 2018). Currently, the cosmetic research are developed to fix skin problems such as aging, skin inflammation, acne and skin disorders. In cosmetics, human skin cells are used as role model for demonstrating the therapeutic potential. The use of *in vitro* tests are the first step to development the research of new products and treatment of skin disorders. Human skin cell studies have increased the hope for a successful cell therapy in aesthetic medicine and cosmetics. Various types

of skin cells are responsible for synthesizing and organizing the skin with three layers including (i) epidermis containing keratinocytes, melanocytes, and Langerhans cells; (ii) dermis that is populated with fibroblasts, vessels and dendritic cells; and (iii) subcutaneous tissue in the deepest layers (Luger *et al.*, 1981). Human dermal fibroblasts have the unique of the first human somatic cells to be induced into a pluripotent stem cells. HDFs contain several distinct layers, including the dermal papilla cells, the base of the hair follicle, the capillary blood, elastin and collagen fibers. These cells are used to study wound-healing and aging skin phenomena which play a vital role in maintaining skin function. However, a major bottleneck of using dermal fibroblast cells in the therapeutic application is their limited proliferative capacity *ex vivo* and tendency to undergo senescence. In some cases, the cells may overcome this replication block due to defective or virus-suppressed p53 and pRb function. However, the continued proliferation results in further shortening of telomeres, extensive chromosome damage, and genomic crisis leading to widespread apoptosis (Petkov *et al.*, 2018).

Cellular immortalization is a technique to solve the limits of cellular senescence. Several pathways are implicated in regulating spontaneous immortalization and senescence, including loss of tumor suppressors, in particular p53, which is necessary, but not sufficient to induce immortalization (Fridman and Tainsky, 2008). The telomere shortenning has a definitive role in the loss of chromosomal integrity during culture expansion (Hanzelmann *et al.*, 2015). Telomeres are proteins or DNA complexes at the end cap of chromosome and the maintenance of their length is essential for genomic stability and cell viability (Sandin and Rhodes, 2014). Human telomerase reverse transcriptase (hTERT) are used to create immortalized cells by

maintaining telomere length. The catalytic protein subunits telomerase plays role in the control of mammalian cell lifespan. Telomerase activation is highly increased in most human cancer but reduced in most human somatic cells. Therefore, the role of telomerase activation in human cancer, and the possibility of using anti-telomerase drugs as anticancer drugs, remain to be studied (Meyerson, 1998). On the other hands, the immortalization techniques have many regulator as well as DNA tumor virus such as simian virus 40 (SV40) large T antigen. SV40 large T antigen virus is directly suppress tumor retinoblastoma protein (pRB) and activate cell cycle arrest of host cells. The generation of immortal cell lines, by using SV40 large T antigen has provide vital tools to examine the mechanism of immortalization (Hubbard and Ozer, 1999). SV40 large T antigen has many effects on cellular gene expression, including induction of cellular DNA synthesis such as quiescent and senescent cells. The extension of life span is attributed largely to large T antigen forming complexes with pRB and p53 (Hubbard and Ozer, 1999). As known, cell proliferation and cellular senescence are regulate by sirtuin activation that promote cell viability. SIRT1 in mammalian cells are studies about aging and age-related diseases, it's direct in another for a variety of proteins, including p53, p21 and p16. However, the mechanism of immortalized cells by ectopic expression in human dermal fibroblasts is not well understood.

The aim of this studies to establish the immortalized cells of human dermal fibroblasts. Increasing of proliferation and maintaining telomere length are interested for studies the role of immortalized cells. Consequently, the immortalized cells were investigated the characterized and discover the main mechanism. SIRT1 expression are recovered in this studies, gene and protein expression were demonstrated by qPCR, RT-PCR and immunofluorescence. In the present, ectopic overexpression are remain

impose culture in the long times. To improve the cell efficiency for stable expression of increasing proliferates and safety for used to therapeutic applications. Additional, miRNA is remarkable for used to develop the immortalization technique. Hence, to successful spontaneous immortalized cells, miRNAs were studies through SIRT1 and cellular senescence. The spontaneous immortalized fibroblasts can used to *in vitro* model for skin therapy, therapeutic applications and cosmeceuticals.

1.2 Research objectives

1.2.1 To establish and characterize the immortalized HDFs by using hTERT and SV40 large T antigen.

1.2.2 To indicate the function of SIRT1 in the immortalized HDFs.

1.2.3 To studies the involvement of miRNA involving in the immortalized HDFs with SIRT1 activation.

1.3 Research hypothesis

Human dermal fibroblast cell lines can found in middle layer of skin cells and are generally used for wound-healing assays, a tool for developing and determining the potential of clinical applications. In cosmeceutical, human skin cells are used as a role model for demonstrating the efficiency of skincare products and toxicity testing. The lifespan limitations of cells are problem for development of *in vitro* test. Cellular immortalization is a technique to solve the limits of cellular senescence. The key role of cell immortalization is increase cell proliferation through the activation of hTERT and SV40 large T antigen and maintain the telomere length. Overexpression of hTERT and SV40 large T antigen are used to immortalize and behave as non-tumor cells but

the mechanism not clearly understood. The role of immortalized cells have several targets such as SIRT1, tumor suppressor protein and miRNA. Other, overexpression has the limit about transient expression of doubling times. Hence, the aim of this study are characterize the cell properties and investigate the role associations in immortalized cells. In the present, the discovery of cell immortalization still popular to use in 3D-skin of cosmetic and therapeutic application.

1.4 Scope of thesis

In the primary purpose this study is to improve the lifespan of human dermal fibroblasts (HDFs) via hTERT and SV40 large T antigen plasmid. The ectopic overexpression is involve through SIRT1-miRNA activation and maintain telomere length. Thereafter, these cells were demonstrated the characteristics of the immortalized cells. The expression of the fibroblast markers such as *COL1A1*, *COL3A1*, *vimentin*, and *elastin* were demonstrated to characterize the attribute of fibroblasts. For, the gene containing *p53*, *p21*, *KRT18* and *SIRT1* mechanism were discovered role that involve in immortalized cells and confirmed by protein expression. The SIRT1 role was investigated by using shRNA to knockdown SIRT1 activity. Besides, the activator-inhibitor of SIRT1 were used to confirm the SIRT1 suppression. miRNAs were reviewed and analyzed the implicate role of SIRT1 in immortalized cells.

1.5 Expected results

The overexpression by plasmid containing hTERT, SV40 large T antigen and co-transfection plasmid could maintain telomere length and increase cell proliferations of human dermal fibroblasts. The successful immortalize behave as non-tumor cells.

The mechanism of cellular immortalization are revealed and found to involve SIRT1 pathways. SIRT1 are interested role because it is control several pathway and all cell proliferation. These expression was confirmed by shRNAs. Other, miRNAs are reviewed associate with SIRT1 and cellular senescence. The miRNA challenger can regulate the mechanism in the immortalized cells.

CHAPTER II

LITERATURE REVIEWS

2.1 Human dermal fibroblasts

Nowadays, cosmetic researchers are responsible produce the new skin care product. The use of *in vitro* test is used as a role model for demonstrating the efficiency of product development. Human skin cells are studies to cues and develop the aesthetic medicine, cosmetic, cell therapy in patients such as wound care in burn patient, transplant onto a chronic granulation and acne scar. The aesthetic medicines is a one of medicine in scientific research, aim to cosmetically enhance an individual's physical appearance. The three dimensional (3D)-skin assay is first step for develop the aesthetic medicine in laboratory to improve the *in vitro* prediction for applied chemicals testing (Figure 2.1).

Figure 2.1 The 3D skin model.

2.1.1 The structure of the skin

Life of terrestrial animal on earth requires the barrier to protect living cells at the direct interface between an organism and its environment such as air, mechanical stress and desiccation. The human skin is the body, its largest organ, covering 1.6 m^2 of surface area and accounting for approximately 16% of an adult's body weight. The response of skin has effect whole body, it is important part of outer for contact signal in body. The deeper layer of skin compose of numerous sensory to communicate with the skin. Human skin cells are responsible for producing and organizing the skin with three layers including epidermis layer, dermis layer and subcutaneous tissue as shown in Figure 2.2.

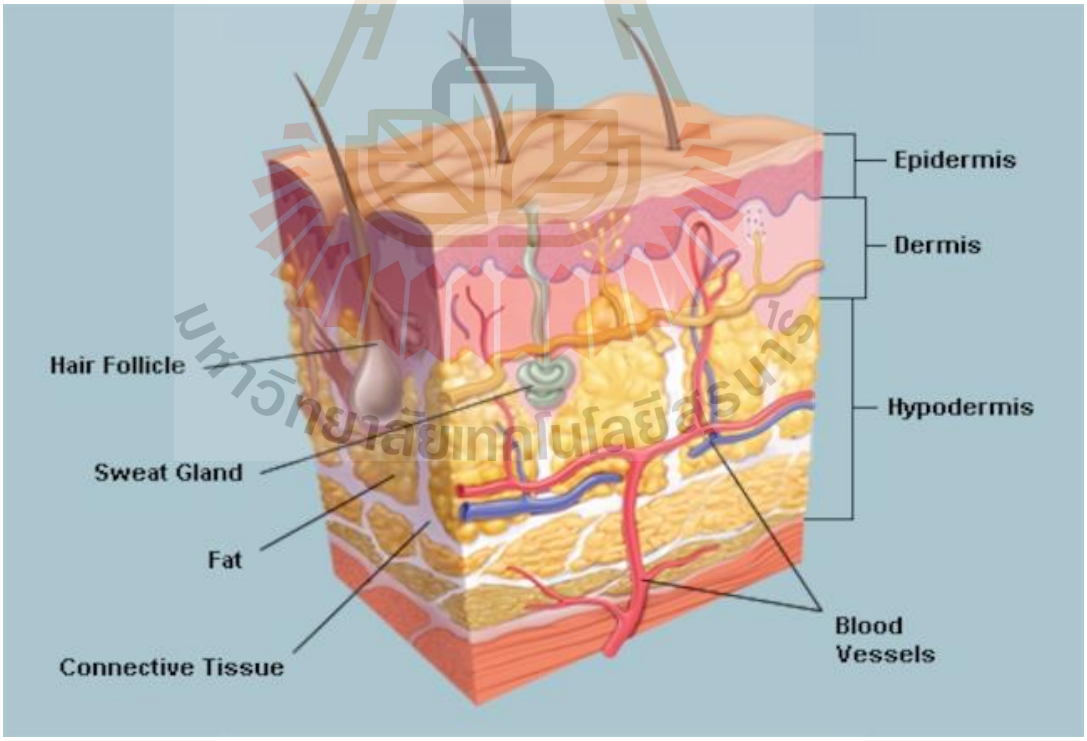


Figure 2.2 Layers of skin (Hoffman, 2014).

2.1.1.1 Epidermis layer

The outermost layer of skin is epidermis act as provide a waterproof barrier and creates our skin. The epidermis is composed of keratinized, stratified squamous epithelium. The epidermis has the miraculous ability to regenerate unlike many other cell types. These cells can suffer from a wound, healthy skin heal and replaces damaged cells with ease. Within the epidermis compose four to five layers in the epithelial cell depending on its location of cell (Figure 2.3).

Stratum lucidum this layer can be found on the palms of the hands and soles of the feet. The stratum lucidum is a smooth, seemingly translucent layer of the epidermis. The keratinocyte on the stratum lucidum are dead and flattened. The relation between keratinocyte and eleiden protein are effects these cells their transparent appearance and delivers a barrier to water.

Stratum corneum the stratum corneum is the outermost layer of the epidermis and a strongest 30 layers of corneocytes. These cells have shown the morphological flat and less nucleus but accumulate of keratin filaments. The increase keratinization or called cornification which comprised three fundamentals (i) the replacement of intracellular organelle and intracellular content by a compact proteinaceous cytoskeleton (ii) the cross-linking of protein at the cell periphery to form a cornified cells envelop and (iii) the linkage of corneocyte into a multicellular, functional but biologically dead structure (Eckhart *et al.*, 2013). Cells in this layer are shed periodically and are replaced by cells pushed up from the stratum granulosum.

Stratum granulosum due to the stratum granulosum close up the layer of stratum spinosum, keratinocytes is changed from these layer. The cells shown flatter, their cell membranes thicken, and produce large amount of the protein keratin,

which is fibrous and keratohyalin. Keratohyalin is accumulated as lamellar granules within the cells (Figure 2.3). Both keratin and keratohyalin make up the amount of keratinocyte mass in the stratum granulosum. The nuclei and other cell organelles disintegrate as the cells die, leaving behind the keratin, keratohyalin, and cell membranes that will form the stratum lucidum, the stratum corneum, and the accessory structures of hair and nails.

Stratum spinosum the stratum spinosum is composed of eight to ten layers of keratinocytes, formed as a result of cell division in the stratum basale. The keratinocyte of this layer has Langerhans cell, which act as a macrophage by engulfing bacteria, foreign particles and damaged cells. These cells begin the synthesis of keratin and release a water-repelling glycolipid that help desiccations from the body and making its waterproof of skin. The stratum spinosum is spiny in appearance that protruding cell with desmosome that is the structure interlocks with each other and strengthen the link between the cells.

Stratum basale this layer is called the stratum germinativum or also known as the basal cell layer. The stratum basale is a deepest of epidermal layer which immediate to dermis layer. Keratinocytes are produce from this state. Other, merkel cell and melanocyte can be found in the stratum basale. Merkel cell act as a receptor and are responsible for stimulating sensory nerves that the brain perceives as touch. These cells are populated on the surface of hands and feet. Melanocyte is produced melanin pigments, which help skin and hairs show its color. This outermost layer is subject to both genetics and external forces that contribute to the aging of this skin. These factors include smoking, alcohol, and excessive UV exposure, all of which contribute to the development of wrinkles and the uneven thickening or thinning of the skin. The next is

second layer of skin is the dermis which populated with fibroblast.

The functions of epidermis are very important for human bodies. Epidermis can resistance trauma and infection from bacterial and fungi which can penetrate the intact skin. The acid mantle of skin can help defense microbial by it is relative dry ness and acidic (pH4-6). Other, the skin necessary in the synthesis of vitamin D, which important for bone development and maintenance. In the skin contain most extensive sense organs to response biological stimulators such as heat, cold, touch, texture, pressure and vibration. These sensory receptors can be found on the face, palm, fingers, soles, nipple and genitals. The important of the skin including thermoregulation or body regulation such as response to overheats. However, the skin are associate with social acceptance of human or called nonverbal communication.

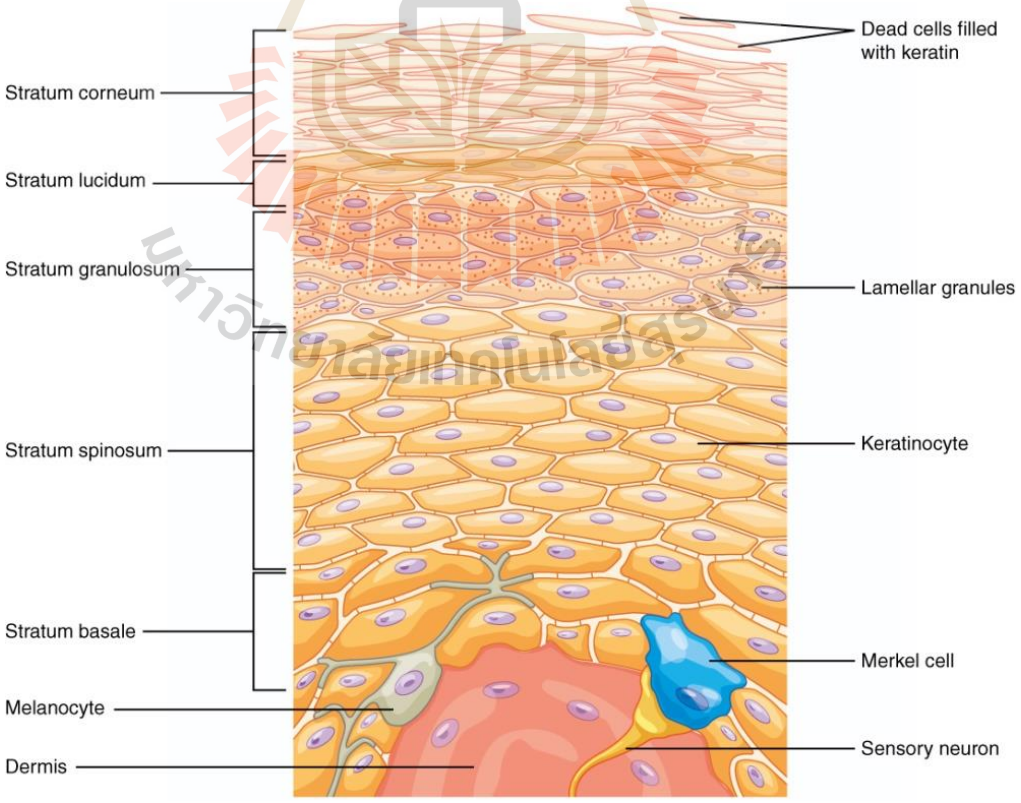


Figure 2.3 Anatomy of the epidermis.

(https://opentextbc.ca/anatomyandphysiology/chapter/5-1-layers-of-the-skin/)

2.1.1.2 Dermis layer

The dermis layer made of dense, irregular connective tissue that houses blood vessels, hair follicles, sweat glands, and other structures. The dermis is comprised of two layers: the papillary dermis and the reticular dermis. The papillary dermis is the more superficial of the two, and lies just beneath the epidermal junction. It is relatively thin and is made up of loose connective tissue containing collagen, reticular fiber, elastic fiber and capillary. The loose connective tissues are responsible strong structure of fibroblast. The extracellular component of the skin including fiber-forming structural molecule, nonfiber-forming structural molecule and matrix cellular proteins. Fiber-forming molecules provide a structure to the extra cellular matrix (ECM) by creating a complex three-dimensional framework of rigid proteins. The nonfiber-forming molecules, mostly proteoglycans and glycosaminoglycans (GAGs), function to create a charged, dynamic, and osmotically active space (Tracy *et al.*, 2014). Collagen is the fiber-forming structural molecule can be found in fat 77% of the fat-free dry weight of the skin. Collagen is the tensile strength which to be the single most abundant protein. Other fiber-forming protein in the skin comprises fibrin, fibronectin, vitronectin, elastin and fibrillin. Proteoglycan in the skin include hyaluronan (HA), decorin, versican, and dermatopontin which are the negatively charged and hydrophilic nature. ECMs component can be found in normal skin and repopulate wounded skin after predictable intervals. The reticular dermis is the deeper and thicker layer of the dermis, which lies above the subcutaneous layer of the skin. It contains dense connective tissue which includes elastic fiber, collagen fiber, fibroblasts, blood vessels, mast cells, lymphatic and nerve ending. Fibroblasts are mesenchymal cells with many vital functions during development and in adult organisms. Human dermal fibroblasts

are responsible for producing the extracellular matrix forming the dense connective tissue of the skin and play major roles in wound healing.

2.1.1.3 Subcutaneous tissue

Subcutaneous tissue or also known as hypodermis is the layer under dermis of skin. It's the deepest layer that rests just above the deep fascism. These tissues are composed of fat and various other types of cells. Subcutaneous fat made up of adipocyte which can form lobules that are separated by connective tissue. The number of adipocyte in the subcutaneous tissue with the area of the body depends on the nutrition of the person.

2.1.2 Fibroblast cultures

Meanwhile, fibroblast cultures isolated from some of these fibrotic conditions have been extensively used to examine the abnormalities of ECM components such as types I and III collagen. These cells are established from skin biopsy keloid and hypertrophic scar tissues. These cells can be obtained from viable skin of debrided burn tissue. The production of dermal fibroblasts for therapy begins with a trained professional obtaining a skin biopsy. The biopsy is then sent to a good manufacturing process (GMP) cell culture processing unit for dermal fibroblasts isolation by enzymatic methods, culture, expansion and purification. Advancements in cell culture techniques to expand the cells without losing the unique cellular identity and regenerative capacity of the cells, is important for successful therapeutic application (Thangapazham *et al.*, 2014). These include advancements in isolating and culturing contamination free homogenous fibroblast populations, technology to rapidly grow optimal number of cells with desired potency, optimal harvest site selection based on desired therapeutic indication, and the storage and transport of the fibroblasts to the

clinical site for application. An important advantage of fibroblasts is that they do not carry mutations in oncogenes and tumor suppressors and maintain intact cell cycle checkpoints. Toxicology studies of fibroblast after are injected in mice no report of tumor induction and oncogenic transformation. In addition to this safety profile, there are several animal models in pig and rodent for preclinical evaluation of dermal fibroblasts in the developmental pipeline to bring the dermal fibroblast products to market (Thangapazham *et al.*, 2014). This makes normal fibroblasts a preferred system for the studies of cell cycle regulation, DNA repair, and apoptosis. Cancer cells achieve these phenotypes in large part by reactivating and modifying many existing cellular programs normally used during development. These programs control coordinated processes such as cell proliferation, migration, polarity, apoptosis, and differentiation during embryogenesis and tissue homeostasis. Cell culture technology has become a widely used method in biology, medical research and applications. Establishing primary cultures of fibroblasts allows researchers to obtain representative cells that have conserved most of their original characteristics and functions, which is an important foundation for further cell biology and cell engineering (Siengdee *et al.*, 2017). The word ‘‘fibroblast’’ encompasses any stromal cell that does not express markers for a more specific mesenchymal lineage. This imprecise definition, and our impaired ability to track cellular changes due to a lack of distinguishing cell markers, lends to the misconception of the fibroblast as a homogenous and static cell (Tracy *et al.*, 2014).

2.1.3 The application of fibroblasts

The development of new therapies designed for skin rejuvenation take advantage to understand the ability of fibroblast reprogramming the skin for the purpose of skin rejuvenation in aging skin. Fibroblasts play a vital role in virtually all skin

process such as skin morphogenesis, embryo development and physiological conditions. The realization that dermal fibroblasts have regenerative potential in skin repair and rejuvenation has led to the development of cell therapies for a variety of skin indications. These cells are defined the morphology the spindle-shaped on tissue culture plastics (Figure 2.4). In skin dermis, for example, different dermal layers host diverse subpopulations of fibroblasts with both unique morphologies and physiologic functions. Depending on their resident depth of dermis, fibroblasts express different quantities of collagen and ratios of collagen type I and III mRNA. Fibroblasts involve wound healing by co-functions keratinocytes, immune cells and endothelial cells which promotes the signaling molecule at injury sites. The signaling molecule are secreted by the platelets activate inflammatory leukocytes. This activation of platelets is providing a chemotactic gradient which allows for the leukocytes, fibroblasts and endothelial cells to enhance the healing response. The development of chronic wound depend on the factors including a self-perpetuating inflammatory stage, cellular senescence, bacterial infection, a reduction in oxygenation and a lack of nutrition. Chronic wounds are wounds in which the normal sequence of healing is disrupted which leads to the prolongation of the healing process despite the appropriate therapy. Most fibroblasts present in chronic wounds prove to be prematurely senescent; moreover, they exhibit both abnormal morphologies and decreased migratory and proliferative capacities. Decreased proliferation is caused by the up-regulation of the p38-MAPK pathway. The p38-MAPK pathway is activated by stress stimulation such as ultraviolet, oxidation and inflammation. Cytokine is responsible for transducing signals to the nucleus leading to differentiation, growth arrest and apoptosis (Raffetto *et al.*, 2008). Fibroblast are used as to sense and respond to tissue damage via the cytokine and growth factors secretion,

due to there the efficiency produce a relation between the surrounding cells which participate in the wound healing process.

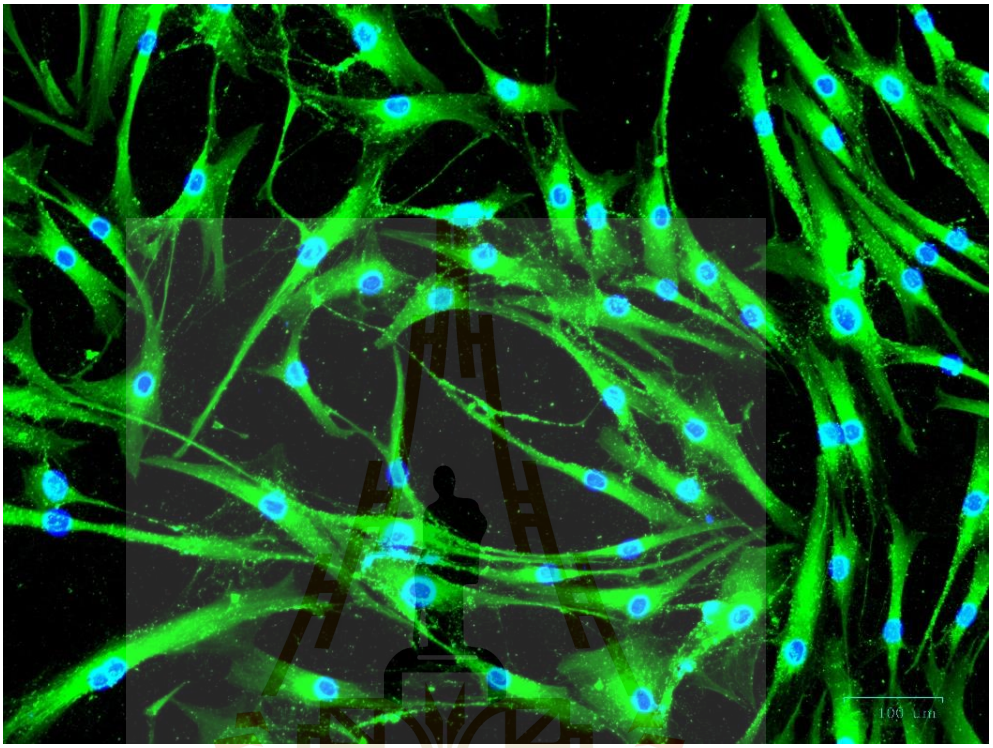


Figure 2.4 The morphology ‘spindle-shaped’.

2.2 Cellular senescence

Normal human cells exhibit a limited proliferative capacity in culture and enter into an irreversible growth arrest called replicative senescence after a set number of cell divisions. Cellular senescence is an irreversible growth arrest that occurs as a result of different damaging stimuli, including DNA damage, telomere shortening and dysfunction or oncogenic stress (Wang and Dreesen, 2018). Senescent cells are characterized by their inability to proliferate, resistance to apoptosis, and secretion of factors that promote inflammation and tissue deterioration. The cellular senescence model of aging was studies by Leonard Hayflick and Paul Moorhead, this phenomenon describe a cell life span into long time period. Leonard Hayflick discovered normal

human fibroblasts *in vitro* can divide cells into the duration times or a finite proliferative capacity. This phenomenon are termed 'Hayflick limit' or 'cellular senescence', are studies to understanding the molecular mechanisms of ageing or cellular senescence. This characteristic of normal cells is in contrast to the cancer cells. Cancer cells can divide to be passage more than normal cells or loss tumor suppressor gene. Since stem cells maintain many tissues during the lifetime of an animal, it follows that stem cell senescence must be prevented to maintain an organ throughout life. Several studies suggest that cellular senescence is accompanied by changes in gene expression, which might be regulated by epigenetic mechanisms. In support of this hypothesis, histone deacetylase inhibitors, which condense chromatin and activate the transcription of some genes, can induce a senescence-like state in human fibroblasts, suggesting that conversion of some heterochromatin to euchromatin may be a feature of replicative senescence (Shay and Wright, 2004). Normal somatic cells have a state of irreversibly arrested growth, is thought to be a replicative senescence mechanism involving ageing. The notion that senescence contributes to ageing, by contrast, stem largely from studies in culture.

2.2.1 The characteristic of senescence

Senescent cells are identified in culture by their failure to synthesize DNA under optimal conditions and exponential increase with passage (Dimri *et al.*, 1995). Several markers of senescence including SA-β-Gal activity, expression of tumor suppressor and cell cycle inhibitors, DNA damage markers, nuclear foci of constitutive heterochromatin and prominent secretion of signaling molecules. The senescence of fibroblasts are detected a β-gal that express in single cells by X-gal, which forms a local blue precipitate upon cleavage, independent of DNA synthesis measurements. To

explore the idea that senescent cells accumulate *in vivo*, skin sample were sectioned and stained for β-Gal. The SA-β-Gal-stained is the histochemical detection of β-galactocidase activity at pH 6.0, were examined blind by a dermatological pathologist for the staining frequency and identity for positive cells. Thus, SA-β-Gal delivers a simple assay for chromosomes that induce senescence and should facilitate the cloning of senescence genes (Dimri *et al.*, 1995). Other markers of cellular senescence through based on a stable cell cycle arrest such as Ki67 protein the proliferative marker or 5-bromodeoxyridine (BrdU) incorporation. On the other hands, the canonical senescence markers comprise the most common mediators of senescence, including p16, ARF, p53, p21, p15, p27 and hypophosphorylated RB (Espin and Serrano, 2014). The characteristic of senescence in *in vitro* culture were morphologically changed such as cell become flat, large, vacuolized, occasionally and multinucleated (Figure 2.5). A deeper understanding of the molecular mechanisms underlying the multi-step progression of senescence and the development and function of acute versus chronic senescent cells may lead to new therapeutic strategies for age-related pathologies and extend healthy lifespan (Deursen, 2014). A senescence stimulation including dysfunctional telomeres, DNA damage, the suppression of tumor suppressor gene as well as oncogenes, perturbation to chromatin organization. Tumor suppressor pathways are activated by the p53 and retinoblastoma proteins, are important for establishing and maintaining the senescence growth arrest (Campisi and Fagagna, 2007). Human cells can express markers of senescence have been shown to accumulate with age and at sites of certain age-related pathologies.

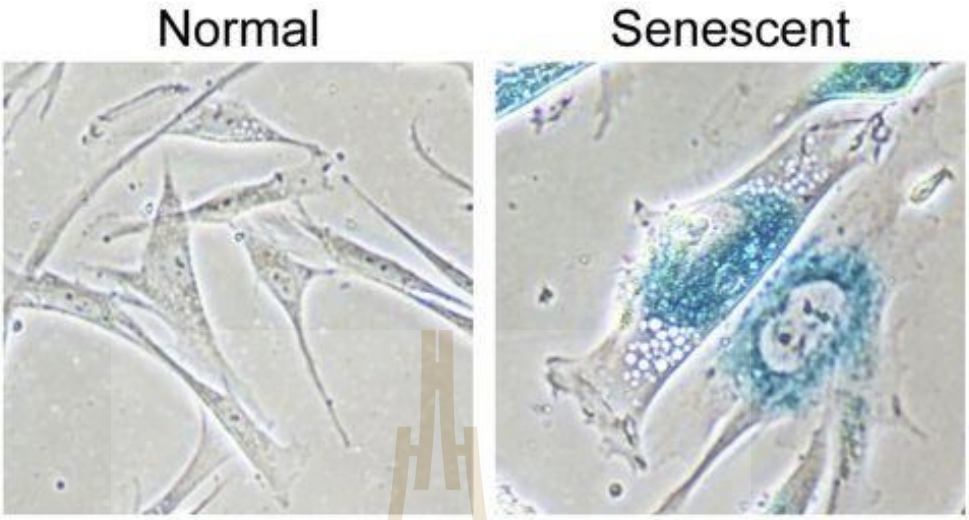


Figure 2.5 The morphology of cell culture comparing normal and senescent cells.

2.2.2 Stimulation on cellular senescence

In addition, cell respond to various types of cellular stresses including telomere erosion, DNA damage, overexpression of tumor suppressor gene or oncogenes, oxidative stress, continuous mitogenic stimuli, and a variety of chemical can also induce senescence (Balducci *et al.*, 2014) (Figure 2.6). Cellular senescence is induced by various stimuli that lead to the accumulation of senescent cells in aged tissues (Deursen, 2014). The senescent state is characterized by activation of the potent tumor suppressor p16INK4A and p53, as well as by secretion of various cytokines, growth factors, matrix-metalloproteinases (MMPs), and extracellular vesicles (EVs) (Schosserer *et al.*, 2017). The replication senescence of typical primary human cells appears to be prevented by two distinct events, mortality stage (M1) and mortality stage 2 (M2). M1 and M2 are often designated as “replicative senescence” and “cellular crisis” stages respectively.

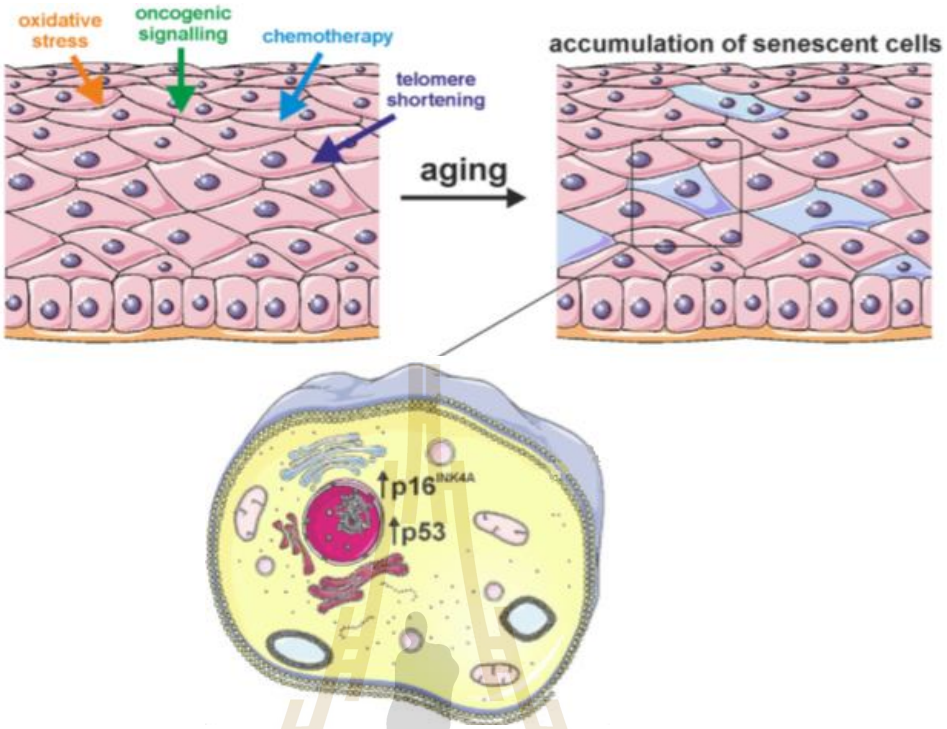


Figure 2.6 Various stimuli induce cellular senescence (Schosserer *et al.*, 2017).

The relationship of senescent are discovered that implicate a role of telomere. Telomere shortening in replicative senescent is a one of role to maintain the lifespan of cells. In fibroblast culture are determine the relationship with telomere length. These results shown telomere lengths is a biomarker of somatic cell aging in humans and are consistent with a causal role for telomere loss (Allsopp *et al.*, 1992). Telomeres are the structure at the end of the chromosome to protect these ends from degradation or joining to one another. The ends of human telomeres consist of repeats of the sequence TTAGGG. A telomere is functionally defined as a region of DNA at the capping of a linear chromosome that is required for replication and stability of the chromosome (Figure 2.7). Telomeres playing a vital role in the maintenance of chromosomal integrity and stability (Moyzis *et al.*, 1988). On the other hands, the regulation of the cell cycle is one critical molecular regulator of replicative senescence.

Experimentally, replicative senescence can be bypassed by expression of viral oncoproteins, such as SV40 large T antigen (LT) or the human papilloma virus (HPV) E6 and E7 proteins, which inactivate the p53 and retinoblastoma (pRB) tumour suppressor pathways. Both replicative senescence and crisis limit the growth of normal human cells. Although a number of oncogenes and tumor suppressors, such as PI3K, Ras, p53, PTEN, pRb, and p16INK4a, are frequently mutated in cancer cells, there also appears to be a large number of low-frequency changes that can contribute to oncogenesis.

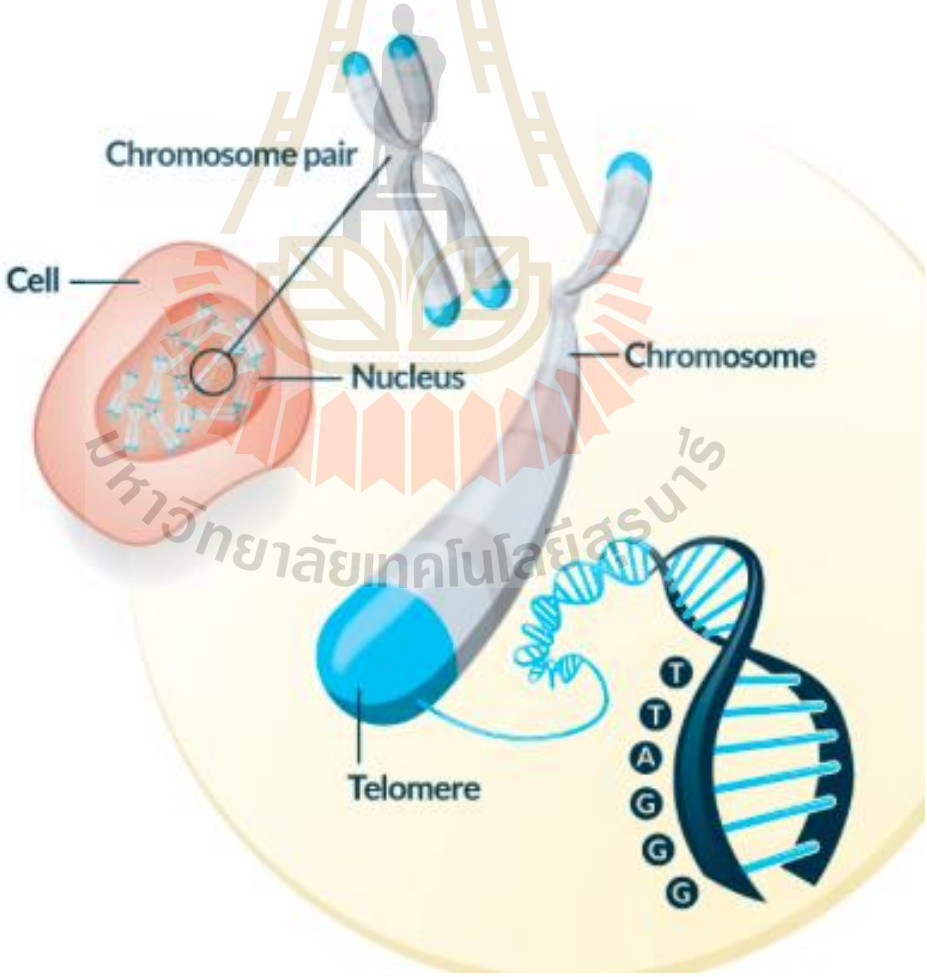


Figure 2.7 The structure of telomere on the chromosome.

(https://thehumanevolutionblog.com/2018/06/11/healthcare-access-stress-telomeres/)

2.2.3 Cellular senescence involve pathogenesis

Many pathologies coordinate senescence and tissue remodeling including fibrotic diseases, vascular disorders, obesity, type 2 diabetes, renal diseases and sarcopenia. In these pathologies, cellular senescence is against roles (Espin and Serrano, 2014). Many health problem worldwide involve senescence such as human fibrotic diseases, is attributed to excess deposition of extracellular matrix components including collagen. Fibrosis is the end result of chronic inflammatory reactions induced by a variety of stimuli including persistent infections, autoimmune reactions, allergic responses, chemical insults, radiation, and tissue injury (Wynn Ta, 2008). However, the unclear knowledge of the fibrotic process and cellular senescence are the main problem to control pathogenesis involve through senescence. The incomplete knowledge of the fibrotic process pathogenesis, the marked heterogeneity in their etiology and clinical manifestations, the absence of appropriate and fully validated biomarkers, and, most importantly, the current void of effective disease-modifying therapeutic agents (Rosenbloom *et al.*, 2017). In the same ways, obesity and type 2 diabetes occurs from the accumulation of senescence. Senescent play a vital role through direct impact on pancreatic β-cells function and involvement in the adipose tissue dysfunctions. In turn, metabolic and signaling changes seen in diabetes, such as high circulating glucose, altered lipid metabolism, and growth hormone axis perturbations, can promote senescent cell formation. Thus, senescent cells might be part of a pathogenic loop in diabetes, as both a cause and consequence of metabolic changes and tissue damage (Palmer *et al.*, 2015). Cardiovascular disease is the risk in aging patients over 65 year old, cause myocardium undergoes degeneration that leads to myocyte death. Others senescent and aging in vitro studies were investigated to understand and developments.

Aging in vivo is required studies to understand the mechanism of aging that involving organisms and cause pathologies. Many studies focus on the rejuvenating aging and novel therapeutic strategies for ischemic heart diseases. Cardiac aging have the three approaches to reverse these including genetic modification, pharmaceutical administration and optimization of extracellular factors (Kaur and Cai, 2018). Genetic modifications can be used to overexpress or knockdown certain genes in cardiac stem cells. The second approach uses to pharmaceutical administration through targeting signaling pathways that control cardiac aging such as proliferation, apoptosis and senescence.

2.2.4 The role of senescence in aging

Aging is accompanied by a general decline in mitochondrial function in all tissues (Kaur and Cai, 2018). This phenomenon is representing in a variety of change overtime, including change in cellular state. The contribution of mitochondria to the aging process can stimulate through several pathways such as age, ROS and chemicals. Mitochondria acts as a regulator in many pathways (Figure 2.8). Loss function of mitochondria has been associated with aging process and general diseases such as chronic inflammatory and age-related stem cells. Mitochondria can modulate cellular aging through the metabolic profile of the cell (Sun *et al.*, 2016). A decline in mitochondrial function has been reported in age-related disease which mutant-mitochondria increase in frequency with age both human and model. Nonetheless, the mitochondria studies are noted that the type and magnitude of mitochondrial mutation do not appear to faithfully replicate what is seen during normal aging (Williams *et al.*, 2010). Thus, the mechanism of the mitochondrial mutation levels increase with age, it

remain unclear whether this increase plays a fundamental role in the aging process (Sun *et al.*, 2016).

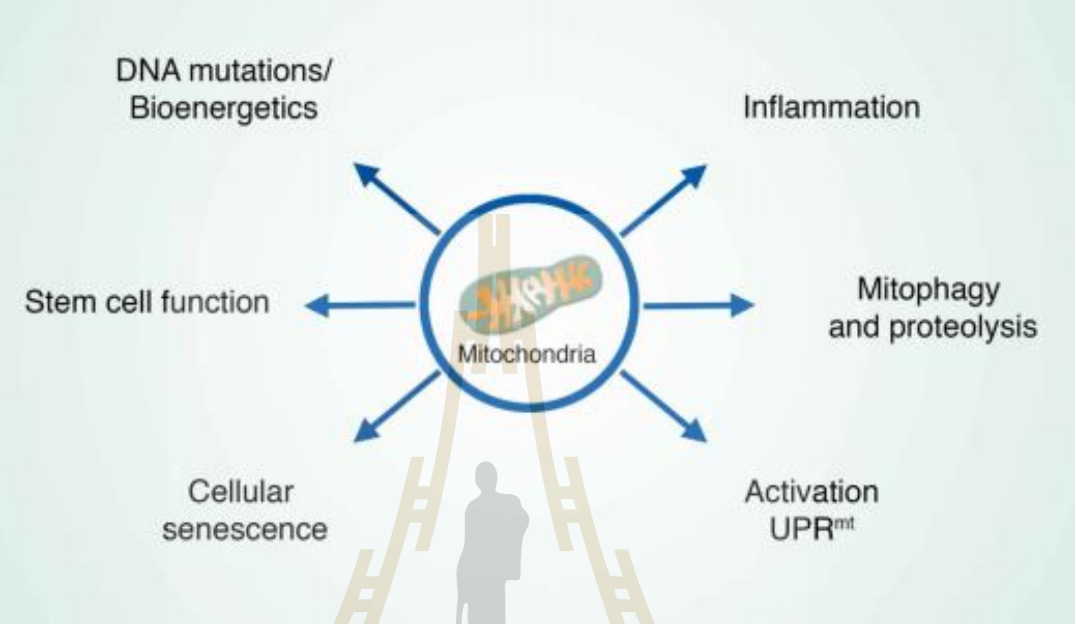


Figure 2.8 Mitochondria as stimulators in organism aging (Sun *et al.*, 2016).

Cellular aging has one key role through oncogene induce senescence which acts as in anti-cancer mechanism. As know cellular senescence is effective of constant change in the metabolome and the metabolic profile of oncogene-induced senescence (OIS) and replicative senescence appear distinct. The relation of senescence and mitochondria metabolic is observed in oncogene-induce senescence. OIS is a robust and sustained anti-proliferative response brought about by oncogenic signaling resulting from an activating mutation of an oncogene, or the inactivation of a tumor-suppressor gene (Chandeck and Mooi, 2010). The advantage of OIS exhibit activating RAF and BRAF mutation which response in whole organisms cause effect cell proliferation and its progeny. However, the mechanism mediating OIS still understood but the proliferative arrest involves activation of both the pRB and p53 pathways.

2.3 Immortalization

The ‘immoltal’ word is meaning living forever or never dying that is believe of Greek ancient. These word can refer a supernatural being who is worshipped by others or god in human of Greek methodology. To apply in cell culture that meaning of cells can be growth for prolonged period *in vitro* but not yet a cancerous cells.

The limited proliferative lifespan of primary cells known as ‘Hayflick limit’ is a major restriction for primary cultures (Sidler *et al.*, 2017). They found that cells have limited capacity to divide, after into senescence (Figure 2.9). Cellular proliferation beyond the Hayflick limit is defined as immortalization. Immortalized cells are valuable for basic research as well as biotechnological applications. For example, the short lifespan of primary somatic cells limits their ability to be genetically modified and is considered to be a major obstacle to gene targeting in livestock (Choi and Lee, 2015). Establishment of immortalized somatic cell lines may resolve such problem and increase the efficiency of genetic modification in animal cells (Giri and Bader, 2014).

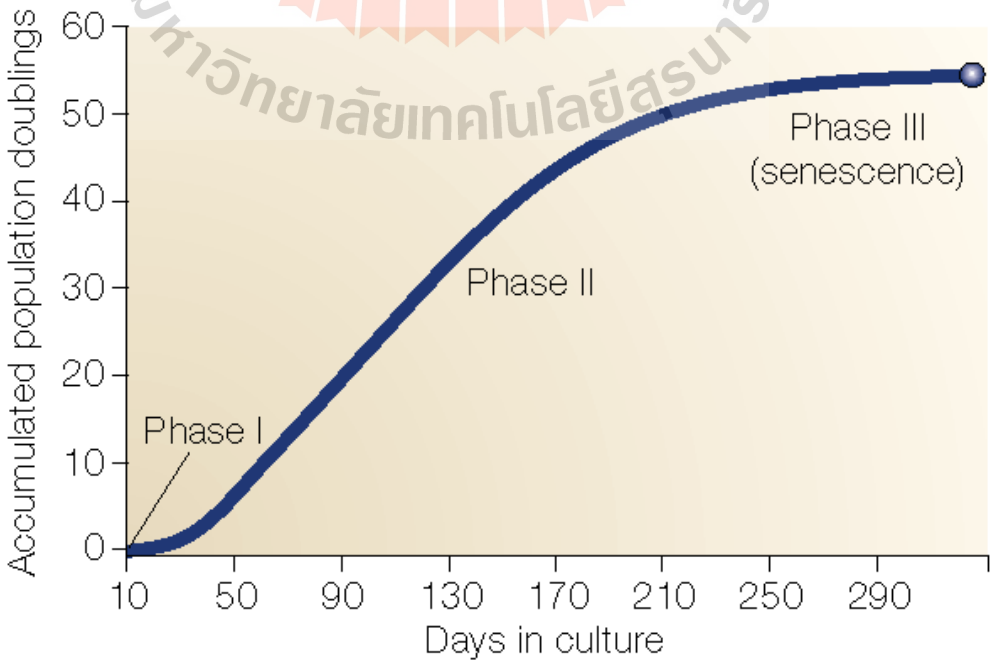


Figure 2.9 Hayflick’s limit (Shay and Wright, 2000).

2.3.1 Role of telomere and human telomerase reverse transcriptase (hTERT)

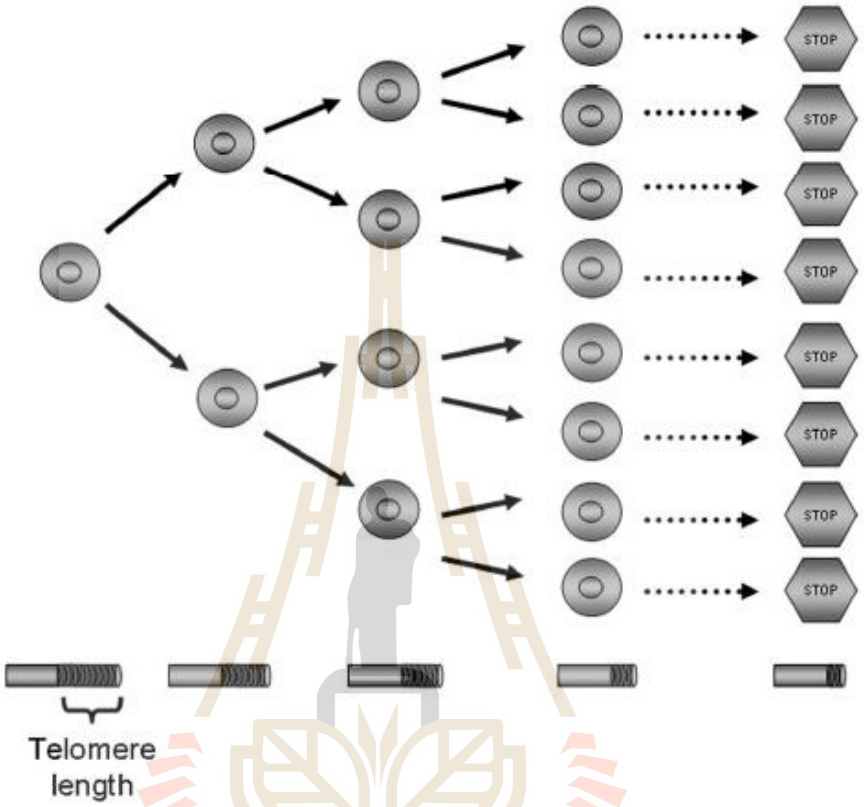


Figure 2.10 Role of telomere length (Shay and Wright, 2005).

Human telomerase reverse transcriptase (hTERT), which is specifically activated in most cancer cells and germ cells, plays an essential role in the immortality of cancer cells via regulation of telomere length by telomerase enzyme activity (Lee *et al.*, 2004). The ends of linear eukaryotic chromosomes contain specialized structures called telomeres (Figure 2.10). Human telomeres consist of tandem repetitive arrays of the hexameric sequence TTAGGG, with overall telomere sizes ranging from ~15 kb at birth to sometimes 55 kb in chronic disease states (Cawthon, 2002). The telomeric repeats help maintain chromosomal integrity and provide a buffer of potentially expendable DNA. The ends of telomeres are protected and regulated by telomere-binding proteins and form a special lariat-like structure called the t-loop (Allsopp *et al.*,

2003). This packaging or protective cap at the end of linear chromosomes is thought to mask telomeres from being recognized as broken or damaged DNA, thus protecting chromosome termini from degradation, recombination and end-joining reactions (Barsov, 2011).

Additional forms of senescence have been described in the literature that are independent of telomere size and cannot be bypassed by hTERT alone. Mortality stage (M0) is a well-documented form of telomere-independent senescence (Shay and Wright, 2004). It limits the *in vitro* lifespan of certain types of primary human cells, including keratinocytes and mammary epithelial cells, and cannot be overcome by hTERT alone. M0 is accompanied by a mobilization of the p16INK4a /pRB pathway, and can be overcome by viral oncogenes, such as HPV16 E7, that can block pRB function (Zhu *et al.*, 1991). Escape from M0 can occur spontaneously through clonal events, involving the loss of p16INK4a expression and the acquisition of aneuploidy, that are reminiscent of cancer development. Whether M0 is an artifact of *in vitro* cultivation or a true physiological process is still debated. Recent findings are most consistent with the notion of M0 representing a delayed reaction to inadequate culture conditions. Most significantly, M0 can be avoided when keratinocytes and mammary epithelial cells are cultured on feeder layers rather than plastic dishes (Choi and Lee, 2015). Human cell types that require feeder layers for hTERT immortalization include mammary epithelial cells, keratinocytes, and oesophageal squamous cells (Bulducci *et al.*, 2014). These observations reiterate the need for an optimized environment capable of sustaining long-term cultivation as a prerequisite for the establishment of human cell lines. This need may especially be pertinent to the immortalization of primary human cells, as these cells tend to have growth requirements that are higher than those of cancer

cells (He *et al.*, 2016). For many cell types, the main obstacle to immortality is their inability to proliferate *in vitro*. This failure to proliferate can be an intrinsic property of the cells, as in the case of post-mitotic terminally differentiated cells, which have lost all proliferative capacity upon differentiation. Alternatively, this failure can result from 35 our inability to emulate *in vivo* conditions that support growth. Indeed, there are several examples of cells with a capability for *in vivo* proliferation that are unable to divide in the artificial environment of the laboratory (Ikbale *et al.*, 2016). hTERT alone should not be expected to overcome these obstacles, as the enzyme does not appear to inhibit differentiation, alter phenotypic properties, or decrease growth requirements.

2.3.2 Role entering cell immortalization

2.3.2.1 *Bmi1*-induced

The cellular proto-oncogene c-*myc* was found recently to induce telomerase activity and immortalize certain human MECs and fibroblasts. Direct support for *c-myc* as an inducer of telomerase came from studies of the hTERT promoter. The *hTERT* promoter contains several *c-myc*-binding sites (CACGTG) through which c-Myc can activate *hTERT* transcription (Dimri *et al.*, 200). Despite being the only cellular gene identified thus far that can activate telomerase, c-*myc* is amplified or overexpressed in only 15% of breast cancers. However, the majority of breast cancers express telomerase. These findings suggest that additional pathways of telomerase induction must exist. *Bmi-1* was cloned as a c-*myc* cooperating oncogene in murine lymphomas. It was shown subsequently to be a transcriptional repressor belonging to the PcG of proteins (Zhang *et al.*, 2017). Consistent with its oncogenic potential, *Bmi-1* also regulates cell proliferation. *Bmi-1*-deficient MEFs overexpressed the *INK4a* encoded genes *p16* and *p19ARF* (mouse homologue of human *p14ARF*) and

underwent premature senescence in culture. Conversely, overexpression of *Bmi-1* reduced expression of p16, and to a lesser extent p19ARF, and immortalized MEFs. *Bmi-1* overexpression in human fibroblasts also extended the replicative life span but did not result in immortalization (Kumar *et al.*, 2017). The mechanism by which *Bmi-1* represses *p16* and *p19ARF* in MEFs appears to be transcriptional but is not well understood. Recently, it was shown that by repressing p19ARF, *Bmi-1* inhibits the proapoptotic function of *c-myc* and thus collaborates with *c-myc* in tumorigenesis (Sharov *et al.*, 2014). *Bmi-1* contains a conserved ring finger (RF) domain at the NH2 terminus, which is required for its ability to cooperate with *c-myc* in tumorigenesis and regulate cell proliferation (Zhang *et al.*, 2017). *Bmi-1* also contains a conserved centrally located helix-turn-helix-turn (H-T-H-T) motif, which is required for transcriptional repression but not transformation, in rat embryo fibroblasts. *Bmi-1* was recently reported amplified in certain mantle cell lymphomas, providing evidence for a role in human tumorigenesis. In addition, *Bmi-1* is overexpressed in other human cancers, such as non-small cell lung cancer and B-cell non- Hodgkin lymphoma (Park *et al.*, 2004). In human fibroblasts, where senescence mechanisms have been studied extensively, intact pRb/p16 and p53/p21 checkpoints are required to establish and maintain the senescent phenotype. Inactivation of either checkpoint results in an extension of replicative life span but not immortalization. These checkpoints appear to contribute to the limited replicative life span of MECs as well. *Bmi1* maintains the stem cell pool by preventing premature senescence, either through repression of genes involved in senescence or perhaps through induction of telomerase to prevent telomere shortening (Shay and Wright, 2005). It is very likely that *Bmi1* is important for maintenance of multiple types of somatic stem cells, since it is widely expressed and

Bmi1-deficient mice have developmental defects in other organs. *Bmi1* is also important for maintenance of leukemic stem cells and perhaps other tumorigenic stem cells; therefore, *Bmi1* could be used as a molecular target to induce senescence in cancer stem cells (Kumar *et al.*, 2017).

2.3.2.2 Pathway inactivation

Recently reports, overexpression of hTERT alone can immortalize certain human fibroblasts and retinal epithelial cells without inactivating the pRb/p16 or p53/p21 pathways. It has therefore been argued that inactivation of these checkpoints is not necessary for immortalization by telomerase (Choi and Lee, 2015). When mammary tissue is explanted into tissue culture medium, a heterogeneous population of MECs, termed preselection cells emerges. This population proliferates for 10–15 doublings times (PDs) before the majority of cells undergo senescence. However, regular feeding of preselection cells eventually gives rise to a homogeneous population, termed postselection MECs, which appear to arise from relatively rare cells in the preselection population. The emergence of postselection MECs is associated with *p16* and believed attributable to, progressive methylation of the *p16* gene. Thus, p16 is silenced and not expressed in postselection MECs (Foster *et al.*, 1998). Nonetheless, postselection MECs eventually undergo replicative senescence after an additional 30–40 doubling times and do not spontaneously immortalize. Recently, it was reported that postselection MECs display signs of genetic instability as they approach senescence. However, consistent with earlier reports, neither the preselection nor the postselection MECs underwent spontaneous immortalization. Ectopic expression of hTERT failed to immortalize preselection MECs or keratinocytes that contained an intact p16/pRb pathway but readily immortalized postselection MECs and keratinocytes in which the

p16/Rb pathway was inactivated because of p16 methylation or expression of the HPV *E7* oncogene (Ganguly and Parihar, 2009). Immortalization induced by a number of oncogenic viruses is intimately associated with inactivation of cell cycle checkpoint proteins such as p16INK4A, p14ARF, and p21CIP1. SV40 T antigen, HPV E6 or E7, and adenovirus E1A or E1B are well- characterized viral oncogenic proteins, whose transforming activities depend on disruption of pRb and p53 tumor suppressor proteins. This virus-induced inactivation of tumor suppressor proteins leads to restoration of telomerase activity, resulting in telomere ends stabilization. There is also a link between pRb inactivation, cell aneuploidy, and chromosome instability. The signaling pathways activated by various cellular stresses are also funneled to the pRb and p53 proteins. Therefore, pRb and p53 can be regarded as two central players governing replicative senescence (Egbuniwe *et al.*, 2011). pRb is found at senescence in its active hypophosphorylated form in which it binds to the E2F protein family members to repress their transcriptional activation of several target genes involved in G1/S phase transition. Growth-suppressive activity of pRb is apparently maintained independently of p53. Therefore, p53 can induce senescence of human cells through a pathway independent of the pRb family. p16INK4A is an inhibitor of cyclin D/cyclin-dependent kinase (CDK) 4, 6 complexes. p16INK4A is solely responsible for the induction of an early, stress-induced, senescence stage in keratinocytes. Of note, p16INK4A is one of the most frequently inactivated genes in human tumors. While p53 and p21CIP1 act to initiate the senescence response, p16INK4A seems to act to maintain this state. The p16INK4A response is found to be more enhanced in human cells than in mouse cells, and provides an additional safety layer to prevent tumor development (He *et al.*, 2016). Similarly, a constitutively active p16-insensitive CDK4 mutant was shown to overcome

replicative senescence. The p14ARF activates p53 by sequestering MDM2 (Mouse double minute 2 homolog), an E3 ubiquitin ligase, in the nucleolus, thereby preventing MDM2-mediated targeting of p53 for proteolytic degradation. Mouse embryo fibroblasts may preferentially rely on the p14ARF/p53 pathway, whereas human keratinocytes employ the p16INK4A/ pRb pathway to enforce the senescence program. Therefore, regardless of species and tissue tropisms, the integrity of both the p16INK4A/pRb and p14ARF/p53 pathways appears to be essential for oncogene induced senescence (Egbuniwe *et al*., 2011).

2.3.2.3 Simian virus large T antigen 40 (SV40 large T Antigen)

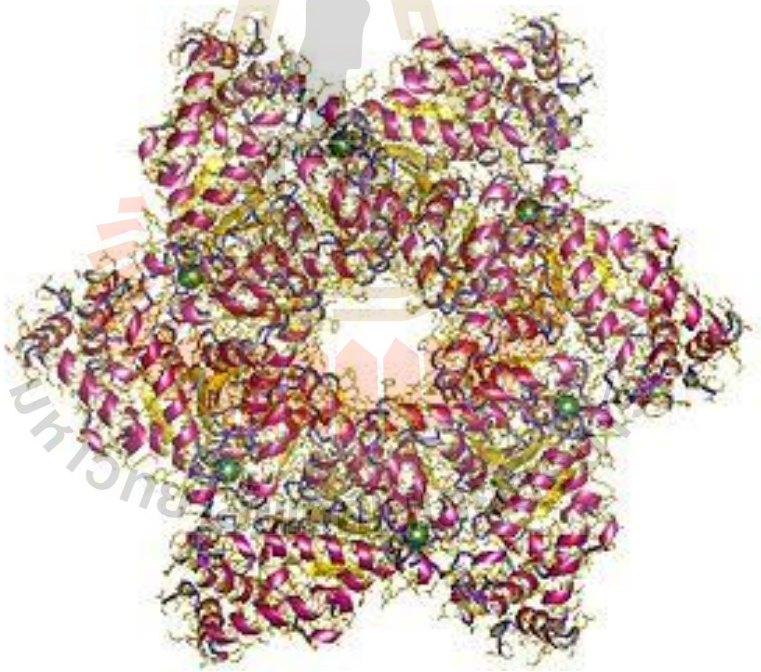


Figure 2.11 Simain virus large tumor antigen.

One of the most commonly used immortalizing genes is SV40 T antigen. Previously studies used retroviral vector-mediated expression of SV40 T antigen to immortalize primary cells (Borowiec *et al.*, 1990). However, the immortalization efficiency was relatively low, largely due to the low viral titters of large

cargo size for retroviral packaging (Cui *et al.*, 2002). Thus, the bottleneck of efficient immortalization is to effectively deliver the immortalizing factors into the targeted primary cells. The transforming proteins of DNA tumor viruses have long been model systems for studying oncogenesis. Adenovirus ElA proteins resemble SV40 large T antigen in that both are transcriptional regulatory proteins and are able to alter cellular growth control. ElA proteins can immortalize primary cells and cooperate with adenovirus E1B proteins and other oncogene proteins, such as activated ras gene products, to bring about full transformation of primary rat cells (Allsopp *et al.*, 2003). ElA proteins can also activate and repress viral and cellular gene expression. Two regions of ElA, aa residues 30 to 60 and 121 to 127, respectively, were found to be responsible for the immortalization and ras cooperation functions. These regions are conserved among different adenovirus serotypes and have homology to portions of SV40 large T antigen and human papillomavirus E7 protein. This region of homology has been shown to be essential for binding to p105Rb. Studies of simian virus 40 (SV40), a tumor virus containing 5.2 kb of circular double stranded DNA (Figure 2.11). It has been particularly useful because the presynthesis reactions are catalyzed predominantly by a single protein. SV40 large T antigen is a multifunctional 82 kd phosphorprotein, is the sole viral protein required for SV40 DNA replication; all other factors are provided by the host cell. In addition to its role in SV40 DNA replication and regulation of viral gene expression, T antigen can also lead to the transformation of susceptible cell lines. Studies of various mutant T antigen proteins have shown that the replication and transformation functions of T antigen can be separated (Dean *et al.*, 1987). The initial step in the pathway of SV40 DNA replication is binding of T antigen to DNA sequence elements comprising the ori. T antigen can bind to two strong sites in

the vicinity of SV40 ori (sites I and II), and under certain conditions to weaker sites (collectively termed site Ill) on the late-gene side of the ori region. From the three sites, only site II is contained within the minimal 64 bp region comprising the core ori; this region is both necessary and sufficient to allow initiation of viral DNA replication. The presence of sites I and Ill can augment core ori activity to various degrees depending on replication conditions. A common feature of each site is the presence of 5'-G&GGC-3' sequences, although the number and orientation of the sequences and their spacing differ at each site. The four conserved sequences in site II are found in a 27 bp perfect inverted repeat that contains two GAGGC sequences in each arm of the palindrome. The critical GAGGC sequences in site I are organized as two direct repeats separated by an AT-rich tract. Biochemical and genetic evidence shows that the GAGGC repeats serve as the recognition site for T antigen (Dean *et al.*, 1987). Scanning transmission electron microscopy has further demonstrated that a minimum of a single T antigen monomer can bind to each GAGGC repeat. The SV40 core ori has been subject to detailed genetic investigation, and these studies indicate the presence of three domains critical for SV40 DNA replication the central GAGGC element, a 10 bp region partially overlapping an imperfect inverted repeat (early palindrome), and a 17 bp region rich in adenines and thymines (AT-tract) (Zhu *et al.*, 1991). The latter two flanking regions undergo structural transitions during initial stages of SV40 DNA replication. DNAase I protection analysis shows that T antigen covers the complete core ori sequence. The cleavage pattern is striking, because loss of DNAase I cleavage sites on both strands over the entire region indicates close apposition of T antigen to all faces of the DNA helix. Moreover, electron micrographs of the ATP-dependent complex show that T antigen is organized into a two-lobed structure at the ori region. The complex of largest

mass found in significant numbers by scanning transmission electron microscopy contains 12 monomers of T antigen, although complexes of lower mass were found. That this number is significant is supported by the observation that ATP can cause T antigen to form multimers even in the absence of DNA, with a mass equivalent to six T antigen monomers. This suggests that the ATP-dependent complex is composed of two lobes, each containing a hexamer of T antigen (Zhu *et al.*, 1991).

Immortality of cells is a recessive phenotype, and immortal human cell lines have been assigned to at least four complementation groups. All SV40-immortalized cell lines except one belong to one complementation group, suggesting that SV40 disrupts one of several distinct cellular functions whose alteration can lead to immortalization of primary cells (Takenouchi *et al.*, 2017). Assays for the immortalization function of T antigen generally have used primary mouse or rat cells. Primary C57/BL6 mouse embryo fibroblast (MEF) cells can be immortalized by transfection of the gene encoding large T antigen, and the amino-terminal 626 aa of T are sufficient for immortalization (Choi and Lee, 2015). Deletion mutants which encode T antigens missing aa 1 to 127 or 121 to 250 were also able to immortalize primary MEF cells (Dean *et al.*, 1987). Therefore, specific and nonspecific DNA-binding activities; DNA helicase activity; binding to p1o5Rb, 107K/120K, and hsp73; and the adenovirus helper-host range function are not essential for immortalization of primary MEF cells. In contrast, the aminoterminal 135 aa of large T appear to be sufficient for immortalization of primary REFs.

2.3.2.4 HPV E6/E7

A number of high risk type HPVs play an etiological role in development of most of cervical cancers. Among HPV proteins, E6 and E7 are regarded

as central players for the oncogenic conversion of host keratinocytes through their specific inactivation of p53 and pRb tumor suppressor genes via their accelerated ubiquitin-mediated degradation. HPV E7 is able to immortalize human keratinocytes at a very low efficiency when cultured in serum-free synthetic medium (Ganguly and Paihar, 2009). Abrogation of pRb function by HPV E7 blocks p16INK4A-mediated senescence. Human corneal cells were also immortalized by using the HPV16 E6/E7 oncogenes for ophthalmologic issues. Use of HPV E6 and E7 turned out to have advantage over other immortalization methods since tissue-innate features have been shown to be better preserved in the descending cell lines, which were immortalized by HPV E6 and E7 than those by other methods. In addition, human mammary epithelial cells (HMECs), which were immortalized by HPV E6 and E7, are shown to stay at pre-neoplastic stage (Ganguly and Paihar, 2009). Therefore, they do not grow in an anchorage-independent manner or produce tumors when implanted in immune-deficient mice. For an efficient immortalization of primary keratinocytes, overexpression of HPV E6/E7 by retroviral transduction has been a method of choice so far thanks to its capacity to preserve normal cell-like properties in immortalized cells (Choi and Lee, 2015). A schematic diagram on how to use a retroviral transduction to induce immortalization of target cells. First, cDNA encoding HPV E6/E7 is cloned into a retroviral vector. Then, this cloned retroviral vector is transfected into 293T packaging cells together with other retroviral envelope or packaging vectors, followed by harvesting of retroviruses, which are encoding HPV E6/E7, in the supernatant. Then, primary keratinocytes, which are typically isolated from human foreskin tissues, are infected with these harvested retroviruses (Shay and Wright, 2004). Through a drug

selection, immortalized keratinocytes, which are stably expressing HPV E6/E7, are isolated and propagated.

2.3.3 Immortalization and biomedical discoveries

A major advantage in using hTERT alone to immortalize primary human cells is that the enzyme telomerase can immortalize without causing cancer-associated changes or altering phenotypic properties (Takenouchi *et al.*, 2017). Conventional methods from establishing human cell lines, such as the cultivation of tumor biopsies and the use of immortalizing oncogenes, have been used successfully to generate most of the human cell lines currently available to researchers, and these lines have been instrumental to the analysis of human biology (Ikbale *et al.*, 2016). Unfortunately, these conventional methods have in common that they almost invariably give rise to cells that display cancer associated changes. These aberrations may include a loss of contact inhibition, reduced growth factor requirements, inhibition of differentiation, genomic instability, aneuploidy, as well as disruptions of cell cycle checkpoints (Giri and Bader, 2014). These characteristics pose significant limitations to the analysis of many cellular functions, particularly those related to genomic integrity and cell cycle regulation. In contrast, primary human cells immortalized with hTERT alone tend to have a relatively 'normal' phenotype. Following their immortalization with hTERT, primary human cells remain diploid, differentiated, contact-inhibited, non-tumorigenic and anchorage dependent (He *et al.*, 2016). They are genomically stable, possess functional cell cycle checkpoints, and express functional p53, pRB and p16INK4a. Thus, aside from their unlimited lifespan, hTERT-immortalized cells have more in common with primary human cells than with cancer cells. This apparent 'normality' of hTERT-immortalized

cells and their capacity for unlimited divisions make them ideal for applications in tissue engineering and as model systems for cancer research (Yang *et al.*, 2017).

Cell-based therapies and tissue engineering share the promise of new cures for degenerative and age-related diseases (Choi and Lee, 2015). Basic science and pharmaceutical researchers use the 3D-skin model to replace animal tests for the assessment of skin irritation due to chemicals, skin-cream-based drugs and cosmetics. 3D-skin is first tools to develop the cosmeceutical in many drug and toxicity testing. It's high efficacy for using in models, safe time in laboratory and non-ethical issue in animals. Through these approaches, replacement parts are created from cells taken from a patient, by the *in vitro* cultivation of these cells and their expansion to sufficient numbers to allow their genetic manipulation and rearrangement into a working tissue. Until recently, a major obstacle to rapid progress in this field had been the limited lifespan of normal human cells. This limitation can now be circumvented by the transient expression of hTERT (Zhang *et al.*, 2016). Cancer development is driven by the acquisition of genetic and epigenetic alterations, which act in concert to override endogenous mechanisms regulating cell proliferation. The better definition of the role of these endogenous mechanisms in the physiology of primary human cells is vital to our understanding of cancer development (Yang *et al.*, 2017). Until recently, two obstacles had limited the use of primary human cells in cancer research: their limited lifespan and their spectacular resistance to *in vitro* carcinogenesis. The stable expression of exogenous hTERT can alleviate both of these obstacles, as telomerase renders cells susceptible to oncogenic transformation without causing cancer associated changes (Balducci *et al.*, 2014). Either as normal controls for comparative studies or as *in vitro*

models for studies of carcinogenesis, hTERT-immortalized cells have become invaluable tools to cancer researchers.

2.4 Sirtuin and SIRT1

Mammalian histone deacetylases (HDACs) are grouped into four categories, of which three contain non-sirtuin HDACs which include the yeast histone deacetylases, RPD3 (class I HDACs), HDA1 (class II HDACs) and the more recently described HDAC11-related enzymes (class IV HDACs), while one category is composed of the sirtuin protein deacetylases (class III HDACs), which are orthologs to the yeast Sir2 protein (Mahlknecht *et al.*, 2006). The (Silent Information Regulator 2) Sir2 gene has been shown to regulate the life span of several model organisms (Kuningas *et al.*, 2007). Sirtuin or Sirt1 is regulated with NAD+ dependent deacetylase by direct NAD+ and indirect in another for a variety of proteins, including p53, p21 and p16 (Haigis and Guarente, 2006). However, protein that are directed by SIRT1 that involved in diverse cellular processes such as cellular metabolism, extended cellular life span, and differentiation. NAD or Nicotinamide adenine dinucleotide is a coenzyme which involved in redox reaction in living cells and important in many metabolism enzymes. Increasing the SIRT1 level through genetic manipulation extends the lifespan of yeast, nematodes and flies (Kanfi *et al*., 2012).Other, in mammalian have been reported SIRT1 is regulated age-related disease and aging, but in mice SIRT1 expression no have the successful reports. Therefore, a key role of SIRT1 is not clear for mechanism through mammalian proliferation.

2.4.1 The family of Sirtuin

The sirtuin family of proteins is highly conserved, both functionally and structurally. Its members are integrated into most forms of life including eubacteria, archaea and eukaryotes, and therefore predate both histone and chromatin formation (Vassilopoulos *et al.*, 2011). The silent information regulator 2 gene (Sir2) was first discovered in *Saccharomyces cerevisiae*. In yeast, however, sirtuin proteins are known to regulate epigenetic gene silencing and to suppress recombination of rDNA (Dryden *et al.*, 2003). Next, sirtuins were studied in yeast, bacteria, plants and mammals. The Sirtuin members in mammalian family are classified as class III histone deacytelase and consist of seven members (Vassilopoulos *et al*., 2011). The 3 members as SIRT1, SIRT6 and SIRT7 are classified as the nucleus. SIRT3, SIRT4 and SIRT5 reside in the mitochondria, whereas SIRT2 is localized predominantly in the cytoplasm. SIRT1, SIRT6 and SIRT3 and SIRT5 are NAD-dependent deacetylases (DAC), whereas SIRT4 and SIRT6 are primarily mono-ADP-ribosyl transferases (ART) with no deacetylase activity on histone substrates *in vitro* (Pillarisetti, 2008) (Figure 2.12). As such, these novel findings for the potential role of sirtuins in human illnesses should allow for the identification of potential molecular targets and biomarkers to determine risk and the development of agents that may be chemopreventive (Vassilopoulos *et al.*, 2011) (Table 2.1).

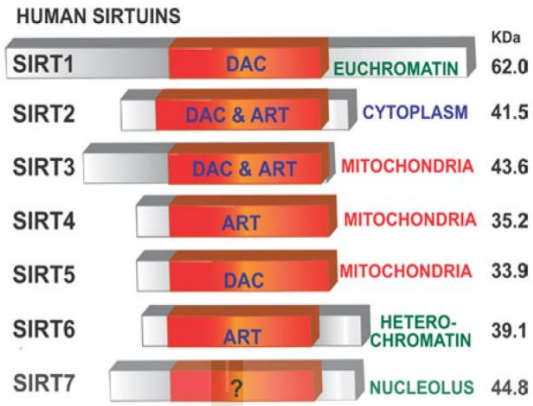


Figure 2.12 Mammalian Sirtuin (Michan and Sinclair, 2007).

Table 2.1 The sirtuin family in human

Gene name	Chromosomal location	Cellular function
SIRT1	10q21.3	Cell proliferation/ Cellular senescence/ Fat metabolism/ Glugenesis and Cell cycle arrest
SIRT2	19q13.3	Tubulin acytelation/ Mitosis/Neuropotection and Inflammatory response
SIRT3	11p15.5	Metabolic regulation/ Energy homeostasis and Oxedative metabolism
SIRT4	12q	Metabolic regulation/ Fatty acid oxidation/ Mitochondrial metabolism and Caloric restriction
SIRT5	6p23	Energy metabolism and Urea cycle
SIRT6	19p13.1	Glycolysis, Triglyceride synthesis/ Fat metabolism and β-oxidation
SIRT7	17q25	Cell proliferation/ Apoptosis/ Inflammatory/ Genotoxic stress and Tumorigenesis

2.4.1.1 SIRT1

The SIRT1 human gene is located at chromosome (Chr) 10q21.3. The role of Sirt1 has been the most extensively studied among the sirtuin family members (Vassilopoulos *et al.*, 2011).

2.4.1.2 SIRT2

Human SIRT2 is located at Chromosome 19q13.3. The SIRT2 protein is similar in sequence to yeast Hst2p and both proteins are located in the cytoplasm, making it the first cytoplasmic sirtuin found (Vassilopoulos *et al.*, 2011). Human SIRT2 is a cytoplasmic protein that increase during mitosis (M phase) of cell cycle. This is associated with the centrosome of mitotic spindle and the midbody, its role in completion of mitosis. Additionally, recent studies SIRT2 are involved in cellular processes such as neuroprotection and the inflammatory response. The neuroprotective role of Huntington's disease is activated sterol level by SIRT2. By contrast SIRT2 inhibitors rescue a-synuclein-mediated cytotoxicity in models of Parkinson's disease (Outeiro *et al.*, 2007).

2.4.1.3 SIRT3

Human SIRT3 is located at Chromosome 11p15.5 and is located primarily in the mitochondrial matrix, where it is proteolytically processed after entry into the mitochondria (Vassilopoulos *et al.*, 2011). Recently reports are suggested that SIRT3 is synthesized as an inactive precursor within the cytoplasm and then transported to the mitochondrial matrix. This report can confirm the safe transfer of a latent enzyme and its selective activation when the proper destination in the mitochondrial matrix has been reached (Schwer *et al.*, 2002). The role of SIRT3 in oxidative stress has been further highlighted after finding that it regulates enzymes that play a key role in the

antioxidant defence mechanism (Vassilopoulos *et al.*, 2011). Intriguingly, SIRT3 are predicted role in cancer. Because of the gene encoding SIRT3 is located close to the telomere subjected to genomic imprinting. It was reported that at least one copy of the SIRT3 gene is deleted in 20 per cent of all human cancers and 40 per cent of breast and ovarian cancers; this is similar to the deletion frequency of the well-known breast cancer tumour suppressor genes, breast cancer susceptibility gene1 (BRCA1) and 2 (BRCA2) (Vassilopoulos *et al.*, 2011).

2.4.1.4 SIRT4

The human SIRT4 gene is located at Chromosome 12q, and the encoded SIRT4 protein is localized within the mitochondria and has no detectable deacetylase activity (Vassilopoulos *et al.*, 2011). Therefore, it is solely an NAD+-dependent protein ADP-ribosyl transferase. SIRT4 is highly expressed in many tissue including brain, kidney, pancreas, liver, thyroid, vascular smooth muscle and striated muscle which suggests a role in global metabolic function. These proteins are implicated in the regulation of insulin secretion by modulation of glutamate dehydrogenase. Taken together SIRT4 inhibition increase fat oxidative capacity in liver and mitochondrial function in muscle, which could provide beneficial in developing therapeutic for many diseases such as type2 diabetes (T2DM) (Nasrin *et al.*, 2010).

2.4.1.5 SIRT5

The human SIRT5 gene is located at Chr 6p23, and the gene product functions as a mitochondrial NAD+-dependent deacetylase with well-defined substrates (Vassilopoulos *et al.*, 2011). SIRT5 plays a role in controlling cellular function by two isoforms with different intracellular localizations or stabilities (Matsushita *et al.*, 2011). The mitochondrial proteins can be found both in the cytoplasm

and nucleus, which proteins are released into the cytoplasm by apoptotic stimuli. The signal of apoptotic stimuli is induced by Cytochrome c, AIF and SMAC/Diablo. This difference in intracellular localization is because of the number of amino acid residues in their C-termini, 25 amino acids for each isoforms. Two isoforms of SIRT5 as $SIRT5^{iso1}$ and $SIRT5^{iso2}$, which are generated from the SIRT5 gene locus by alternative splicing (Matsushita *et al.*, 2011). As SIRT5 found in mitochondria that involve ATP

2.4.1.6 SIRT6

The human SIRT6 gene is located at Chromosome 19p13.3 (Vassilopoulos *et al.*, 2011). Initial investigation into SIRT6 activity supported a lack of NAD+-dependent protein deacetylase activity and showed that it is a broadly expressed nuclear ADP-ribosyltransferase. SIRT6 is predominantly a nuclear chromatin-associated protein which aids in the protection of DNA damage and suppresses genomic instability through association with base-excision repair and DNA-end resection (Mostoslavsky *et al.*, 2006). The Sirt6 protein is a nuclear protein and associatation with chromatin, which identified as ADP-ribosyltransferase. SIRT6 play a role in metabolic stress which associate metabolic syndrome. The metabolic syndrome is main epidemics of the twenty-first century which include a cluster of disorders comprising abdominal obesity, dyslipidemia, glucose intolerance, insulin resistance, and premature appearance of age-related diseases, such as diabetes type II, hypertension, generalized inflammation, and a propensity towards developing neurodegenerative diseases (Kanfi *et al.*, 2010).

2.4.1.7 SIRT7

The human SIRT7 gene is located at Chromosome 17q25.3 (Vassilopoulos *et al.*, 2011). SIRT7 is a broadly expressed protein, which play roles

with heterochromatic regions and nucleolus. SIRT7 expression is founded in the cell nucleus (56.5%), cytoplasm (26.1%), mitochondria (13.0%) and cytoskeleton (4.3%). Interestingly, silencing of SIRT7 expression stops cell proliferation and triggers apoptosis. SIRT7 also directly interacts with the rDNA transcription factor, upstream binding factor (UBF) (Vassilopoulos *et al.,* 2011). Human SIRT7 is involved in pathogenesis of hematological malignancies and acute myeloid leukemia. Recently studies, the SIRT7 protein could potentially be either missing, dysfunctional or exhibitits enzymatic activity at wrong times in the wrong places and therefore contribute to an imbalance of the intracellular acetylation status and to the development of disease (Dryden *et al.,* 2003). Additionally, SIRT7 expression is demonstrated in the tumorigenesis potential of numerous mouse cell lines (Figure 2.13). The result suggested that SIRT7 can maintain a host of critical metabolic pathways through the inhibition of cell growth under stress environment (Vakhrusheva *et al.,* 2008).

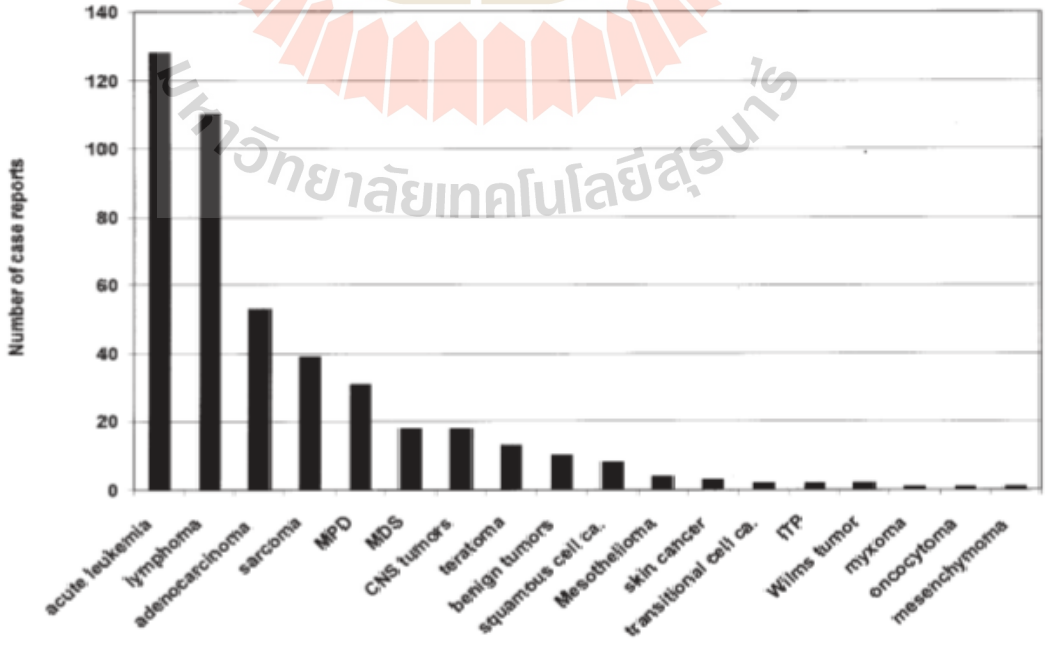


Figure 2.13 The chromosome region, 17q25.3, is a section which has been found to be involved in various malignancies (Mahlknecht *et al.*, 2006).

2.4.2 The role of SIRT1 in cellular regulation

SIRT1 can deacetylate a variety of substrates which involved in a biological system, including control of gene expression, metabolism and aging (Rahman and Islam, 2011). SIRT1 catalyzes an enzymatic reaction that generates nicotinamide by independent from NAD+ and the acetyl group of the substrate is transferred to cleaved NAD, which can generating a distinct metabolite, O-acetyl-ADP ribose (Figure 2.14) (Pillarisetti, 2008). The list of SIRT1 substrates is continuously growing and includes several transcription factors: the tumor suppressor protein p53, members of the FOXO family (forkhead box factors regulated by insulin/Akt), HES1 (hairy and enhancer of split 1), HEY2 (hairy/enhancer-of-split related with YRPW motif 2), PPARγ (peroxisome proliferator-activated receptor gamma), CTIP2 [chicken ovalbumin upstream promoter transcription factor (COUPTF)- interacting protein 2], p300, PGC-1a (PPARγ coactivator), and NF-ĸB (nuclear factor kappa B) (Rahman and Islam, 2011). SIRT1 can also indirectly modulate critical players involved in insulin signaling and lipid metabolism including AMP-activated protein kinase (AMPK), PPARγ, PTP-1B and UCP2.

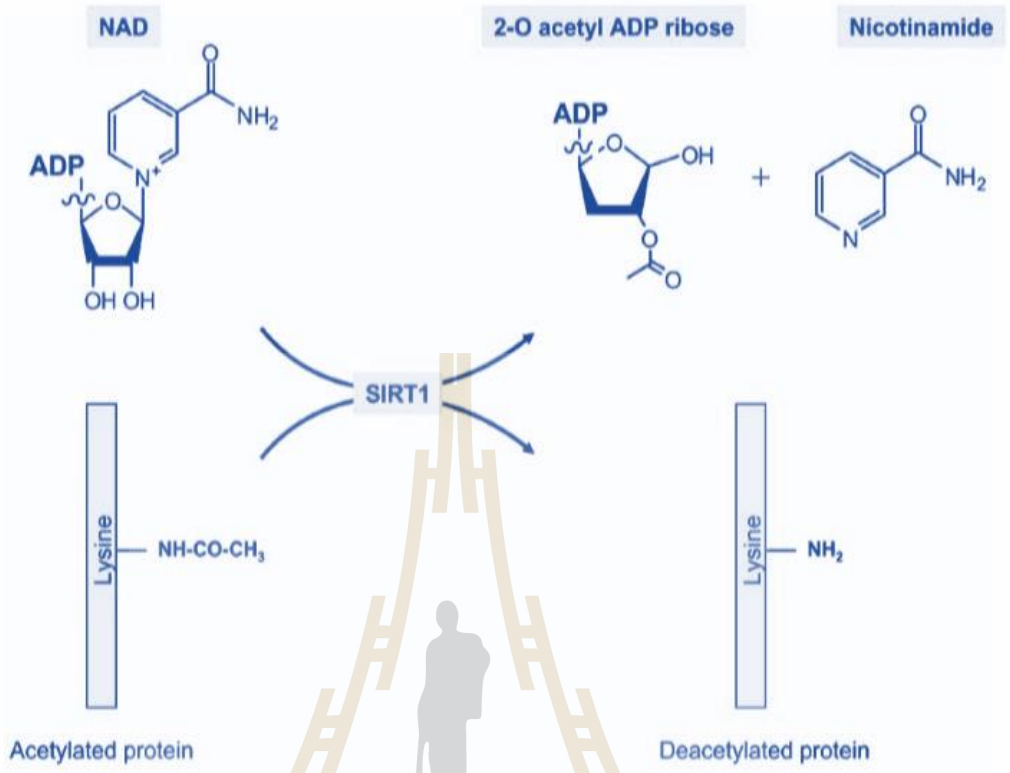


Figure 2.14 Deacetylation reaction catalyzed via SIRT1 (Pillarisetti, 2008).

SIRT1 is regulates aging and longevity in higher mammals that has been shown to regulate metabolic response to change in nutrient availability and calories restriction. For, SIRT1 is tested the role in age-related disease, cognitive functioning, and mortality in human. These finding suggested that SIRT1 play a vital role in a broad spectrum of biological processes. SIRT1 function to slow aging and various disorders associated with aging including metabolic diseases, cancer, and neurodegenerative conditions (Chang and Guarente, 2015). SIRT1 is linked with Wallerian regeneration, age-related neurodegeneration, Alzheimer's disease, and Parkinson's disease. Spontaneous immortalization of these cells restored the level of SIRT1 protein to that of early passage, highly proliferative mouse embryonic fibroblast (MEFs) (Milner, 2009). Interestingly, the phosphorylation status of SIRT1 is determined the associate

with JNK2 that plays role in the inverse correlation between SIRT1 protein level and senescence. In addition, increased SIRT1 activity also promotes hepatic gluconeogenesis and inhibits glycolysis via peroxisome proliferator-activated receptor c co-activator 1-α (PGC1-α) during fasting (Kuningas *et al.*, 2007). Fasting is mediated the metabolic switch by SIRT1. Fasting also associates muscle and liver oxidation of fatty acids produced by lipolysis in adipose tissue. Recently reports suggest SIRT1 interacts with deacetylates PGC1-α at specific lysine residues in an NAD1 dependent manner. SIRT1 induces glucogenesis genes and hepatic glucose output through PGC1-α, but does not regulate the effect of PGC1-α on mitochondrial gene. Inaddition, SIRT1 modulates the effects of PGC1-α repression of glycolytic genes in response to fasting and pyruvate (Rodger *et al.*, 2005). Furthermore, SIRT1 has an effect on fat metabolism, via inhibition of peroxisome proliferator-activated receptor c (PPARγ) (Kuningas *et al.*, 2007). In addition, SIRT1 appears to be important for the development of heart, as SIRT1 knockout mice presented cardiac abnormalities.

2.5 Micro-RNA

MicroRNAs or miRNAs are endogenous 20-23 nucleotides that regulate post-transcriptional repression by targets the complementary messenger-RNA (mRNA). miRNAs a class of non-coding RNAs, play role in animal and plant. Their major roles of miRNAs is in posttranscriptional regulation of protein expression regulate the cleavage of target mRNAs or just repress their translation. The number of identified miRNA gene has surpassed 110 in the nematode (*Caehabditis elegans*, 140 in the fly *Drosophila melanogaster* and 400 in humans. The nematode is initially discovery of miRNAs that found lin-4 and let-7, development through incomplete base pairing to the

3’untranslated regions (3’UTRs) of the target mRNAs to repress their translation (Cai *et al.*, 2009). The regulatory role of miRNAs associate with cell differentiation, apoptosis, proliferation, tumorigenesis and host-pathogen interaction.

miRNAs are named with the prefix ‘mir’ followed by a dash and a number. As well as, hsamiR-25 involve esophageal cancer (EC), which ‘hsa’ represent the species Homo sapiens. Next, ‘miR’ (with capital ‘R’) for mature miRNA and mir (with small ‘r’) for pri-miRNA, pre-miRNA or genomic locus. The abbreviation of pri-miRNA is instead primary microRNA transcript, are cleaved to a shorter sequence called the ‘microRNA precursor’ (pre-miRNA). The short sequence pre-miRNA displays a hairpin-like secondary structure.

2.5.1 Mechanism of miRNAs

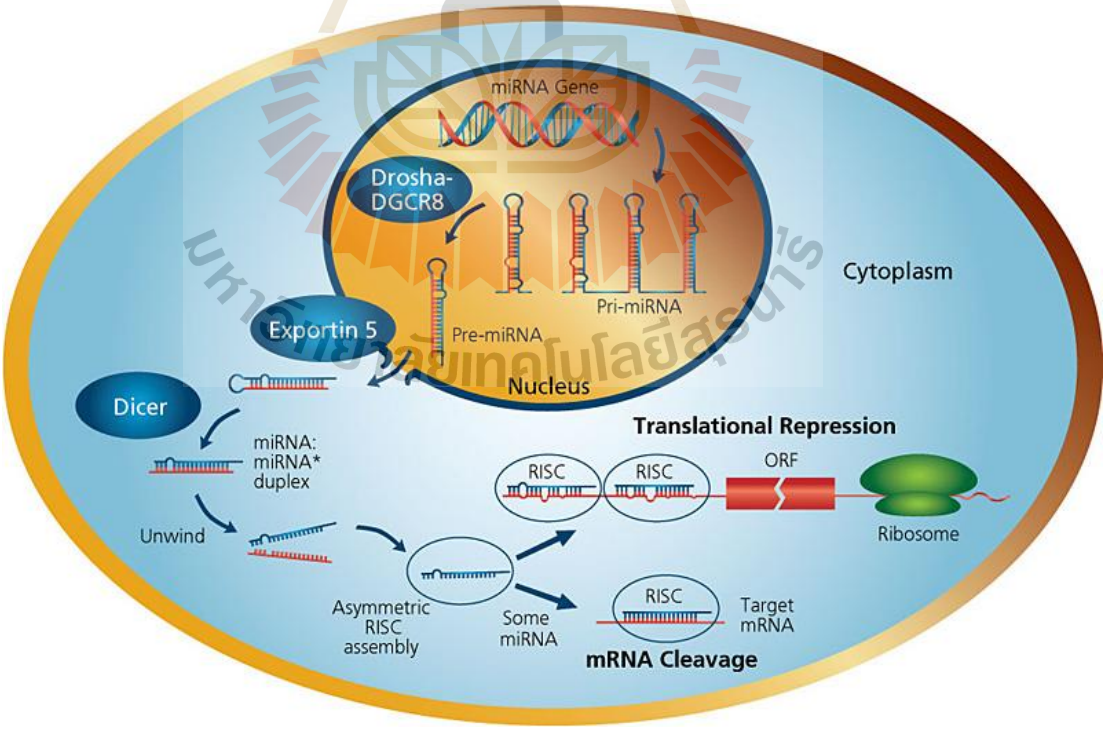


Figure 2.15 The mechanism of miRNA.

As known, miRNAs function as posttranscriptional regulators of gene expression by interacting with target mRNAs (Neudecker *et al.*, 2016). Majority of miRNAs originate from independent loci in the genome but some are processed from within the introns and exons (Ramanathan *et al.*, 2018). miRNA genes are transcribed by RNA polymerase II into a long transcript called primary microRNA transcript (pri-miRNA). For miRNA genes organized in clusters, the pri-MiRNA can be monocistronic or polycistronic. Within the nucleus, the DROSHA and DGCR8 (known as Pasha in *D. melanogaster* and *C. elegans*) microprocessor complex cleaves the pri-miRNA into a shorter sequence, called the 'microRNA precursor' (pre-miRNA), which displays a hairpin-like secondary structure (Desvignase *et al.*, 2016). The pre-miRNA is translocated to the cytoplasm by Exportin-5 in a complex with RANGTP, and then DICER1, an RNase III enzyme (hereafter called Dicer), cleaves off the loop of the pre-miRNA and generates a mature miRNA duplex (Desvignase *et al.*, 2016). The two strands of the miRNA duplex pair has one strand is known as the guide-strand. This strand is incorporated into the RNA-induced silencing complex (RISC), other strand is degraded. The mature miRNA as part of the RISC, bind the 3'UTR of specific messenger RNAs and mediates mRNA degradation, destabilization, or translational inhibition according to sequence complementarity to the target (Bartel, 2009) (Figure 2.15).

2.5.2 The biological function of miRNAs

The mechanism of miRNA is studies to understand function that regulate miRNA biogenesis. MiRNAs have predicted tendency to multiple target gene with related functions, which can provide insight into biological roles of these miRNAs. These were demonstrated to act on several key biological function such as cell

differentiation, cell cycle arrest, cell proliferation and cell death. MiRNAs control the target expression by base pairing to sequence motifs in the 3’ UTR of mRNAs with perfect or near perfect complementarities (Cai *et al.*, 2009). In cancer, miRNAs plays role in oncogene and tumor suppressor gene. Otherwise, the tumor protein p53 modulate p68 and Dorcha-mediated miRNA processing. Both Drosha and p68 associate with miRNAs and their processing is enhanced in the presence of the p53 transcription factor (Shukla *et al.*, 2011). These report can suggested that why TDNA damage leads to posttranscriptional induction of several miRNAs as p53 is induced in DNA damage. The activation of miRNAs active certain mRNA targets in stress conditions such as hypoxia and nutrient deprivation. Whether these up-regulation phenomena happen in special conditions or in ubiquitous regulatory mechanisms remains to be further illustrated (Cai *et al.*, 2009).

2.5.3 miRNAs in therapeutic medicine

miRNAs regulate posttranscriptional gene expression and inhibit protein translation under cellular processes, cellular homeostasis and diseases. These regulations are responsive to pharmacological intervention and miRNA expression is modified by various pharmacological agents. Dysregulation of miRNAs may alter gene networks in disease states such as, metabolic diseases, cancers, neurodegenerative diseases; thus, miRNA therapy could restore gene expression in the cells to reverse back in normal state (Kandhro, 2016). The level of miRNA can influence drug efficiency and toxicity. Thus, the involvement of miRNA level in key biological processes can be used to biomarker for predicting efficacy and toxicity of drug. Promising preclinical studies in the biomedical and clinical field demonstrated constructive therapeutic strategy for treating disorders ranging from metabolic diseases, cancers, neurodegenerative

disorders to organ failure, although several challenges related to tissue specificity, targeted delivery remain to be overcome. Moreover, ratio of successful experimental studies is low, due to expensive technologies and approaches used in miRNA discovery, identification of biomarkers and therapeutic markers. Until now, miRNAs have been extensively studying, but their specific functions remain largely unknown (Kandhro, 2016). In addition, elucidating the mechanistic significance of miRNA alterations can aid in the identification of therapeutic targets, patient stratification and personalizing therapy (Ramanathan *et al.*, 2018).

CHAPTER III

MATERAIL AND METHOD

3.1 Materials

Human dermal fibroblasts [AG07871(A)] was obtained from cell based assay and innovation (CBAI), biotechnology, SUT. SHSY-5Y cells were purchased from ATCC (www.atcc.org). The hTERT (pBABE-neo-hTERT) and SV40 large T antigen (pBABE-neo largeTcDNA) plasmid were purchased from Addgene, Cambridge, MA.

3.2 Methods

In this study the human dermal fibroblasts were immortalized by using hTERT (human telomerase reverse transcriptase), SV40 large T antigen and co-transfection compare three conditions. The immortalized cells were characterized gene and protein expression during cell play role in immortalization. Figure 3.1 was presented the overall in this study.

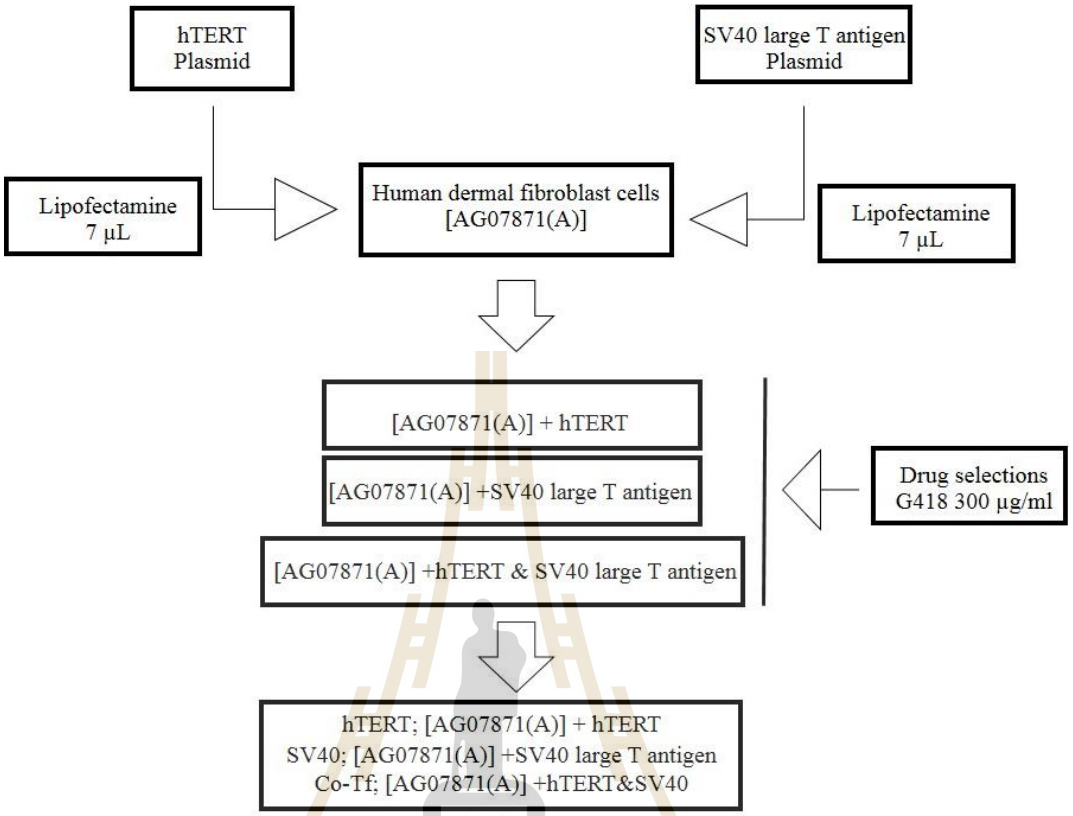


Figure 3.1 The overall of cell immortalize technique.

3.2.1 Cell culture

Human dermal fibroblast cell lines (HDFs) were stored in liquid nitrogen (stock cell). Thawing cells from stock, were washed twice with 1X Phosphate Buffered Saline (1X PBS, pH7.4) and seeded into T25 flask (NEST). The cells were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM/HG; Hyclone, Logan, UT, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco BRL, Grand Island, NY), 1mM L-glutamine, 1 mM Minimal essential medium (MEM; Sigma-Aldrich, St. Louis, MO, USA), and 100 U/ml Penicillin, and 100 g/ml Streptomycin (Sigma-Aldrich) incubate at 37°C 5% CO_2 (Thermo scientific CO_2incubator, USA).

3.2.2 Immortalization process

To immortalize HDFs for increase the proliferative capacity, were transfected by plasmid hTERT (pBABE-neo-hTERT; Addgene, Cambridge, MA, https:// www.addgene.org) (Figure 3.2) and SV40 large T antigen (pBABE-neo largeTcDNA; Addgene, Cambridge, MA, https:// www.addgene.org) (Figure 3.3). These cells were seeded on 6-well plate density 2.5 x 10^5 cells per well. In the same time, hTERT and SV40 large T antigen plasmid 1 µg were prepared in medium free serum (0% DMEM) by incubate with Lipofectamine2000 (Invitrogen) 7 µl incubate at room temperature (RT) 30 mins. Then, after cells reach 70% confluence, the cells were added reagent containing plasmid and lipofectaminje2000, incubate at 37°C 5% CO_2 for 24 h. After another 24 h, neomycin-resistance cells or geneticin were selected by adding 300 µg/mL G418 sulfate (Invitrogen) into the medium. G418-resistance cells were isolated and subculture.

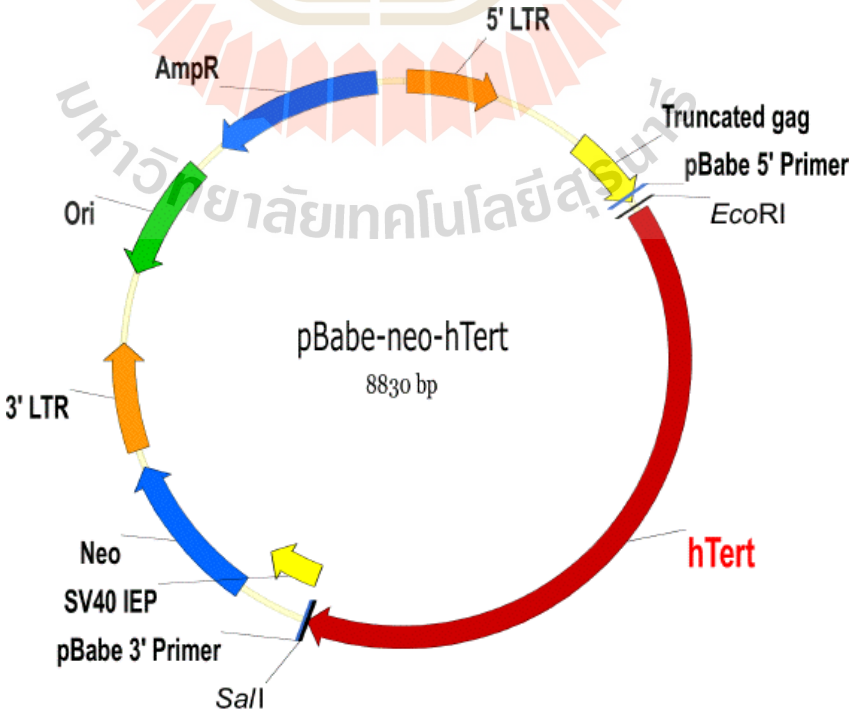


Figure 3.2 Map image of pBABE-neo-hTERT.

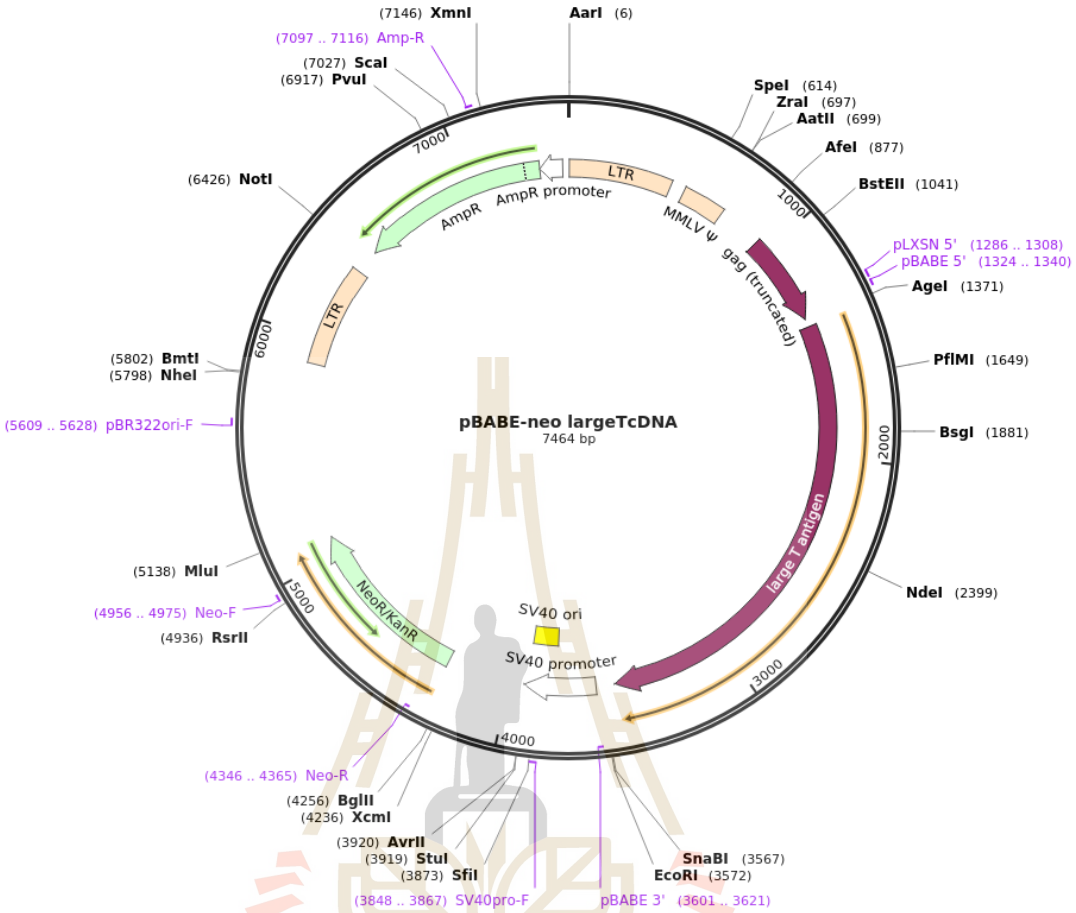


Figure 3.3 The sequence map image of pBABE-neo largeTcDNA.

3.2.3 Proliferation rates

Measurement of cell viability and cell proliferation via MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide). The cells approximately 3 x 10^3 cells were seeded on 96-well microplates (NEST) and cultured in 10 per cent DMEM for 9 days. Cells were counted every 48 h until 9 days. At each time point, cells were discarded medium, washed twice 1X PBS and added MTT 0.5 mg/µl 200 µl incubated 2 h at 37°C 5% CO_2. After 2 h, cells were discarded MTT reagent and added DMSO (dimethyl sulfoxide) 200 µl. The microplate was measured absorbance at 570 nm in a microplate reader (SPECTROstar nano, Germany).

3.2.4 Gene and miRNA expression

Total RNA from the cells was extracted by using NucleoSpin RNA kit (Macherey-Nagel, Dueren Germany) in accordance with the protocol of the manufacturer. For, miRNAs were extracted from dermal fibroblast by miRNeasy minikit (QIAGEN, Germany) in accordance with the protocol of the manufacturer. The OD260/280 ratio of the RNA samples was measured, and the samples with a ratio of 2.0 were used for reverse transcription. In total, 0.5 mg RNA was reverse-transcribed by using ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). The 4X DNA master mix reactions were performed at 65°C for 5 min and then add 5X RT master mixes were performed at 37°C for 15 min, 98°C 5 min.

3.2.4.1 Reverse transcriptase PCR (RT-PCR)

Addition, cDNA was determined by RT-RCR and shown in Table 3.1 including *KRT18, Vimentin, Elastin, COL1A1* , *p53, p21* and *SIRT1*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as the endogenous housekeeping gene. Then, PCR product was separated by gel electrophoresis (1-1.5 % agarose gel, Vivantis, Maleysia), 100 volt using time 30 min. After, gels were detected by Gel Doc machine (Gel Doc EZ Imager, BIO-RAD, U.S.A) and measured gene expression levels.

Table 3.1 Primer sequences

Gene	Sequence (5'→ 3')
GAPDH	Forward: 5'- TCACCACCACGGCCGAGCG -3'
	Reverse: 5'- TCTCCTTCTGCATCCTGTCG-3'
Vimentin	Forward: 5' CCAGGCAAAGCAGGAGTC 3'
	Reverse: 5' CGAAGGTGACGAGCCATT 3'

Table 3.1 continued.

Gene	Sequence (5'→ 3')
Elastin	Forward: 5' CCGCTAAGGCAGCCAAGTATGGA 3'
	Reverse: 5' AGCTCCAACCCCGTAAGTAGGAAT 3'
COL1A1	Forward: 5' GGGCAAGACAGTGATTGAATA 3'
	Reverse: 5' ACGTCGAAGCCGAATTCCT 3'
COL3A1	Forward: 5' AGGTCCTGCGGGTAACACT 3'
	Reverse: 5' ACTTTCACCCTTGACACCCTG 3'
p53	Forward: 5'- CCCCTCCTGGCCCCTGTCATCTTC-3'
	Reverse 5'- GCAGCGCCTCACAACCTCCGTCAT -3'
P21	Forward: 5' CATGTGGACCTGCACTGTCTTGTA 3'
	Reverse: 5' GAAGATCAGCCGGCGTTTG 3'
SIRT1	Forward:5' TGCTGGCCTAATAGAGTGGCA 3'
	Reverse: 5' CTCAGCGCCATGGAAAATGT 3'
KRT18	Forward: 5' CAAAGCCTGAGTCCTGTCCTT 3'
	Reverse: 5' CTCCCGGATTTTGCTCTCCA 3'
B-actin	Forward:5' CTCTGCTCCTCCTGTTCGAC 3'
	Reverse:5' TTAAAAGCAGCCCTGGTGAC 3'
Tel	Forward: 5' TGTGCACCAACATCTACAAG 3'
	Reverse: 5' GCGTTCTTGGCTTTCAGGAT 3'
Ki67	Forward: 5' TGTATCCTTTGGTGGTCGTCTA 3'
	Reverse: 5' GCTGGAGTGTGAGTGGTGAG 3'
36B4A	Forward: 5' AAG CGC GTC CTG GCA TTG TCT 3'
	Reverse: 5' CCG CAG GGG CAG CAG TGG T 3'

3.2.4.2 Quantitative PCR (qPCR)

After RNAs and miRNAs were converted to cDNA by reverse transcription. The expression level of *Tel*, *36B4*, *Ki67*, and *β-actin* were examined. The primers for reference gene β-actin and primers design for interesting genes were shown in Table 3.1. The QuantStudio 5 Real-Time PCR System (Thermo fisher scientific) were used to carry out Real time PCR experiments which were conducted in 96-well plates. To monitor cDNA amplification, qPCRBioSyGreen Mix Low-Rox (PCR BIOSYSTEMS, London, UK) were used in a 20 µl reaction volume. Cycling conditions according to the specific SYBRGreen protocol were: 95 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 45 sec (40 cycles). Melting curve analysis of the PCR products were performed by heating at 60 °C for 60 sec, 95 °C for 15 sec, and continuous measurement of the fluorescence to verify the PCR product. Relative expression was determined by 2^ (-delta delta C(T)) method with the expression of β-actin as housekeeping reference gene.

For miRNAs, were reviewed and 5 miRNAs were analyzed by quantitative PCR technique which the level expression in immortalized cells containing miR-22, miR-93, miR-217, miR-34a, miR-449 and U6. Primers were designed and shown sequence in Table 3.2. The amplification steps consisted of denaturation at 95 ◦C, followed by 40 cycles of denaturation at 95◦C for 15 s and then annealing at 60◦C for 1 min. Relative expression was determined by 2^ (-delta delta C(T)) method with the expression of U6 as housekeeping reference.

Table 3.2 Primer sequences of miRNA

miRNA	Sequence
U6	5’CGCAAGGATGACACGCAAATTC
miR22	5’AAGCTGCCAGTTGAAGAACTGT
miR34a	5’TGGCAGTGTCTTAGCTGGTTGT
miR93	5’CAAAGTGCTGTTCGTGCAGGTAG
miR217	5’CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTTGGC ATT
miR449	5’TGGCAGTGTATTGTTAGCTGGT

3.2.5 Immunofluorescence staining

For immunofluorescence analysis, cells were cultured on coverslips in 24-well plates and fixed in 4% Paraformaldehyde (PFA) in 1X PBS for 20 minutes at RT. The fixed cells was washed twice 1X PBS and blocked in blocking solution supplemented with 1% Bovine serum albumin (BSA, EMD Millipore, and U.S.A.), 0.2% TritonX-100 and dilute in 1X PBS. After incubation 30 minutes in blocking solution at 4°C, the cells were incubated in blocking buffer containing primary antibodies (anti-Collagen1 Type I (1:500; MERCK), anti-SIRT1 (1:200; DSHB) and anti-Vimentin (1:500; DSHB) overnight at 4°C. The cells were washed in washing solution supplemented 0.2% TritonX-100 in 1X PBS. Then, cells were incubated in secondary antibodies anti-mouse (1:500; Invitrogen) and anti-rabbit (1:500; Invitrogen) for 2 h at RT. Next, cells were washed 1X PBS 5 times each 5 min, nucleus cells was stained with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen) on the slide, and were coated by nail polish on the coverslips. Slide was observed using fluorescence microscope (ZOE ^{TM} Fluorescent Cell Imager Bio-Rad Laboratories,CA, USA).

3.2.6 Soft agar assay

The immortalized HDFs were investigated tumor formation after plasmid transfection. The soft agar assay can detect colony formation for malignant and tumor immortalized HDFs. The cells were cultured in Dulbecco's modified Eagle's medium DMEM supplemented with 10% FBS, 1% penicillin and 1% NEA incubate at 37°C 5% CO_2. Base agar was prepared 1% agarose gel and malted in microwave 2 min at 700 Watt. The medium 2X DMEM were prepared contain 20% FBS, 1% l-glutamine, 1% penicillin and 1% NEA blend with 1per cent agarose gel and were placed into 35 mm petri dish. Top agar was prepared 0.3% agarose gel mix with 2X DMEM and 20% FBS. The immortalized cells were counted ~5000 cells/plate and blended in recipe 2X DMEM. The cells in recipe were transferred on base agar incubate 37°C 5% CO_2 for 10-30 days. During 10-30 days, the cells were seeded on top agar every 5 days. After 15-30 days, the soft agar was stained via 0.005% crystal violet for 1 h at RT and counted colonies formation using microscope (Nikon, Japan).

3.2.7 Knockdown SIRT1 expression by shRNA

3.2.7.1 shRNA preparation

The oligonucleotide was designed by using Basic local alignment search tool (NCBI database, https://blast.ncbi.nlm.nih.gov/Blast.cgi) and show in Table 3.2. The pSUPERIOR.neo vector (OligoEngine) (Figure 3.4) was used vector for insert oligonucleotide (shRNA1 and shRNA2), the 5' end corresponds to the BglII site while the 3' end contains the T5 sequence and any HindIII corresponding nucleotides. First, the oligonucleotides were annealed in annealing buffer incubate 90°C for 4 min, then at 70°C for 10 min. Slowly cool the annealed oligo to 10°C. Second, linearize the vector with BglII and HindIII restriction enzyme (NEW ENGLAND BioLabs; NEB) incubate 37°C 3 h and heat inactivate 80°C for 20 min.

Third, ligation to ligate oligo into pSUPRIOR vector ratio 3:1 in reaction supplemented T4 DNA ligase buffer (NEW ENGLAND BioLabs; NEB), T4 DNA ligase and nuclease-free water incubate 37°C 10 min, 65°C 10 min.

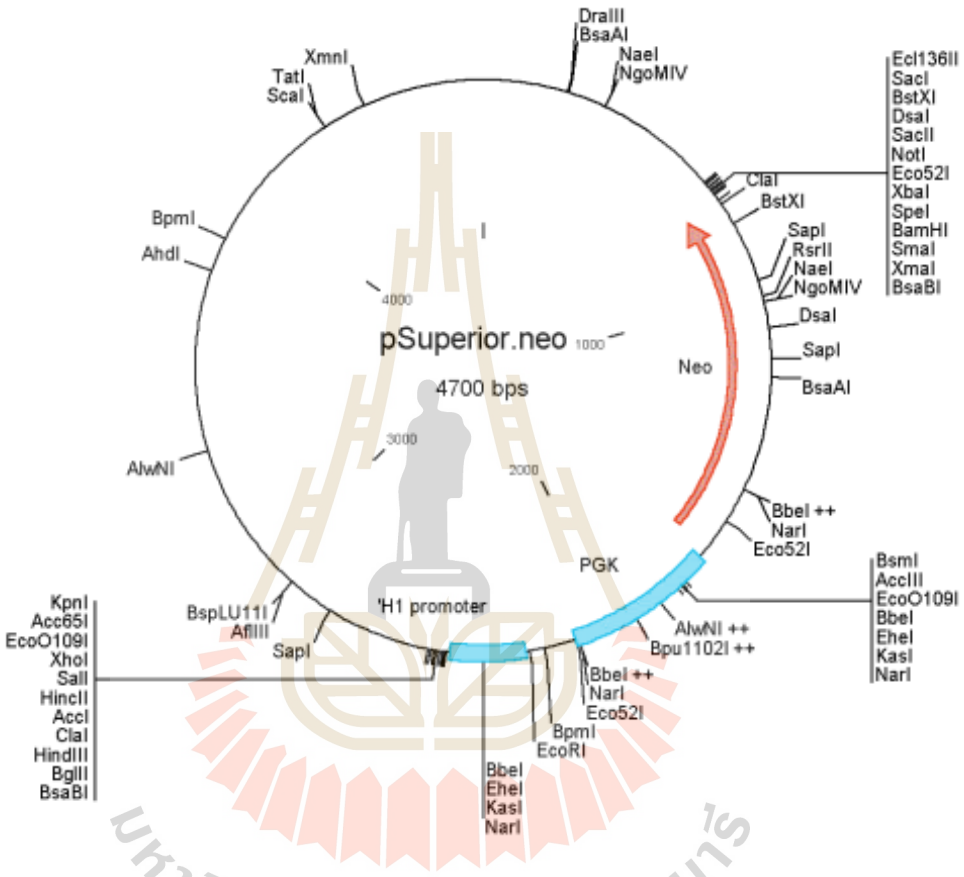


Figure 3.4 Image map of pSuperior.neo vector.

3.2.7.2 Vector amplifacation

The vector contain insert was amplified by transformed in bacteria (competent cells), DH5α was used in this experiments. The oligo ligated were transformed in DH5α competent cell incubate on ice 30 min, then heat shock 42°C 50 sec and on ice 2 min. The mixture was added lysogeny broth (LB) shake 200 rpm 37°C for 60 min and spread plate on lysogeny agar plate contain ampicillin incubate 37°C for 16 h. The colonies on agar were selected and picked up into lysogeny broth contain

ampicillin shake 200 rpm 37°C for 16 h. Plasmid was extracted from bacteria using plasmid extraction kit (GF-1 plasmid DNA extraction kit, vivantis).

Table 3.3 The shRNA design

shRNA	**Sequence**
shRNA 1	Forward:5'GATCCCCCTGAAGATGACGTCTTATCCTTTCAAGA GAAGGATAAGACGTCATCTTCAGTTTTTA3' Reverse:5'GGGGACTTCTACTGCAGAATAGGAAAGTTCTCTTC CTATTCTGCAGTAGAAGTCAAAAATTCGA3'
shRNA 2	Forward:5'GATCCCCAACAAATCATAGTGTAATAATTTCAAG AGAATTATTACACTATGATTTGTTTTTTTA3' Reverse:5'GGGTTGTTTAGTATCACATTATTAAAGTTCTCTTA ATAATGTGATACTAAACAAAAAAATTCGA3'

3.2.8 Statistic analysis

Data were coded and entered using the statistical package SPSS version 15. Data were summarized using mean, standard deviation and % change for the quantitative variables. Comparisons between groups were done using analysis of variance (ANOVA) for the quantitative variables. P-values less than 0.05 were considered as statistically significant.

CHAPTER IV

RESULTS AND DISCUSSION

4.1 The immortalized HDFs increase proliferation and maintain telomere length

4.1.1 The morphology of immortalized cells

Human dermal fibroblasts (AG07871 (A)) were successfully immortalized by plasmid transfection of hTERT and SV40 large T antigen. The three conditions including hTERT-transfected alone, SV40 large T antigen-transfected alone and co-transfection hTERT with SV40 large T antigen in ratio 1:1. Human dermal fibroblasts passage 20 were used to transfected plasmid. The aim of immortalized cells was to increase proliferate of cells and maintain telomere length. The cells were transfected by using lipofectamine 2000 to transfected plasmid into human dermal fibroblasts. Lipofectamine 2000 has lipid subunits to help form liposome in an aqueous environment, which entrap the transfection. After transfection, cells were selected by geneticin (G418-drug selection) 24 h in DMEM-free serum. The cells without plasmid were usually died and floating in DMEM. In plasmid contain neomycin-resistance insert for select cell that were transfected plasmid. After drug selection, cells were discarded selective medium and cultured 95% confluents, during culture 10% DMEM was changed every 2-3 days. Then, cells were sub-culture to T25 and T75 flasks respectively, for expansion. The transfected cells were demonstrated and characterized proliferation, mechanisms, telomere length , gene and protein expression, tumor

properties and miRNAs. First of all, cells were sub-cultured over 60 passage after transfected plasmids. The morphology of cells was observed under microscope, human dermal fibroblasts passage 20 show the spindle shaped approximately 100-150 micro mates (µm) in Figure 4.1. The 'spindle-shaped' of fibroblasts are membrane-bound collagen fibrils (Madrid *et al*., 1981). As known, fibroblasts require the presence of serum (fetal bovine serum) in culture medium at the concentration of 10 to 20 per cent. The concentration of serum have an effect on proliferative life span. Fibroblasts have a finite number of divisions, which called process of a replicative senescence. These cells were cultured passage 30 and slow down into senescence.

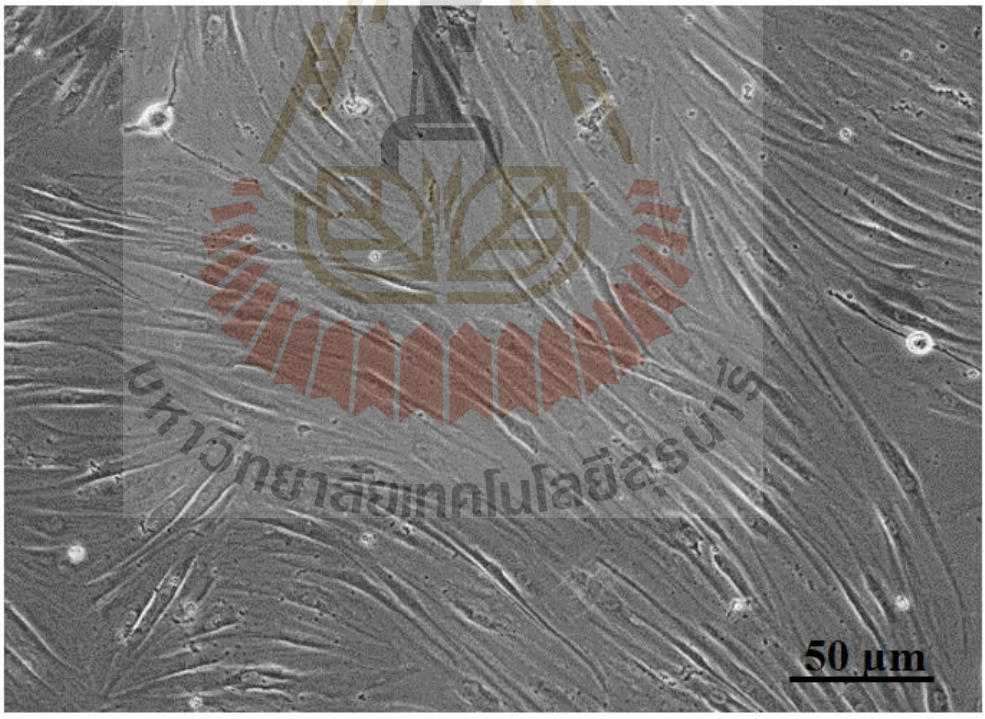


Figure 4.1 Human dermal fibroblasts show the spindle-shaped.

On the other hands, hTERT-transfected cells obviously change the morphology in passage 5, which counted passage 1 after drug selection (Figure 4.2A). The ectopic expression of hTERT plasmid used to create immortalized cells, which

play role through the elongate of telomeres. The catalytic subunit of enzyme telomerase has been implicated as an important participant in the immortalization process (Counter *et al*., 1998). The hTERT-transfected cells after transfect show the spindle-shaped and rapidly decrease the number of cells (Figure 4.2B). Until, cells grow 95% confluents and show clumping spindle-shaped and cytoplasm was reduced (Figure 4.2C). The cell association or cell to cell attachment is important role in determining the shape of the cell. Normally, these cell of fibroblasts show spindle-shaped and distance the surface area with neighbors. Interestingly, the studies about the DNA tumor-virus encoded oncoproteins of simian virus 40 (SV40). The large T antigen of simian virus 40 (SV40), the adenovirus E1A and E1B proteins, and the E6 and E7 proteins of certain strains of human papillomavirus (HPV). These viral proteins distribute the ability to bind and inactivate the retinoblastoma (pRB) and p53 tumor suppressor proteins (Hahn *et al*., 2002). The ectopic expression of SV40 large T antigen has been reported the stimulation of cell proliferation, permits anchorage-independent growth, and confers increased resistance to nutrient deprivation. The morphology of SV40 large T antigen-transfected cells show spindle-shaped in passage 1 (Figure 4.3A) and the number of cells were increase which show triangle-shaped with spindle-shaped in passage 3 (Figure 4.3B). However, cells distance change the morphology to triangle-shaped in passage 5 (Figure 4.3C). The gap of cell is large and cytoplasm decrease that show size of cell approximately 20 to 30 µm. Both two plasmids have the similar properties for create immortalize cells. Therefore, the co-working hTERT and SV40 large T antigen are demonstrated about the relation mechanism. After transfection in ratio 1:1, these cells show the spindle-shaped in passage 1 (Figure 4.4A) like the cell morphology of SV40 large T antigen-transfected Sub-culture to passage 3 show the spindle-shaped and triangle-shaped (Figure

4.4B).The clumping of triangle-shaped is the important change a decrease of cytoplasm. Figure 4.4C show a passage 5 is lost group of triangle-shaped, it's loose and increase the gap between cell-to-cell attachments.

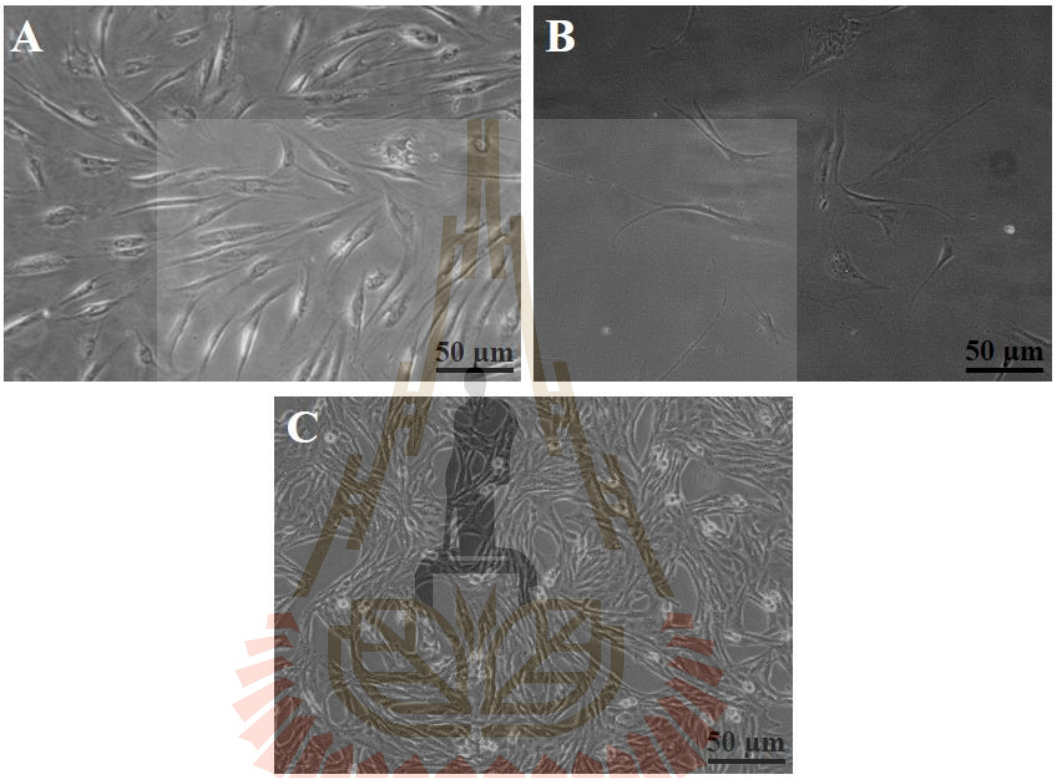


Figure 4.2 The hTERT-transfected cells (A) sub-culture in passage 1 (B) passage 3 (C) passage 5.

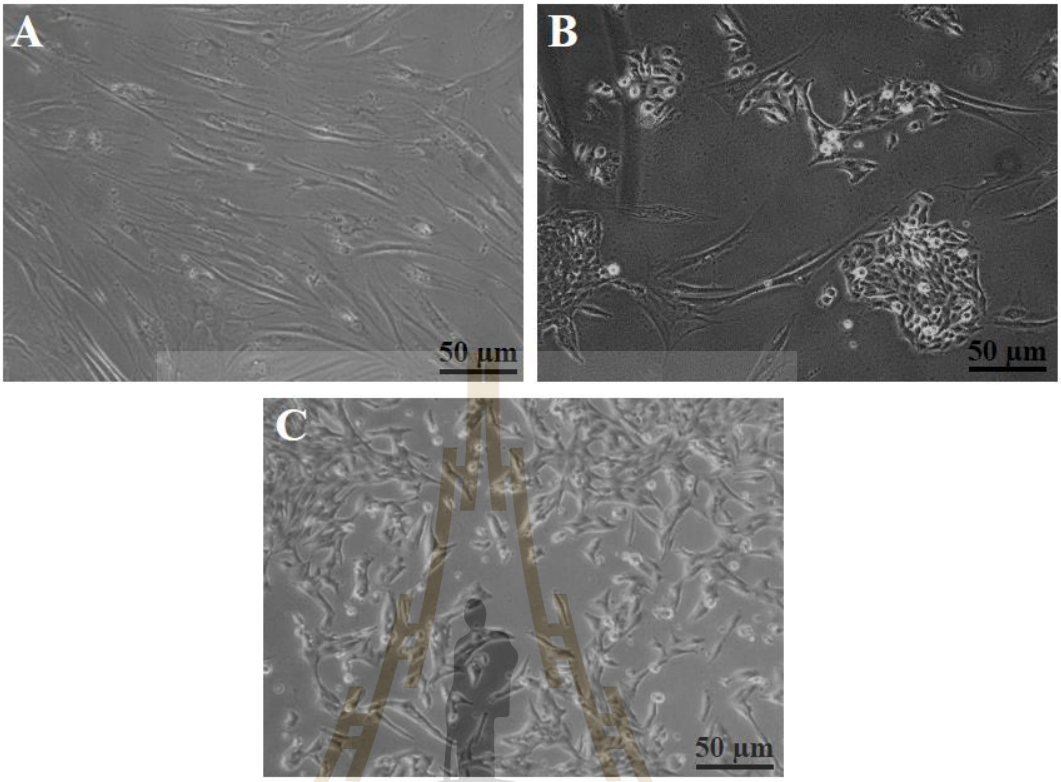


Figure 4.3 The SV40 large T antigen-transfected cells (A) sub-culture in passage 1 (B) passage 3 (C) passage 5.

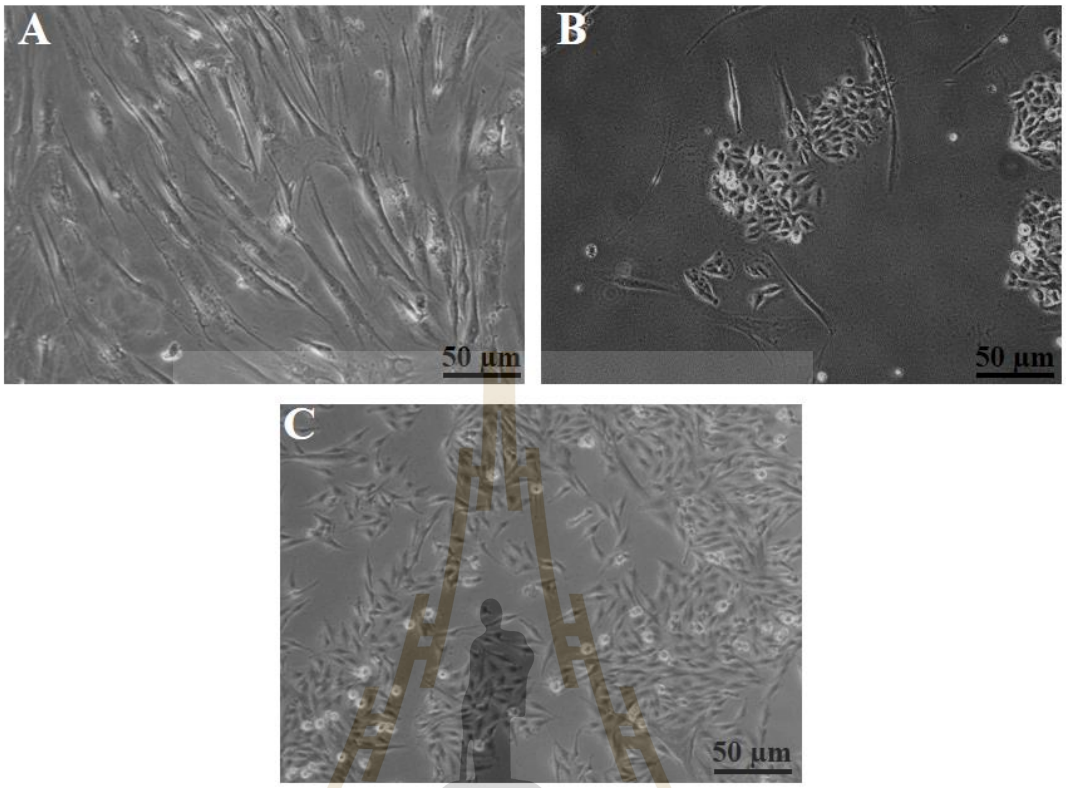


Figure 4.4 The co-transfection hTERT and SV40 large T antigen ratio 1:1 (A) sub-culture in passage 1 (B) passage 3 (C) passage 5.

4.1.2 The cell proliferation

To investigate the cell proliferation of transfected-cells compare with normal human dermal fibroblasts, called control (Ctrl). The 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide or MTT assay is used to demonstrate doubling time and proliferates. The cells were seeded into 96-well plate approximately 3,000 cell/well. Cells were cultured in well and detect the number of cells by MTT assay at various times points 2, 4, 6 and 8 days respectively. Every 2 days, cells were measured relative cell growth show in Figure 4.5. The result is shown highly proliferate of hTERT-transfected cell (hTERT) at day 6 and 8. Surprisingly, the day 2 and 4 of cell culture is shown increasing of SV40 large T antigen (SV40), hTERT, co-transfected

(CoTF) and control respectively. These observation suggested that plasmid transfection of all condition can increase the cell proliferation. Contrast, to confirm the cell proliferation or doubling time by MTT technique. The Ki67 gene expression by using quantitative-PCR is used to validate the cell proliferation. Ki67 is a nuclear protein expressed in all proliferating vertebrate cells, and it is a widely used biomarker to estimate the proportion of dividing cells to grade tumors (Sobecki *et al*., 2017). These result in Figure 4.6 suggested that the Ki67 expression is significantly expressed in SV40 and CoTF. The highly expressed of Ki67 in SV40 and CoTF-transfected cells may be involve cell death. Due to the doubling times are treated in long time, SV40 and CoTF-transfected cells that quickly growth in day 2 and 4. Then, cells slowly into cell death in day 6 and 8 by limit of area culture. Finally, the results of doubling time and Ki67 expression are confused via time of cultures but consistent with day 2 and 4.

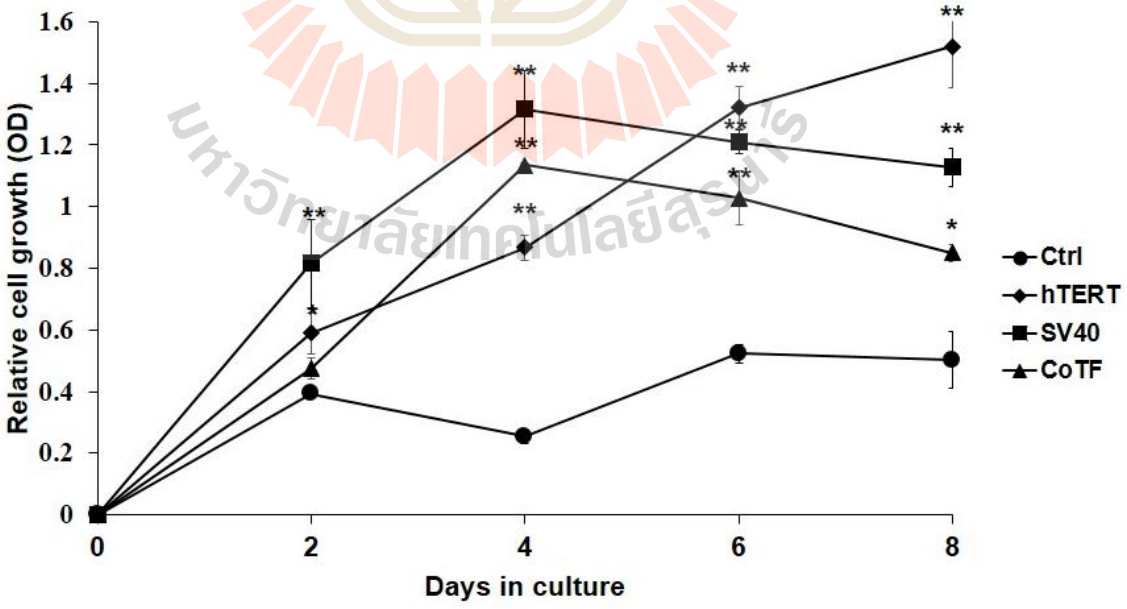


Figure 4.5 The Relative cell growth of immortalized cells. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

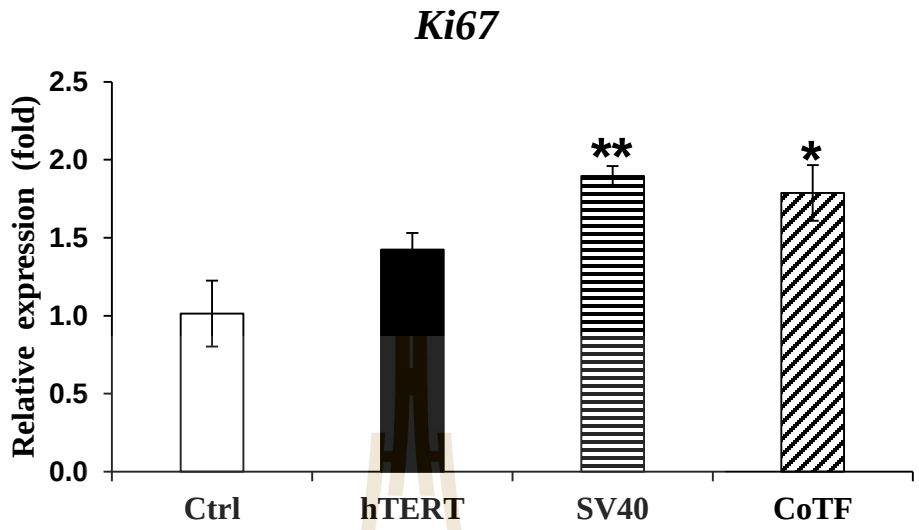


Figure 4.6 The Relative expression of *Ki67*. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

4.1.3 The maintenance of telomere length

The traditional method of measuring telomere length in samples of total human genomic DNA determines a mean terminal restriction fragment (TRF) length (Cawthon, 2002). This technique is developed to repeated minisatellite telomeric units lack of target sites for restriction enzymes. Therefore, telomeres in rather long fragments, which genomic DNA is digested into short pieces. Fragments of telomeric DNA are then envisioned by hybridization with radioactively labeled telomeric probe. As TRF include besides telomeres also a short region of telomere-associated DNA depend on the first restriction site, results are slightly removed towards higher TRFs values. Consequently, the use of frequent cutters is suggested to minimize this difference. Develop to using quantitative realtime polymerase chain reaction (qPCR) to measure absolute telomere length (aTL). This method is based on the Cawthon method for relative measurement of telomere length (Telomere/single copy gene ratio).

The result in Figure 4.7 shown that relative of telomere/single copy gene of CoTF-transfected is highly expressed than the control. Next, both hTERT and SV40 large T antigen is obviously increased but remain less than CoTF-transfected cells. The result suggestion, the ectopic expression of plasmid can increase and maintain the telomere length of cells.

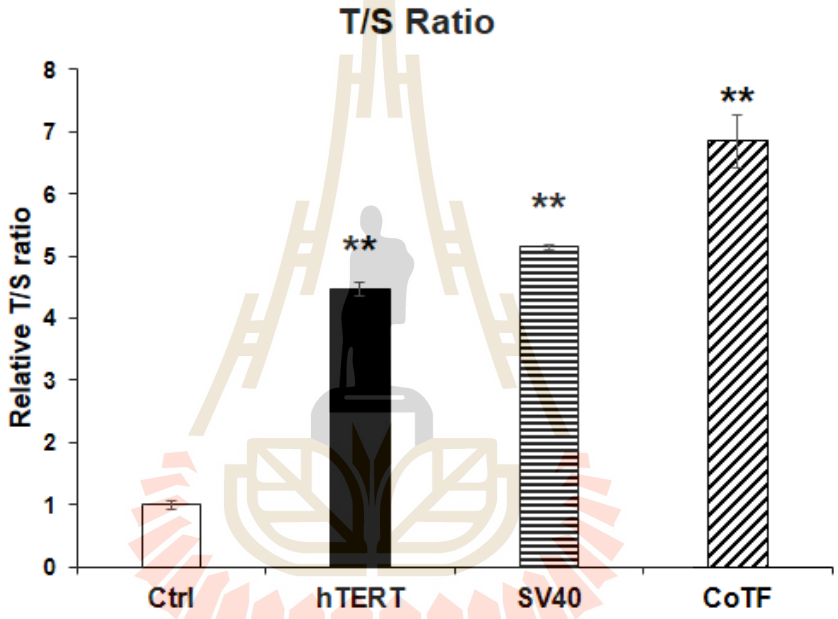


Figure 4.7 The relative ratio of telomeres. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

4.1.4 Tumor properties

Anchorage-independent growth is the ability of transformed cells to grow independently of a solid surface, and is a hallmark of carcinogenesis. The soft agar colony formation assay is a technique widely used to evaluate cellular transformation *in vitro* (Borowicz *et al.*, 2014). The principle of this technique is detected cells spreaded onto a culture plate and grown in feeder cells. Normal cells are prevented from anchorage-independent growth, due to a particular type of apoptotic death, called

anoikis (Borowicz *et al.*, 2014). Nevertheless, these cells have the capability to grow and divide without binding to a substrate. To exploit on this concept, was developed the soft agar colony formation assay. Figure 4.8 shown soft agar assay transfected cells were compared SH-SY5Y cell, are neuroblastoma and used to *in vitro* model of cancer and neuronal functions. The SH-SY5Y cells were used to positive control and human dermal fibroblasts are control in Figure 4.8A that grown on agar after were seeded 20 days. The colony of SH-SY5Y were counted under microscope and calculated colony efficiency (Figure 4.8F) and size approximately 60-100 µm (Figure 4.8B). Control cells are shown tumor formations and size less than 10 µm. The transfected cells have size of colony approximately 10-20 µm and obviously decreased colony efficiency compare positive control. These results suggest that the tumor properties of transfected cells can agreeable for used to model of immortalized cells *in vitro*.

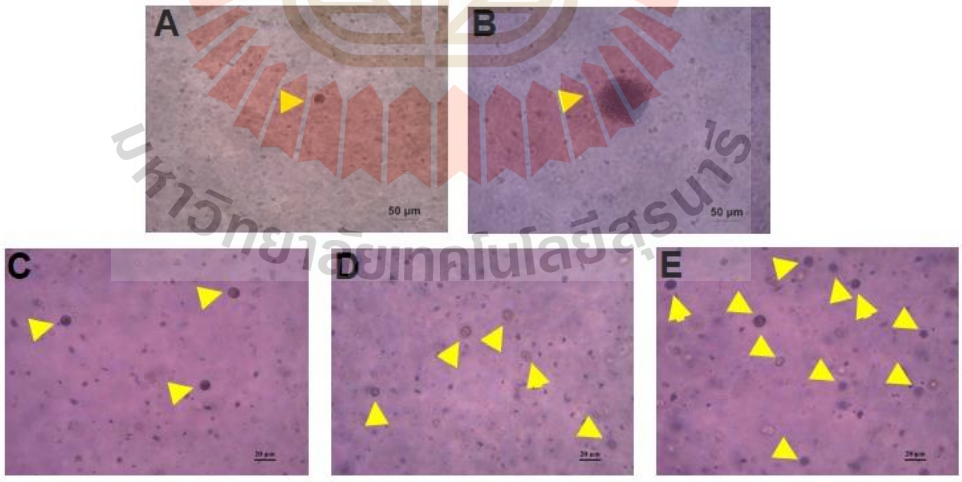


Figure 4.8 Tumor formation of immortalized cells (A) human dermal fibroblasts (ctrl) (B) SH-SY5Y cells (C) hTERT-transfected cells (D) SV40 large T antigen (E) co-transfection hTERT and SV40 large T antigen (F) colonies efficiency of SAA. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

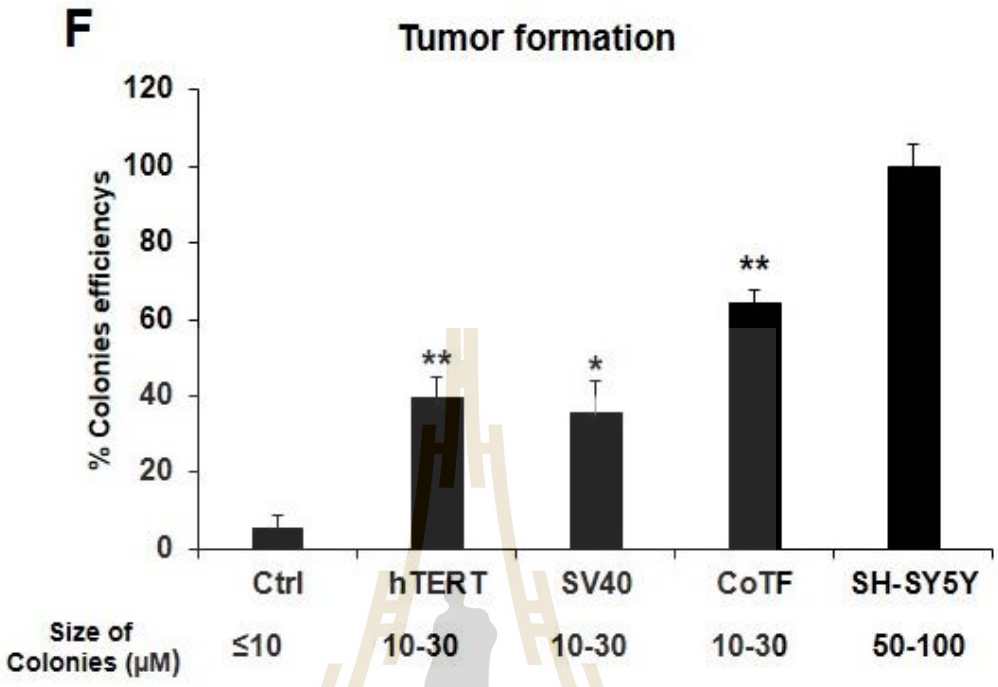


Figure 4.8 continued.

4.2 The characterization of immortalized cells

4.2.1 Gene expression

Otherwise, human dermal fibroblasts were transfected plasmid were investigated characterization of cells. The purpose of this point to studies the role involving in immortalized cells. Gene expression was analyzed using reverse transcriptase PCR on gel based. Primers were designed associate with immortalized cells, role cell cycle and properties of fibroblasts. First, the properties of fibroblasts were investigated 4 genes containing *Collagen 1 type I* (*COL1A1*), *Collagen 3 type I* (*COL3A1*), *elastin* and *vimentin*. Normally, fibroblasts compose of type I collagen which is abundant protein in cells for high tensile strength. Type I collagen is important during development and wound healing. On the other hands, elastin is elastic protein in connective tissue which play roles in the elasticity and resilience of vertebrate tissue

including large arteries, lung, ligament, tendon, skin and elastic cartilage (Mithieux and Weiss, 2006). The tensile strength still remain the intermediate filament (IF), vimentin a major of IF family of proteins. Vimentin is universally expressed in mesenchymal cells and is known to maintain cellular integrity. Increased *vimentin* expression has been reported in various epithelial cancers including prostate cancer, gastrointestinal tumors, central nervous system (CNS) tumors, breast cancer, malignant melanoma, lung cancer and other types of cancers (Satelli and Li, 2011). These genes were determined in immortalized cells and the results of PCR based on gel were shown in Figure 4.9A. The results of relative expression shown that the highly expressed of *COL1A1* and *COL3A1* in hTERT-transfected-cells, it is increase the fibroblast properties after become immortalized cells (Figure 4.9B and 4.9C). The *Vimentin* expression was not shown the different result of relative expression after transfected cells, were compared with control of normal fibroblasts (Figure 4.9F). These result was suggested that transfected-cells still the characteristic of fibroblast and not become the tumorigenesis. Another, elastin is involve wound healing that show the fibroblast markers. *Elastin* expressions were enhanced in transfected cells that is promising to use the immortalized cells for *in vitro* model studies (Figure 4.9G).

Normally, the ectopic expression may indicate role through various pathways, were determined *p21*, *p53*, *KRT18* and *SIRT1*. Recently studies, p53 and p21 protein were increased in immortalized cells compared to normal primary oral epithelial cells (Sdek *et al*., 2006). In some cancers have the increasing of the wild-type *p53* gene but p53 protein level are very low. p53 acts to increase the level of *p21*, a cyclin inhibitor. A reduced p53 level results in less p21 expression with an associated loss of cell-cycle checkpoint regulation (Sdek *et al*., 2006). The direct target of SV40

large T antigen is tumor suppressor Rb (pRb). Nevertheless, the *pRB* gene expression was not detected (not show in result) but *p53* was extremely increased in immortalized cells (hTERT, SV40 large T antigen and co-transfection) (Figure 4.9E). However, the highly expression of *p53* is not consistent with *p21* (Figure 4.9D). The results suggested that *p53* and *p21* are not remain truly target of immortalized cells. Therefore, the *SIRT1* was investigated the relation in cells. The highly expression of *SIRT1* in immortalized cells significantly different from normal fibroblasts (Figure 4.9H). The SIRT1 activation is important for many cellular processes including gene silencing, regulation of p53, fatty acid metabolism, cell cycle regulation, and life span extension (Boora *et al*., 2005). The consistent of *p53* and *SIRT1* activation are important keys to studies the mechanism of immortalized cells by ectopic expression. Several reports, immortalized cells are popular for *in vitro* models. *SIRT1* activation was interested to studies the role of immortalized cells in this studies. On the other hands, to confirm the tumor formation of soft agar assay. The *keratin 18* (*KRT18*) marker were detected tumorigenesis properties. These marker has been used extensively to classify modality of cell death in a number of liver disorders including cholestasis, ischemia-reperfusion, non-alcoholic fatty liver disease, acetaminophen overdose and alcoholic hepatitis (Woolbright and Jaeschke, 2018). Increasing of *KRT18* marker in transfected cells are still problem (Figure 4.9I). The main aim of immortalized cells to increase the proliferation of cells but not yet a cancerous or tumorigenesis. However, *KRT18* may involve thought other role which is not tumor, it's still not clearly roles.

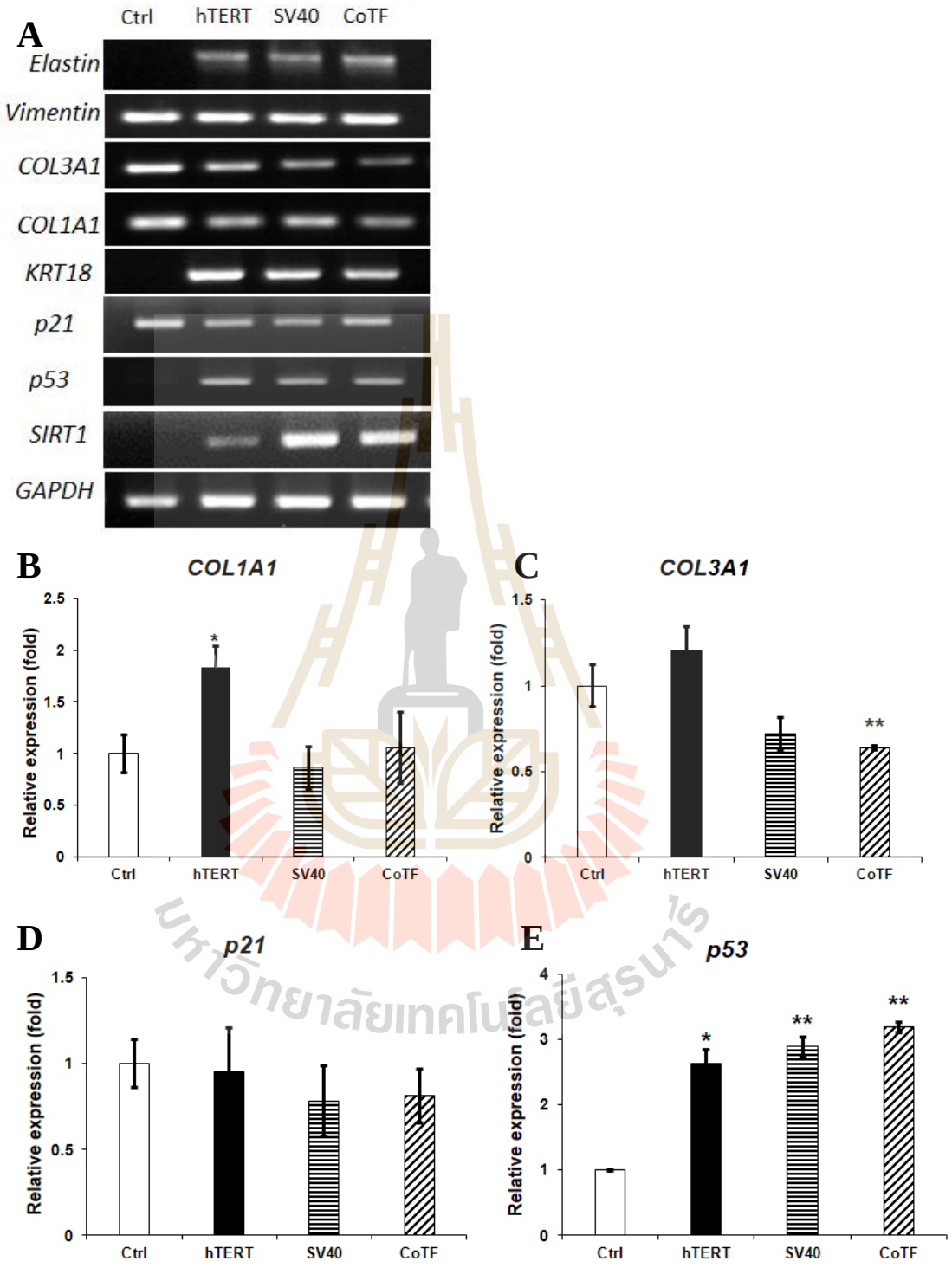


Figure 4.9 Gene expression (A) gel based PCR (B) relative expression of *COL1A1* (C) *COL3A1* (D) *p21* (E) *p53* (F) *Vimentin* (G) *Elastin* (H) *SIRT1* and (I) *KRT18*. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

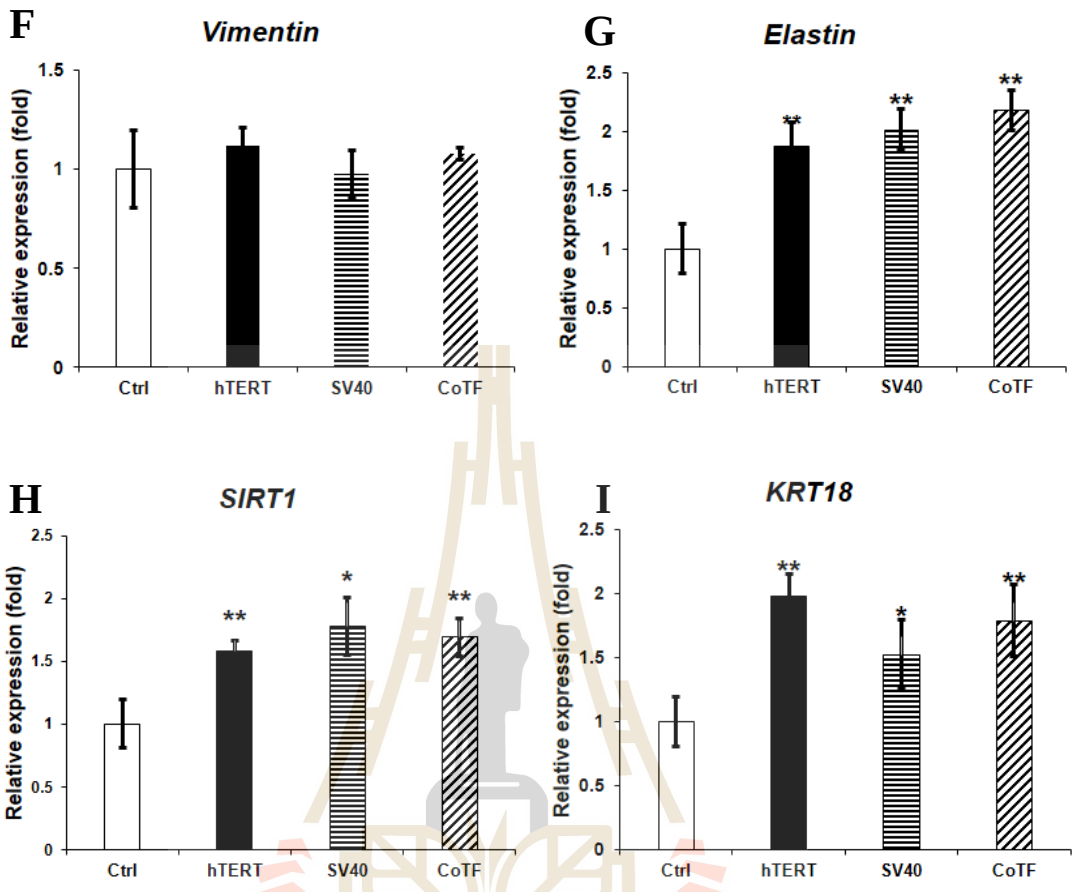
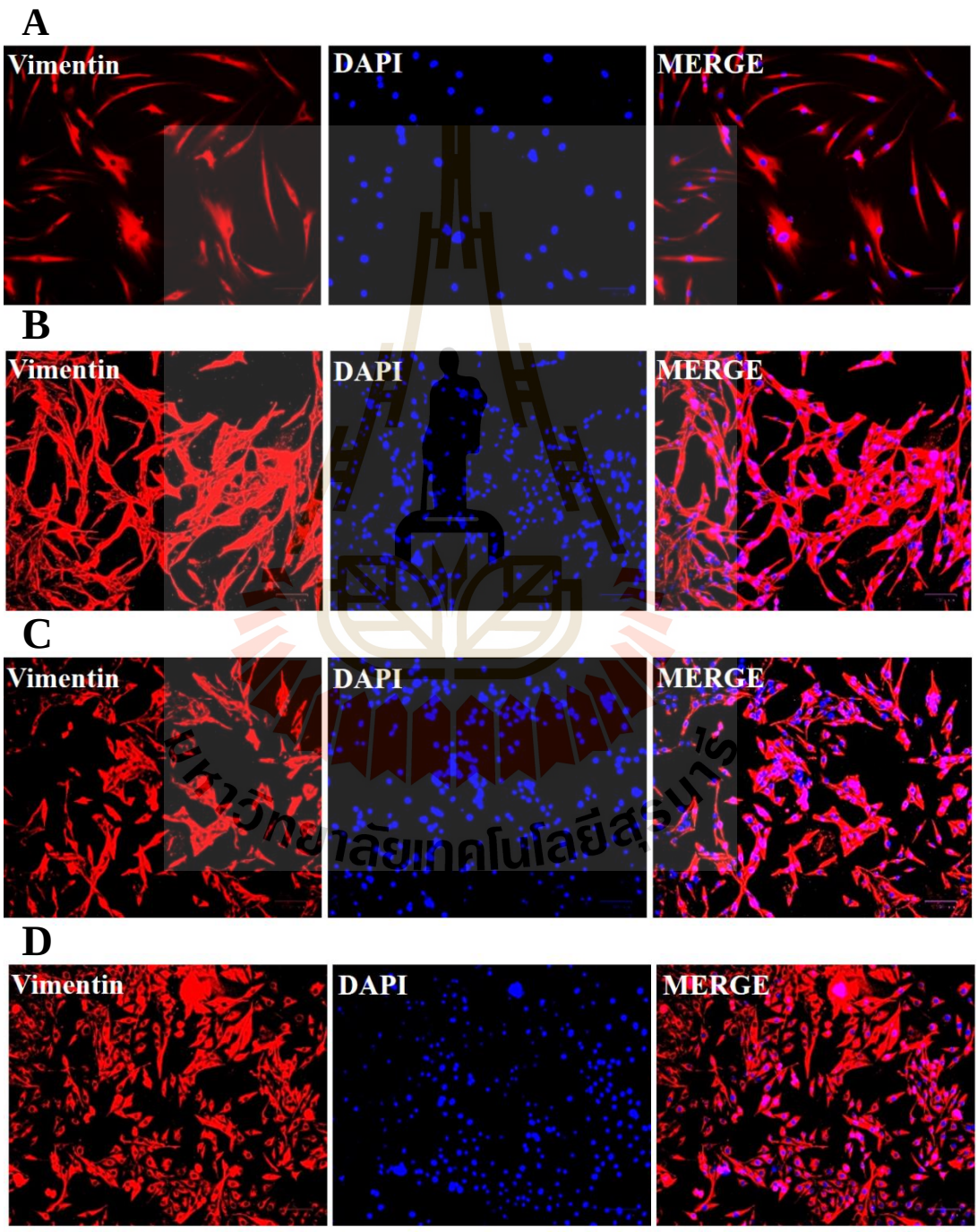


Figure 4.9 continued.

4.2.2 Protein expression

Protein expressions were demonstrated by immunofluorescence to measure protein levels compare with gene expression levels. Vimentin protein was used to studies to confirm the characteristic of fibroblasts. As known, vimentin is expressed in different cancer cell lines and tissues, it is association with increased cancer cell growth, invasion and migration. The results shown that vimentin protein expression that highly expression in hTERT-transfected cells (Figure 4.10). Conversely, gene expression which vimentin not different from control. However, the relation of vimentin protein and gene still not constant. On the other hands, the collagen I type I protein expression were demonstrated immunofluorescence also, highly

expressed in hTERT-transfected cells (Figure 4.11). These protein results associate with *COL1A1* gene expression, that show characterize of fibroblasts after transfection to immortalized cells.



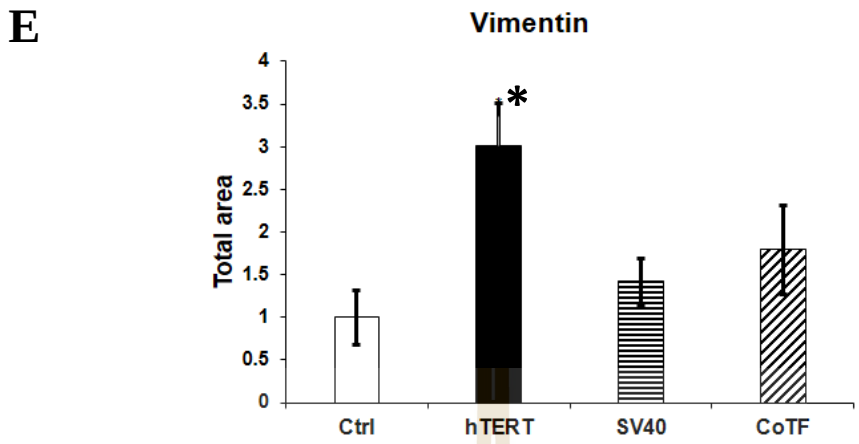


Figure 4.10 Protein expression of vimentin (A) normal fibroblast (B) hTERT-transfected cells (C) SV40 large T antigen-transfected cells (D) co-transfection (E) total area of vimentin expression. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

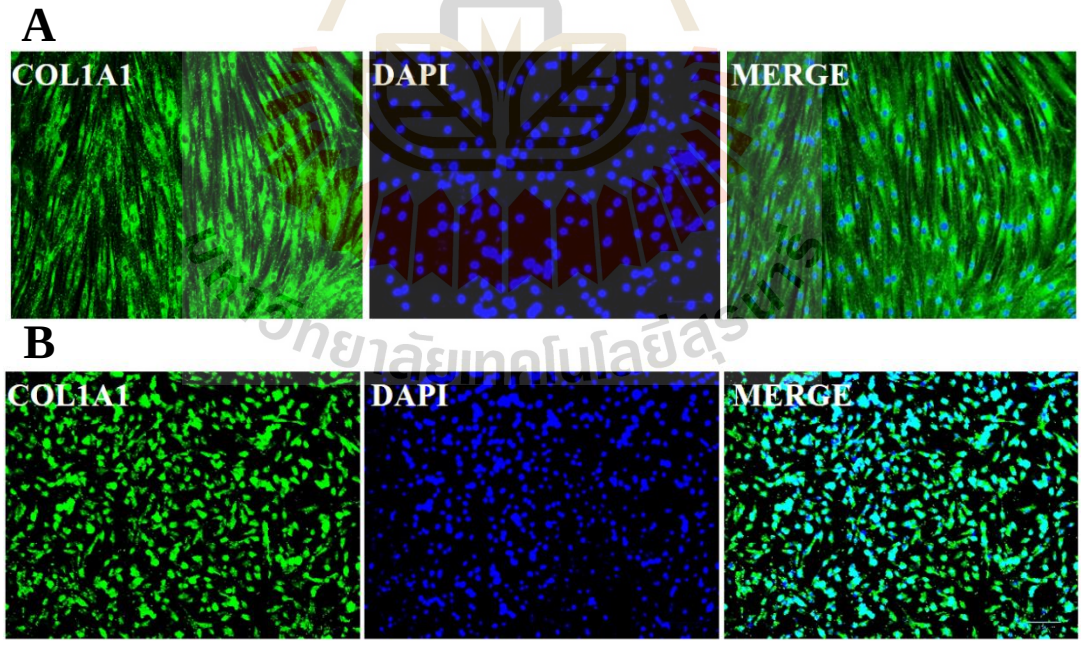


Figure 4.11 Protein expression of collagen 1 type I (A) normal fibroblast (B) hTERT-transfected cells (C) SV40 large T antigen-transfected cells (D) co-transfection (E) total area of collagen1 type I expression. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

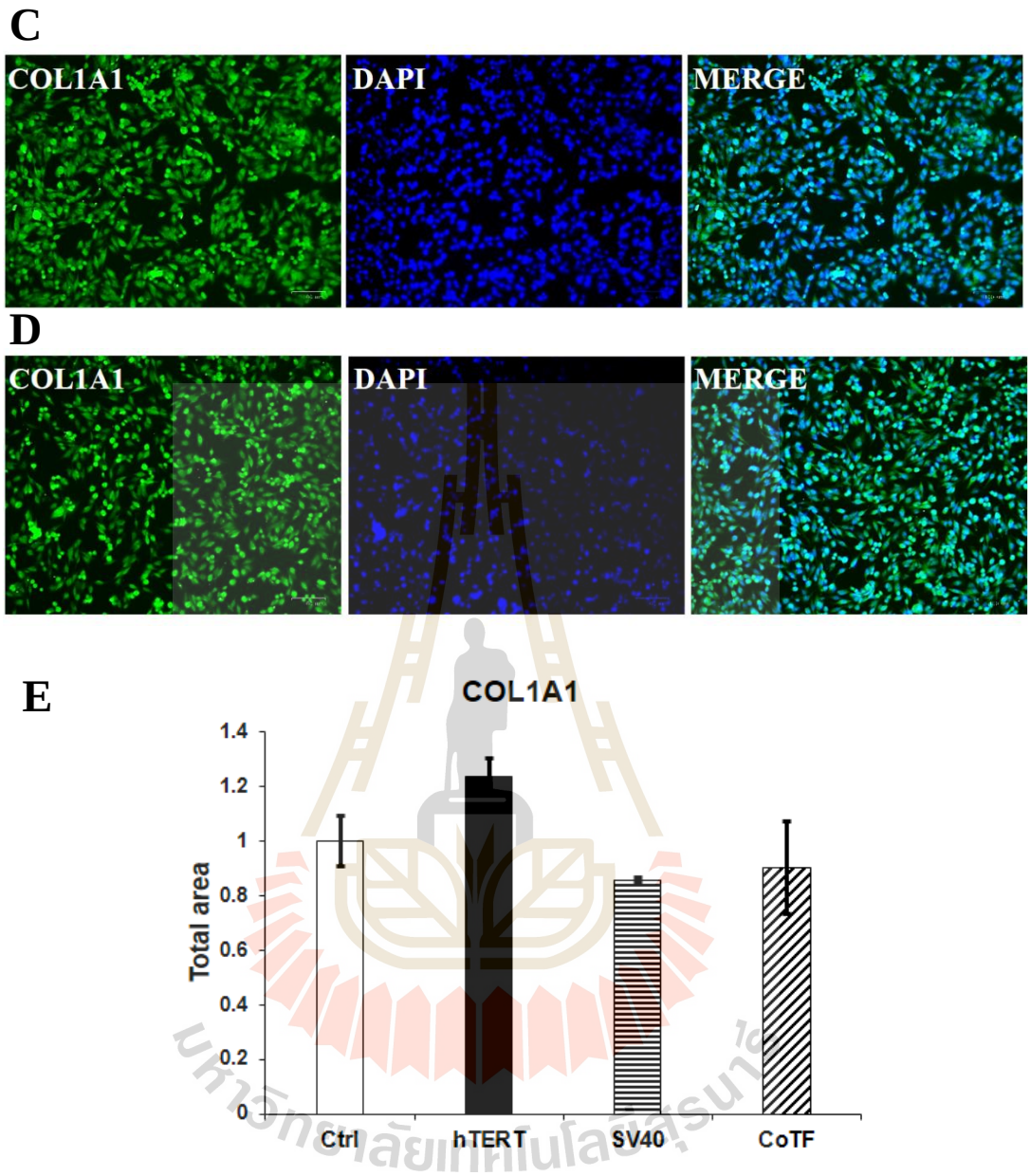


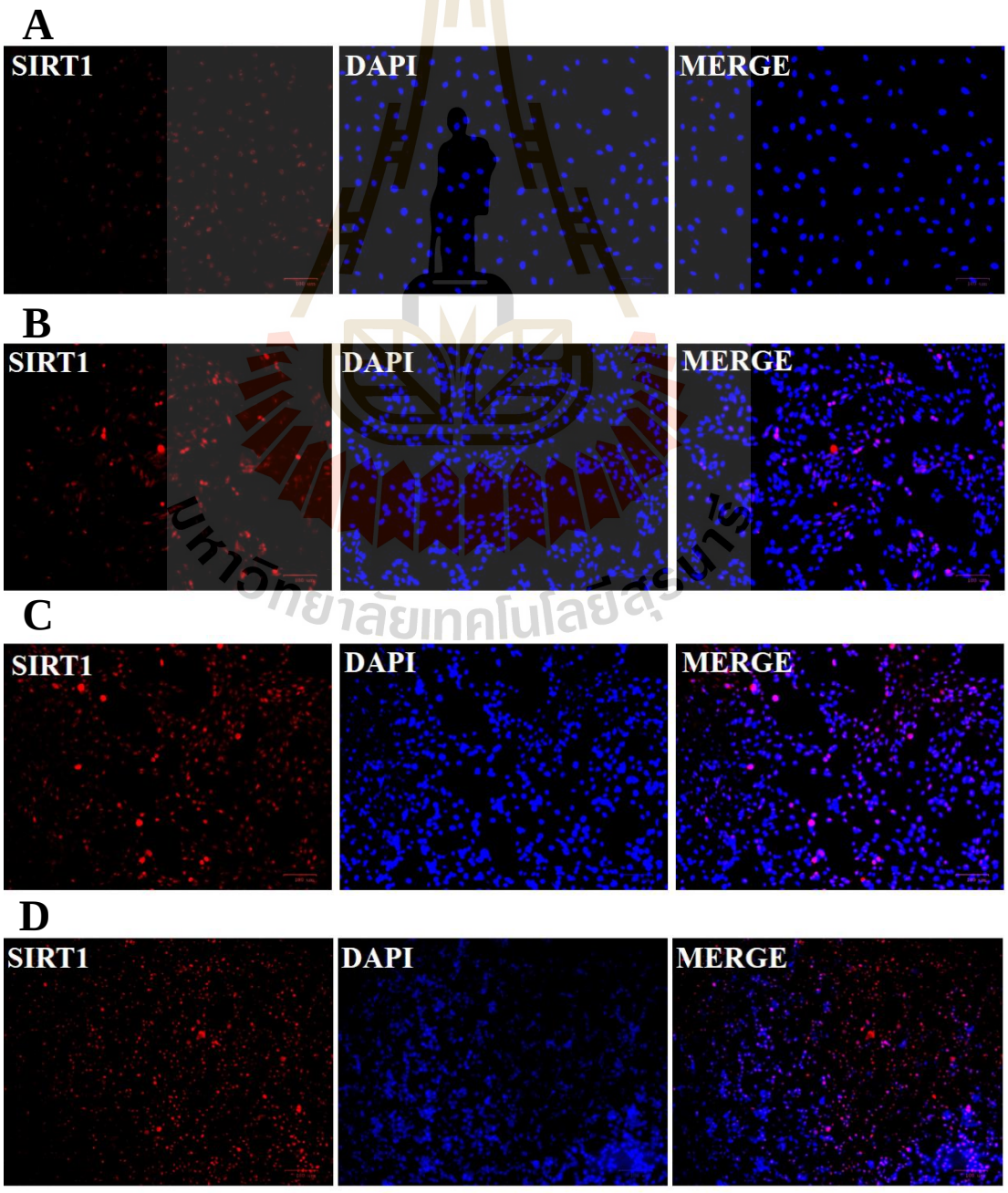
Figure 4.11 continued.

4.3 SIRT1 involving through immortalized HDFs

The aim of this studies focus on SIRT1 play role in immortalized cells, protein was investigated by immunofluorescence. This part to confirm the relation of SIRT1 and cell proliferation in immortalized by ectopic overexpression. First of all, SIRT1 that show total protein and highly expressed in all condition transfected cells (Figure 4.12). As know, SIRT1 protein are accumulated in nucleus and stained anti-sirt1 that

shown red color in cell. Increasing of SIRT1 involve the cell proliferation because the number of cells were increased after culture in the same culture. Therefore, the 848 and 269 cells were used to transfected plasmid that contain shRNA1 and shRNA2. Both shRNA were designed to knockdown SIRT1 expression and ligated into pSUPERIOR vector. After transfected cells were selected by G418 sulfate that containing neomysin resistant in plasmids. Results show that morphology of cells, were clearly shown flatted cytoplasm, increase of nucleus and the accumulation of substrate proteins in cell. The cells shRNA1 and shRNA2 can suggested that the cell morphology was changed to senescence characterization within passage 3 after selected by G418. Senescence characterization can concluded that cells end of the proliferative life span of normal cells after transfected both shRNAs (Figure 4.13). It is accompanied by alteration of SIRT1 protein expression. SIRT1 expression were confirmed after shRNA knockdown in cell. These proteins were decreased protein that shown in Figure 4.14. Ki67 as cell proliferate protein marker were demonstrated relative expression and decreased in both shRNA-knockdown (Figure 4.15). The approach of immortalized cells play role through SIRT1 activation were demonstrated by SIRT1 knockdown. Another, to verify the effect of SIRT1 in normal cells. Activator and inhibitor SIRT1 as resveratrol and sirtinol were treated to measure cell viability. Cell viability was increased at 1 µM concentration of resveratrol and slowly decreased in higher dose-dependent manner (Figure 4.16A). Likeawise, sirtinol reduce cell viability at 5 µM concentration after treated 24 h (Figure 4.16B). Thus, *SIRT1* gene were measured relative expression and it is decreased in dose-dependent manner of resveratrol and sirtinol. Resveratrol at 5 µM was reduced SIRT1 expression which diverse functional of activator. The proliferation marker (Ki67) was measure which resveratrol at 1 and 5 µM can increase Ki67 and sirtinol at 1 and 5 µM reduced marker

of cell proliferates (Figure 4.17). In the same ways, SV40 large T antigen cells were treated with inhibitor which show highly proliferates. These results of SV40 large T antigen relative expression different from hTERT and CoTF which were up-regulated Ki67 after treated resveratrol with 5 µM and down-regulated after treated sirtinol 5 µM. The SV40 large T antigen-transfected cells were treated with SIRT1 inhibitor and show highly proliferates than treated with SIRT1 activator (Figure 4.18). These result suggested that SV40 large T antigen cells may are regulate through other pathways.



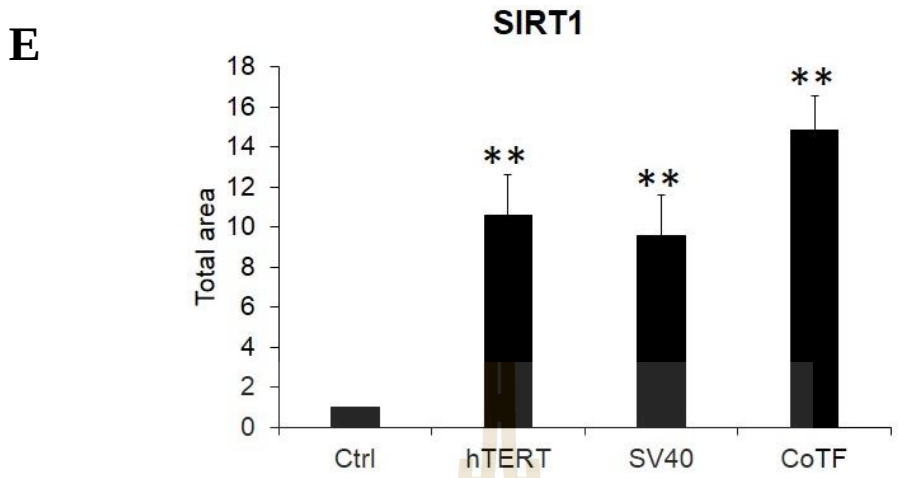


Figure 4.12 Protein expression of SIRT1 (A) normal fibroblast (B) hTERT-transfected cells (C) SV40 large T antigen-transfected cells (D) co-transfection (E) total area of collagen1 type I expression. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

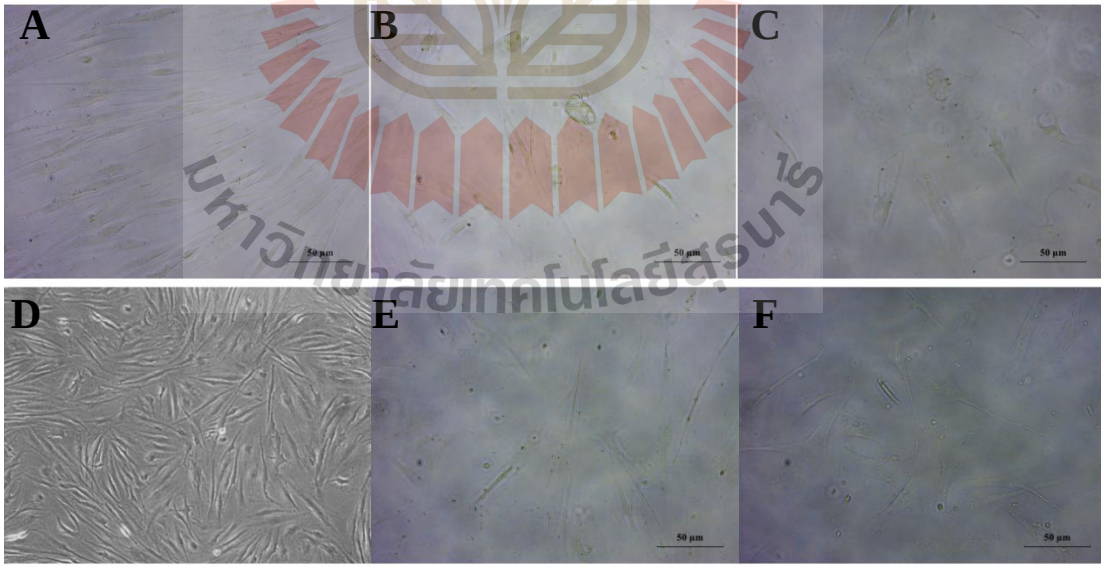


Figure 4.13 The cell morphology of SIRT1-knockdown cells (A) 848 control cells (B) 848 –transfected shRNA1 cells (C) 848-transfected shRNA2 cells (D) 269 control cells (E) 269-transfected shRNA1 (F) 269-transfected shRNA2 cells.

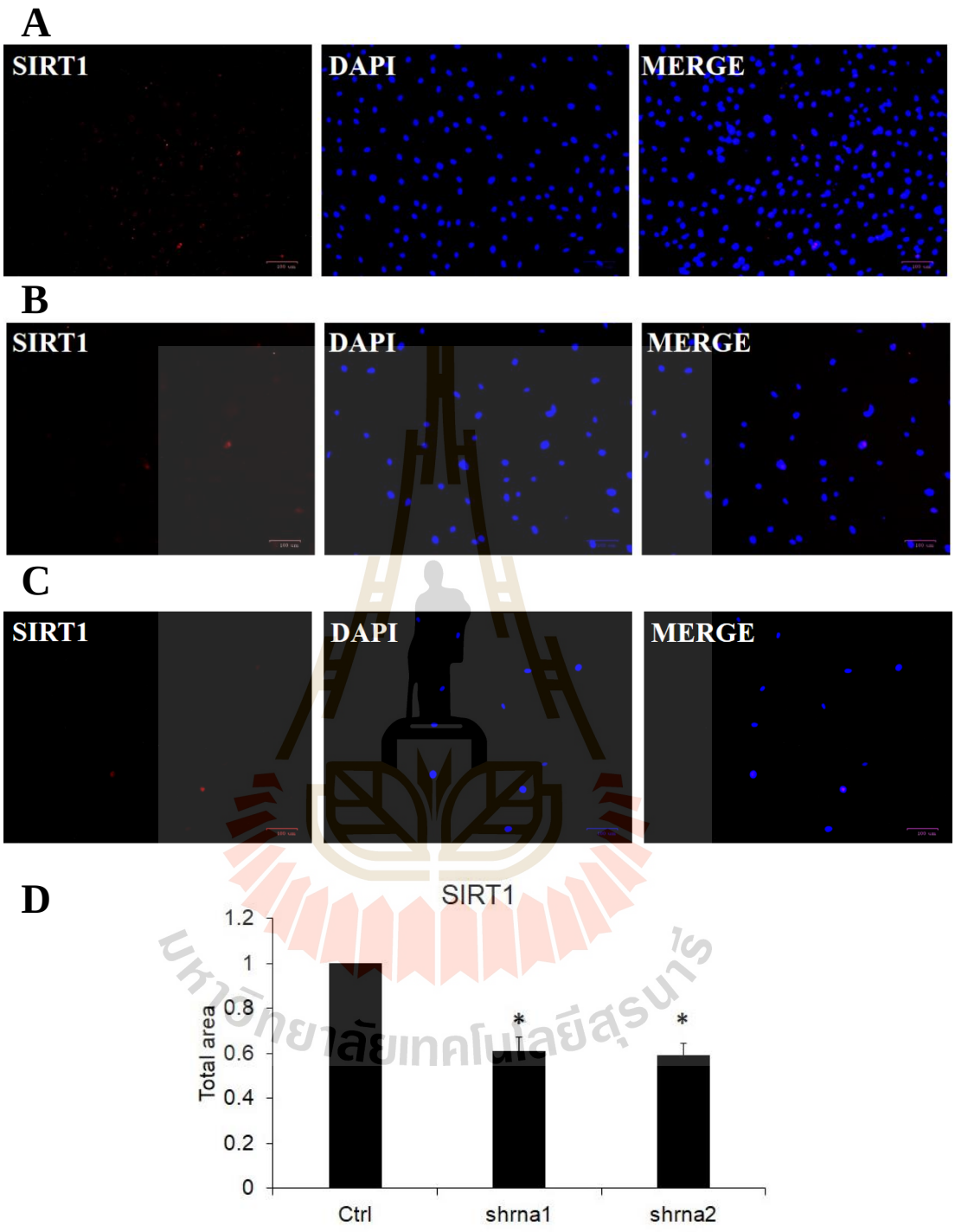


Figure 4.14 Protein expression of SIRT1-knockdown cells (A) 848 control cells (B) 848 –transfected shRNA1 cells (C) 848-transfected shRNA2 cells (D) total area of SIRT1 expression. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

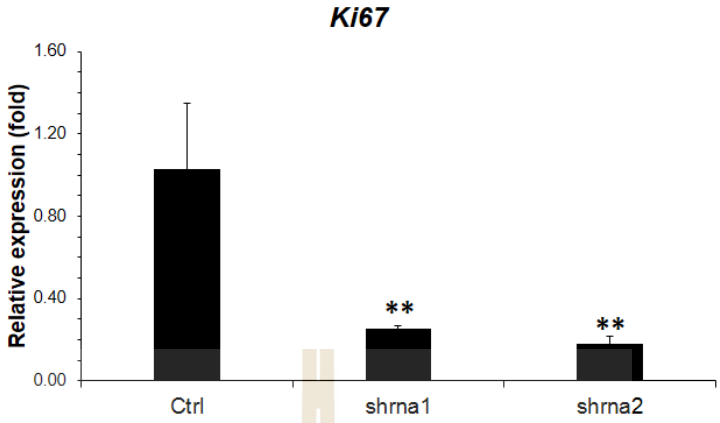


Figure 4.15 Relative expression of Ki67. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

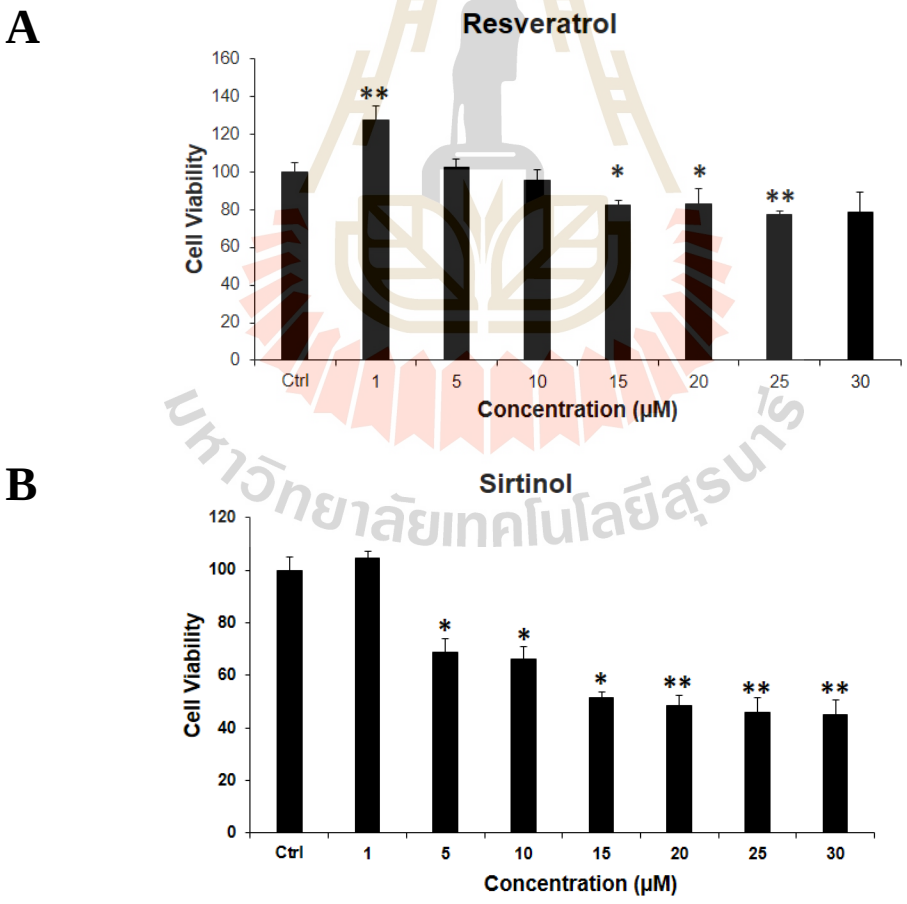


Figure 4.16 Effect of cell proliferation on SIRT1 (A) Resvertrol is activator of SIRT1 (B) Sirtinol is inhibitor of SIRT1. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

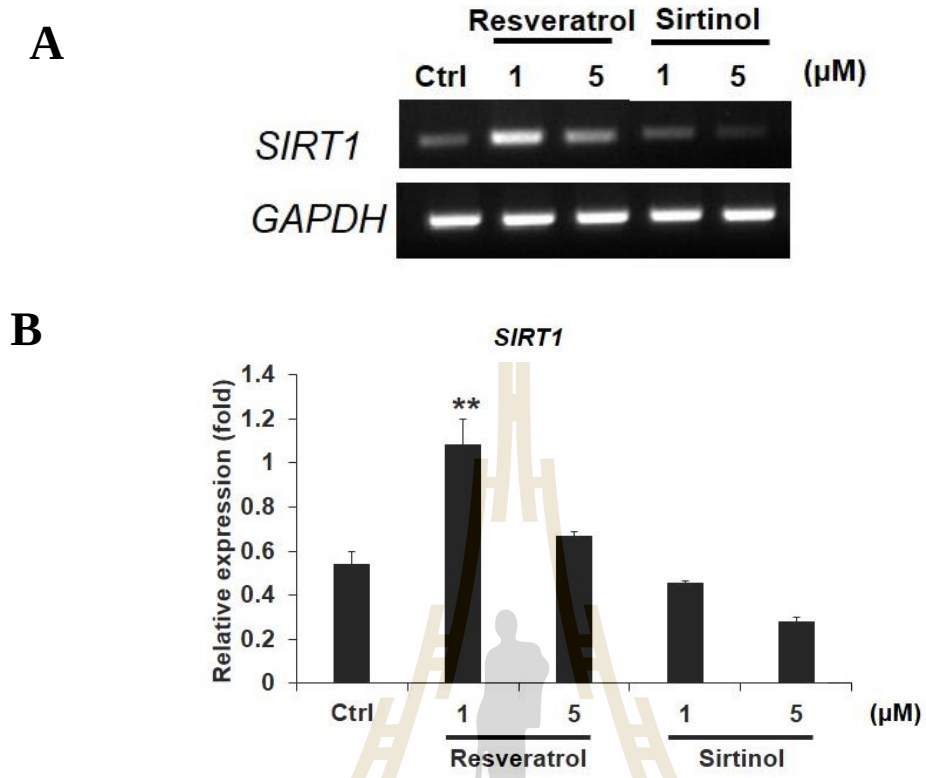
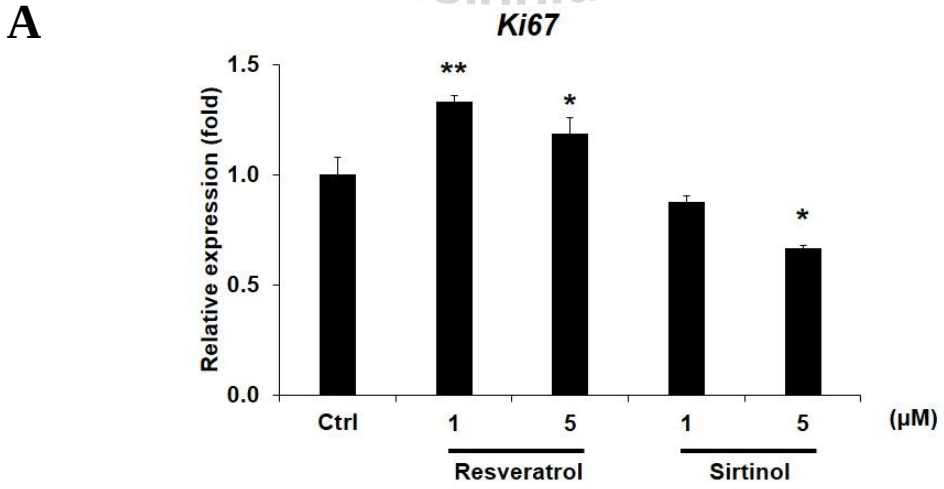


Figure 4.17 Gene expression of SIRT1 after were treated by resveratrol and sirtinol (A) gel based on PCR (B) relative expression of SIRT1. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.



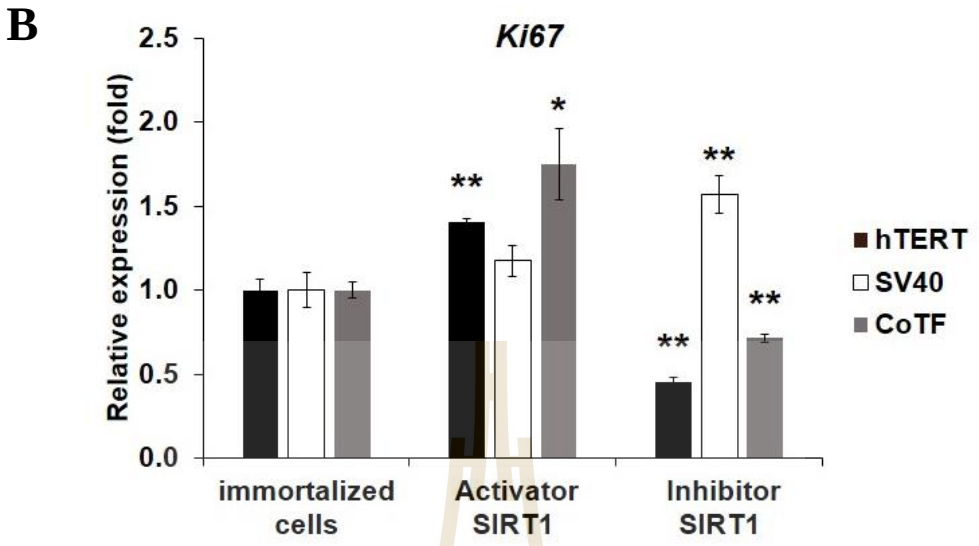


Figure 4.18 Gene expression of *Ki67* after were treated by resveratrol and sirtinol (A) human dermal fibroblasts were treated SIRT1 effectors (B) immortalized cells after treated with SIRT1 effectors. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

4.4 miRNAs play role in SIRT1 up-regulation

In the presence of miRNAs in immortalized cells have been various reported that involve within cell proliferation and cellular senescence. In this studies focus on the miRNAs because it is safe mechanism for develop or manage the role in cells. Truly, immortalization has many techniques to improve cell for *in vitro* model but has limit of mutation and safety to use. As the ectopic overexpression by plasmid can immortalized cells in transient time of culture. Five miRNAs were reviewed and predicted target in immortalized cells, and shown in Table 4.1. The relation of miRNAs containing senescence and SIRT1 were selected for consistent within immortalized cells. miRNAs were designed primer for detect relative expression of miRNA by using qPCR. miR-217 cannot amplified by qPCR in immortalized cells that no expression in

cells. Therefore, 4 miRNAs were analyzed and prrdiced targets. miR-22 is expressed in fibroblast and down-regulated in cancer and regulate inhibition of growth, metastasis and induce senescence. Recent reports, miR-22 induced senescence acts as a barrier to cancer progression *in vitro* and *in vivo* (Xu *et al.*, 2011). Figure 4.20 shown that SV40-transfected was highly up-regulated by miR-22, but co-transfected hTERT and SV40 was down regulated same hTERT-transfected alone. The direct target of miR-22 is SIRT1, sp1 and CDK6 which effect on senescence. In the same way, miR-449 was up-regulated in SV40-transfected alone (Figure 4.21). These result suggested that directly target of SV40-transfected may involve through 2 miRNAs. The link of 2 miRNAs are related with the same target as CDK6 and pRB phosphorylation. pRB are suppressed by SV40 large T antigen and cell cycle arrest at G1 phase. miR-449 is expressed in grastic cancer, regulate senescence and apoptosis. Previous reports, the evidence of epigenetic mechanism in clinical primary tumor samples, the epigenetic repression of miR-449 may be widely present within human cancer cells. miR-449 was hypothesized that these may be necessary to maintain the high E2F1 activity to promote transformation during tumorigenesis (Yang *et al.*, 2019). Futhermore, miR-449 can target HDAC1 or Wnt for growth inhibition, which acts as a tumor suppressor by inhibiting oncogenic situation and pharmacologic reactivation of miR-449 (Noonan *et al.*, 2009). On the other hands, miR-93 is regulate senescence and support angiogenesis in cancer cells. It is has been reported the expression of tumor suppressor FUS1 and p53 induced p53 nuclear protein 1 (Yang *et al.*, 2008). The highly expression of miR-93 can help to proliferate and survive in cells (Fang *et al.*, 2010). Figure 4.19 was shown up-regulated miR-93 in hTERT-transfected cells, which role of proliferate and senescence may indicate through miR-93. However, miR-34a was down-regulated in immortalized cells. The down regulation of miR-34a has been reported increase

proliferate and regulate the cell cycle arrest by direct transcriptional target of p53 (Zou *et al.*, 2017). miR-34a were predicted to analyzed role in many immortalized cells for develop to *in vitro* models. The results was shown that down-regulated in all immortalized (hTERT, SV40 large T antigen and co-transfected cells) and relevance consistent role in cells Figure 4.22. Interestingly, ectopic overexpression by plasmid were regulated role through miR-34a which can elaborate to further immortalize application.

Table 4.1 A list of miRNAs involving SIRT1 and cellular senescence

miRNAs	**Tissue/cells/diseases**	**Regulation/function**
miR-22	Fibroblast and cancer	Inhibition of growth and metastasis, senescence
miR-217	Endothelial cell	Senescence
miR-34a	Pancreatic cancer, Colorectal cancer, Brain and liver cancer	Neural differentiation, senescence, Liver metabolism
miR-93	Liver	Senescence
miR-449	Gastric cancer	Apoptosis, senescence

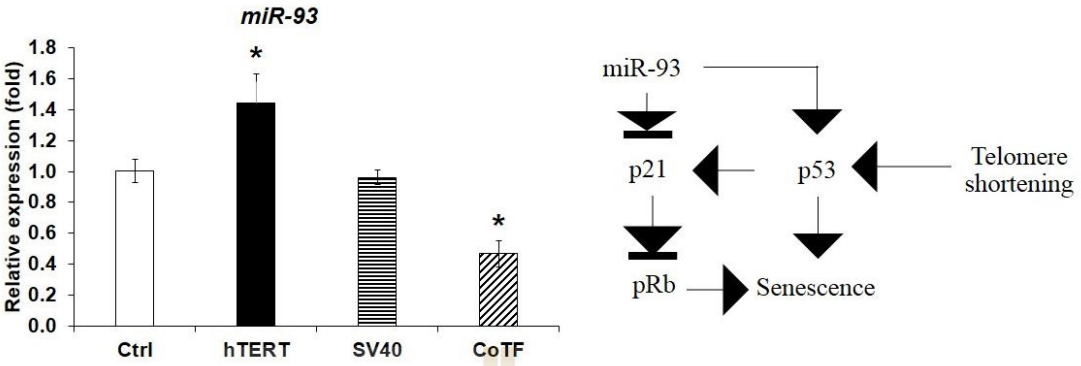


Figure 4.19 The relative expression of miR-93 by qPCR and directly target flowchart of miR-93. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

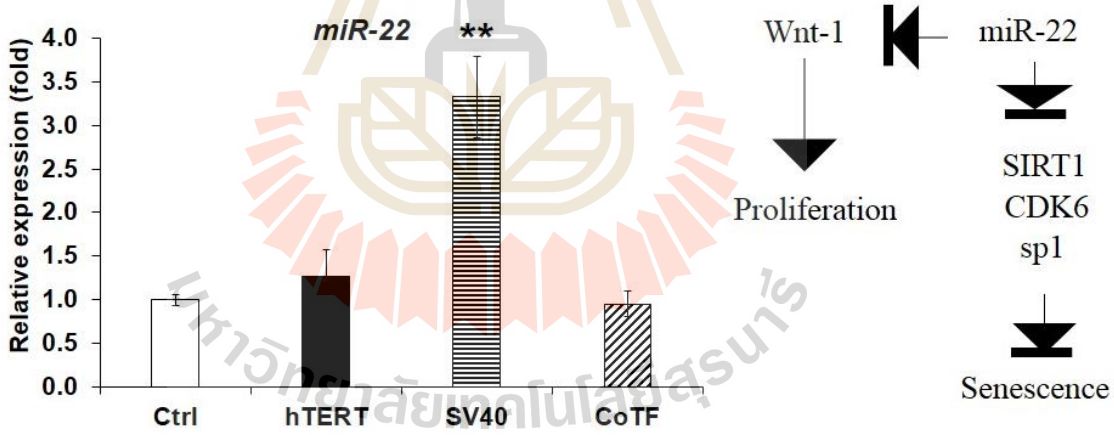


Figure 4.20 The relative expression of miR-22 by qPCR and directly target flowchart of miR-22. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

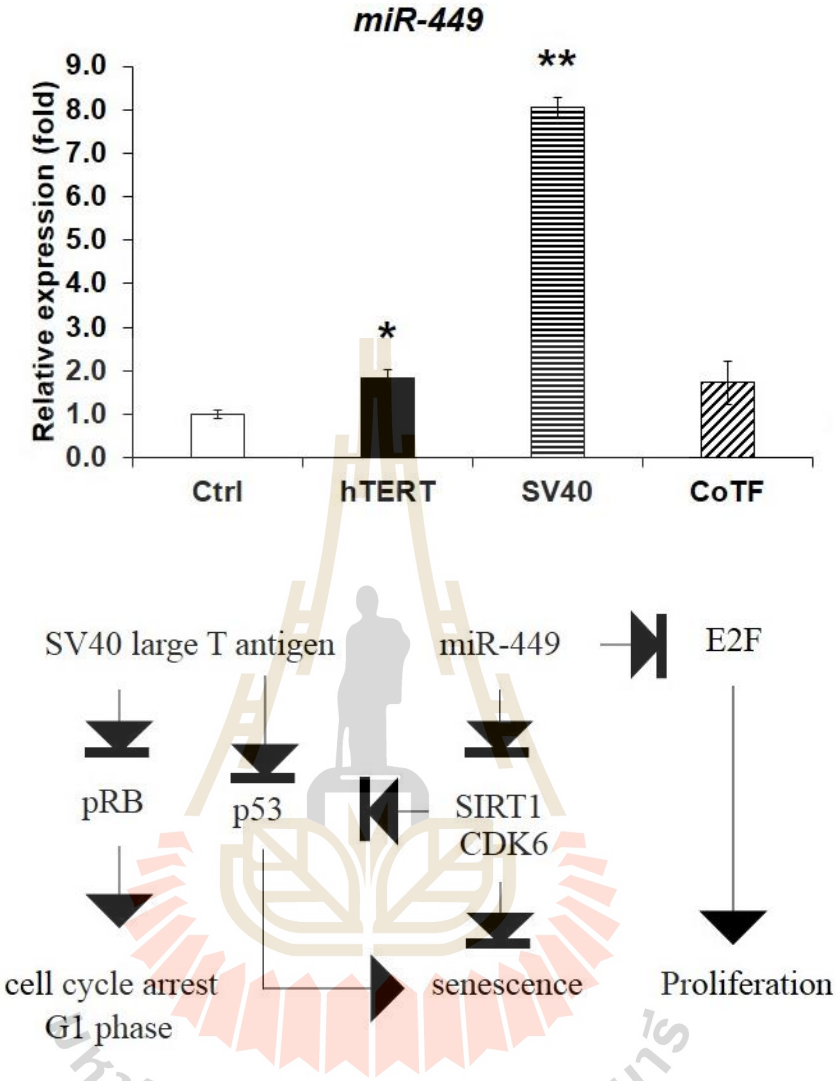


Figure 4.21 The relative expression of miR-449 by qPCR and directly target flowchart of miR-449. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

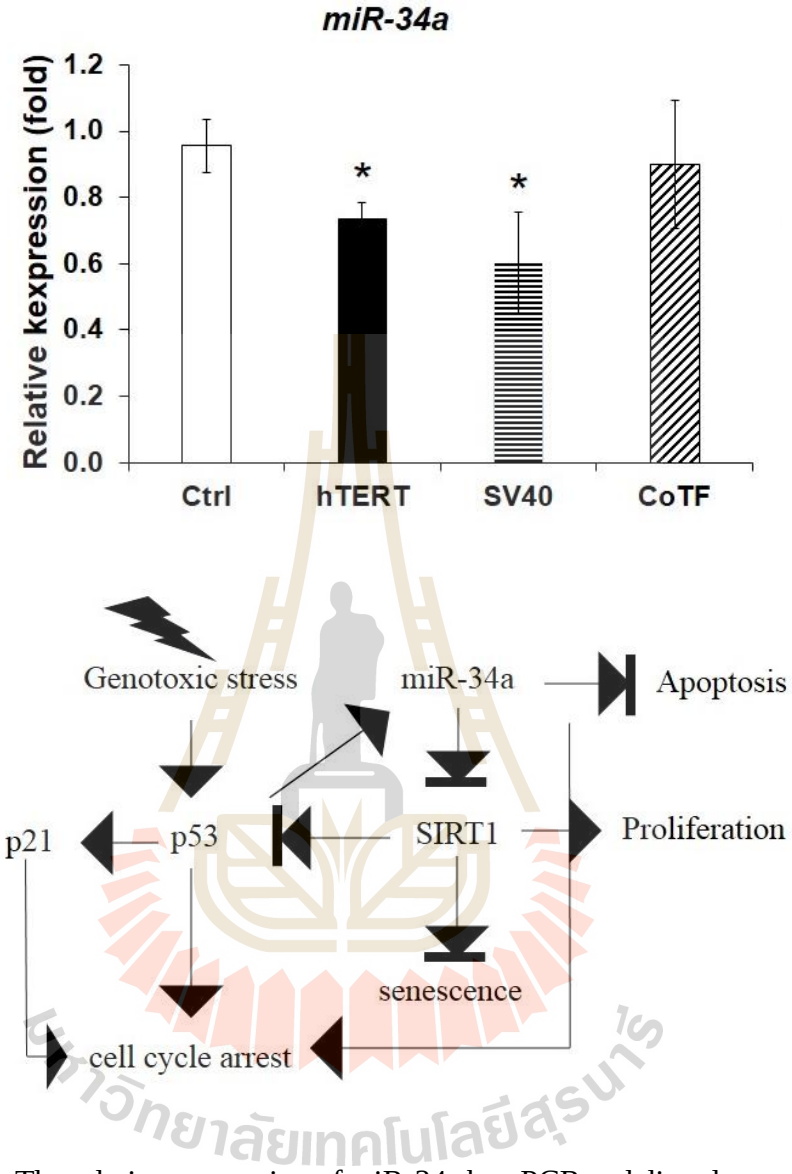


Figure 4.22 The relative expression of miR-34a by qPCR and directly target flowchart of miR-34a. Values are expressed as mean ± SD (n =3). *p < 0.05 and **p < 0.01 versus control cells.

CHAPTER V

CONCLUSIONS

In this study, human dermal fibroblasts were immortalized by using hTERT and SV40 large T antigen plasmid. The properties of both plasmid can used to immortalized through role of cell cycle and maintain cell proliferation. The definition of immortalization are increase cell proliferation and not become to cancer cells. hTERT plasmid are the protein structure protein can maintain the end of chromosome or called telomere. For, SV40 large T antigen is tumor virus acts as a suppressor of pRB and p53 to activate cell cycle arrest. The aim of this study to create the immortalized by using both plasmid which is ectopic overexpression. The characterization of immortalized cells were demonstrated gene and proteins that play role through immortalized cells. The results shown that human dermal fibroblast can increase cell proliferation and maintain the telomere length. Telomere length was measured by T/S ratio using qPCR techniques, were increasing in transfected cells. Ki67 protein marker of cell proliferation was up-regulated in transfected cells. Beside, transfected cells were investigated the properties of tumor and cancer cells by using soft agar assays. SH-SY5Y cells was used to positive control of tumor cells, it is neuroblastoma for compare properties of tumor. The transfected cells were shown colony on agar but size colony smaller than neuroblastoma and the number of cells still less than. Fibroblast markers containing collagen 1 type I, collagen 3 type I elastin and vimentin were expressed in transfected cells, suggested that cells still the characteristic

of fibroblasts. Protein of vimentin and collagen 1 type I were confirmed by immunofluorescence and still up-regulated in cells. On the other hands, SIRT1 and p53 were highly expressed in transfected cells, which interested about mechanism of cells. SIRT1 acts as in cell proliferation and p53 play role in the cell cycle. The co-mechanism may indicate the role of ectopic overexpression of cells. Therefore, SIRT1 protein were demonstrated and up-regulated in immortalized cells. To confirm SIRT1 role in cells, shRNA were designed to knockdown SIRT1 expression. After SIRT1 knockdown, cells show the characteristic of senescence such as the flattened cytoplasm, large nucleus and release protein out of cells. The proliferation protein marker Ki67 was decreased after SIRT1 knockdown. Activator and inhibitor of SIRT1 were used to treat with fibroblasts that inhibited the cell viability in concentration-dependent manner. The complete of SIRT1 play role in cell proliferation can suggested that the mechanism of transfected cells. However, the transient of ectopic expression is not enough to acquire *in vitro* model for 3D-skin phenomenon. The safety of cells are interesting about become to tumor. The miRNA one of choice was predicted in transfected cells. Five miRNAs were investigated by qPCR, miR-217 cannot analyzed in cells. miR-22 and miR-449a were up-regulated in SV40-transfected cells which have same target. SIRT1 and CDK6 are directly target of miRNAs and involve in cell proliferation. miR-93 was activated in hTERT-transfected cells through p53 and SIRT1 proteins. Last, miR-34a were down-regulated in transfected cells, that have been reported promote cell proliferation. The role of each miRNAs not clearly understood in transfected cells. The complete of immortalized cells can used to develop *in vitro* models for skin therapy and therapeutic applications.

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