MOLECULAR BIOLOGY EFFECTS OF VANILLIN ON KERATINOCYTES

BY

MISS SUPAK TABOONPONG

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE (DERMATOLOGY)
CHULABHORN INTERNATIONAL COLLEGE OF MEDICINE
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THESIS

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ENTITLED

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was approved as partial fulfillment of the requirements for
the degree of Master of Science (Dermatology)

on April 10, 2017

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ABSTRACT

Keratinocyte stem cells residing in the basal layer of the epidermis are thought to function in the process of the epidermis renewal and maintain the barrier property of the skin. This study has explored for the first time that vanillin, a natural compound from vanilla cured beans, has the ability to augment the stem cell property and signaling in the human HaCaT keratinocytes. The cell viability effect of vanillin on the keratinocytes was investigated by MTT assay. The western blot analysis was performed to determine the level of stem cell-mediated proteins, mesenchymal and epithelial markers. Results showed that treatment of the HaCaT cells with vanillin at non-toxic concentrations was able to significantly up-regulate the stemness mediators Oct-4, p-Oct-4 and Nanog. Besides, vanillin could increase the expression of epithelial adhesive protein E-cadherin, but has no significant effect on mesenchymal mediator Slug. As the stem cells as well as their stemness properties hold the central functions of skin renewal and repair, information gain from this study may benefit the development of vanillin to be used for skin therapy.

Keywords: Vanillin, keratinocyte, stemness, cell adhesion, Oct-4, Nanog, skin renewal

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Miss Supak Taboonpong

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
%	Percentage
°C	Degree Celsius
µM	Micro Molar(s)
µg	Micro gram(s)
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ALDH	Aldehyde Dehydrogenase
ALDH1A1	Aldehyde Dehydrogenase 1 Family, Member A1
ANOVA	Analysis of variance
CD-133	Cluster of differentiation 133
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
EPU	Epidermal proliferative unit
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
G	Gram(s)
GRAS	Generally Recognized As Safe
H	Hour(s)
HF	Hair follicle
IFE	Interfollicular epidermis
L	Litre(s)
Min	Minute(s)
Ml	Micro litre(s)
MMP-9	Matrix metalloproteinase-9

MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-κB
OCT-4	Octamer-binding transcription factor 4
PBS	Phosphate buffer saline
PPAR	Peroxisome proliferator-activated receptors
S	Second(s)
SC	Stem cell
Sox-2	Sex-determining region Y -related HMG-box-2
S.D.	Standard deviation
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Vim	Vimentin
Wnt	Wingless-type MMTV integration site family member
Wnt/β-catenin	The Wnt family and int1/β-catenin

CHAPTER 1
INTRODUCTION

Introduction

Enhancement of mechanisms in regulation of skin homeostasis has become novel emerging approaches for skin therapy as well as cosmeceutical targets. As human epidermis always has tissue turnover in response to damage or ageing, the ongoing renewal process is required for sustaining a balance status and functions of skin (2). Maintaining the stemness is the key to preserve our skin function as long as possible.

Epidermal stem cells are the key maintaining epidermal homeostasis via the cell renewal process and cytokine production. As we aged, it is hard to avoid the condition of age-associated inflammation skin. In that situation, activities and amounts of epidermal stem cells were found to be dramatically reduced (1). As evidence indicating that the stem cells not only acts as a new cell reservoir for regeneration, but they also produce a number of cytokines and growth factors supporting skin functions (2). Thus, the decrease of stem cells function and population from age-associated inflammation skin would deteriorate skin function (1). So, if we could distinguish the agents that are the stemness booster and might could preserve our skin function. It could be a strategy to solve this problem.

In searching for possible active agents that regulate stemness of keratinocytes, natural product derived vanillin is of interest. Vanillin is the primary aromatic agents, isolated from vanilla cured beans. Vanillin is as an anticlastogen (3) (4-6), from its properties to suppress gene mutations in both bacterial and mammalian cells. In addition, it was found that vanillin has the ability in protection of human single cell DNA from oxidative attack (7). In 2014, Lee et. al(8) found that vanillin could protect human keratinocyte stem cells against ultraviolet B irradiation. These characteristics suggest vanillin as an interesting agent to study its potency in improving stemness in keratinocytes.

CHAPTER 2
REVIEW OF LITERATURE

2.1 Basic knowledge of stem cell

2.1.1 Stem cell types based on cell potency

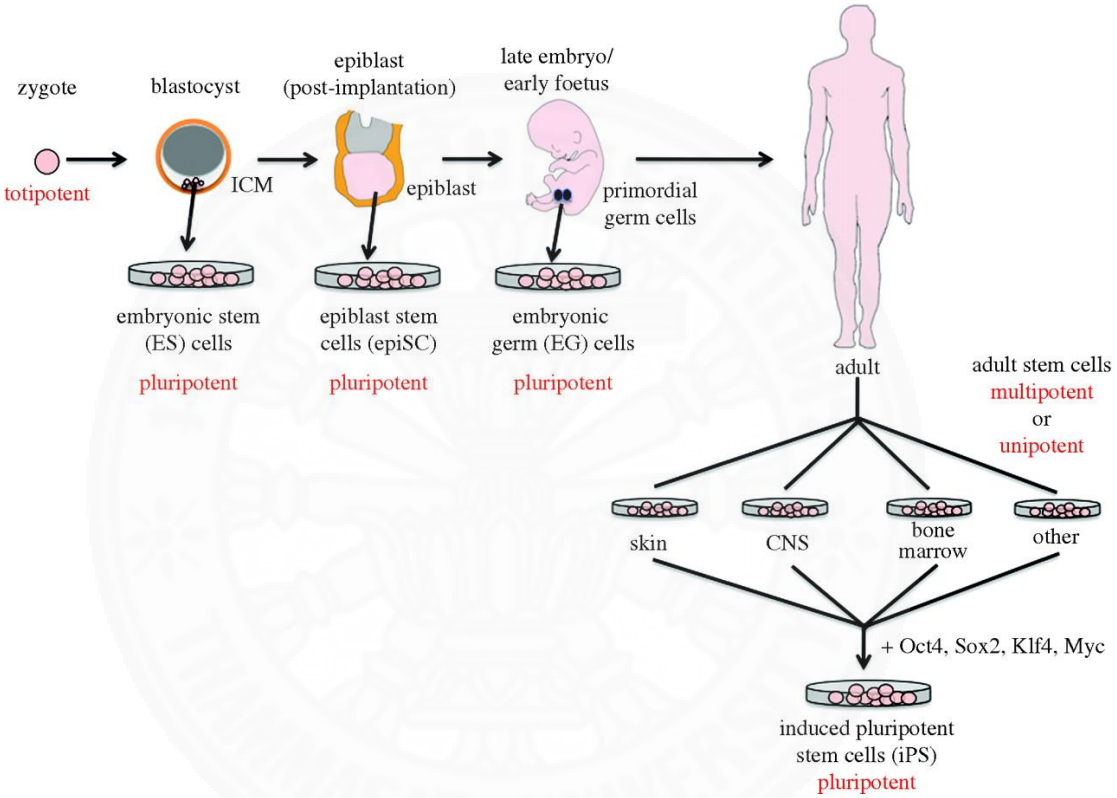

Figure 2.1 Stem cell types based on cell potency(9)

Stem cells are classified base on their abilities to differentiate into different cell types as known as differentiation potential, as the list below.

1. Totipotent (Omnipotent) stem cells Totipotent stem cells are the stem cells which have an ability to differentiate into all lineages of organism. This type of cells could be produced from the fusion of an egg and sperm cell and the first few divisions of the fertilized egg (in mammalians, blastomere of embryo at stage of 2 cells) exclusively.

2. Pluripotent stem cells Pluripotent stem cells are the stem cells which have an ability to differentiate into all lineages of the body.

3. Multipotent stem cells Multipotent stem cells are adult stem cells which have an ability to differentiate into all cell types within one particular lineage, e.g. hematopoietic stem cells that can differentiate into all types of hematopoietic cells, but limits in this lineage.

4. Unipotent stem cells Unipotent stem cells are adult stem cells which have an ability to differentiate into only one cell types, e.g. spermatogonial stem cells that can differentiate into sperm exclusively (10, 11).

2.2 Epidermal Stem cell(12)

2.2.1 Proliferative hierarchy in epithelium

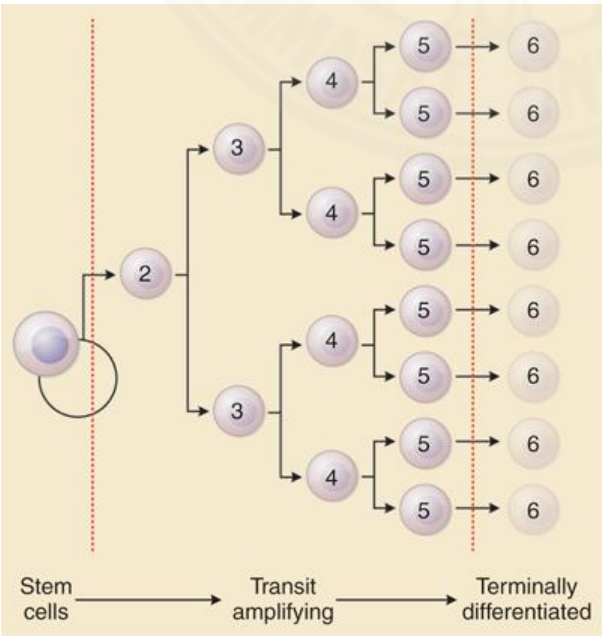

Figure 2.2 Proliferative hierarchy in epithelium (12)

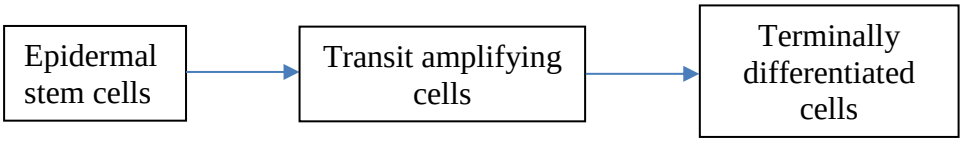

Figure 2.3 Diagram of proliferative hierarchy in epithelium

At epithelium, the hierarchy of cell proliferation and differentiation could be explained from this diagram.

- Epidermal stem cells: They are unipotent stem cells, which is at the top of this hierarchy. They have maximal potency in proliferation. Epidermal stem cells cycle is slow. They are long-living cells, which still have undifferentiated phenotype. They reside in the stem cell niches, and they are a few population of the total epithelial cell.

- Transit amplifying cells (TAC): They are committed progenitor cells which have fast cycling, produce the terminally differentiated daughter cells.

- The terminally differentiated cells: Maintaining mass of epithelium.

2.2.2 Stem cells within the adult skin epithelium

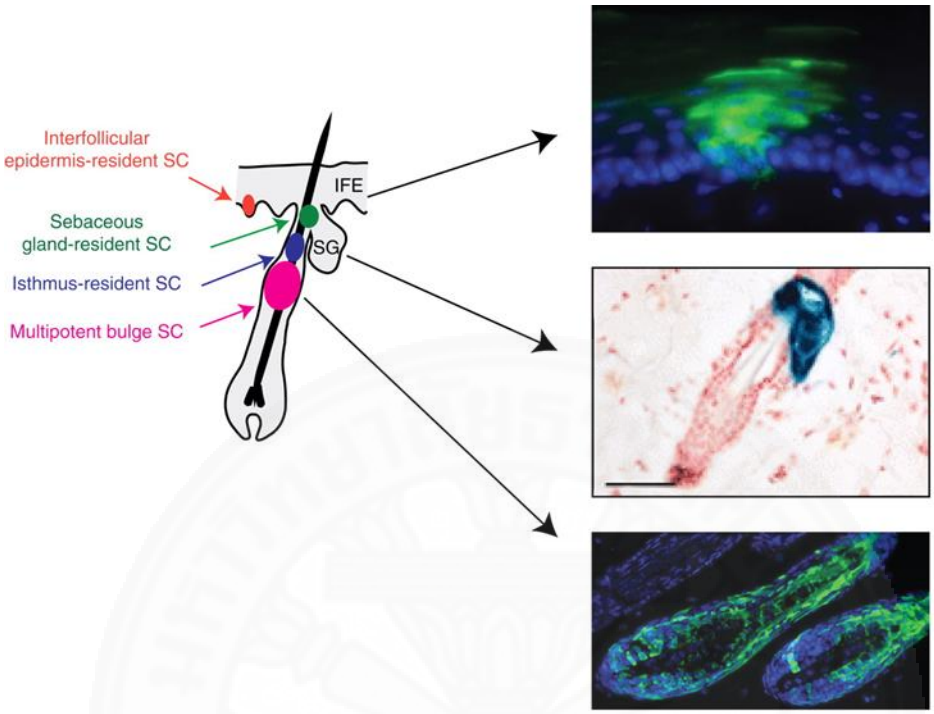

Figure 2.4 Different types of stem cells regulate epidermal homeostasis (13)

The adult skin epithelium is the combined of molecular building units. Each of the unit is composed of a pilosebaceous unit (hair follicles (HF) and sebaceous gland) and its surrounding interfollicular epidermis (IFE). Not only the IFE has its own progenitor cells to serve tissue regeneration, HFs also has multipotent SCs. At the beginning of the new hair cycle or when injury occurs, these multipotent SCs would be activated for HF renewal and the epidermis repairing (14).

The evidences that there are the progenitor cells in the epidermis are the epidermal potency to renew and repair itself. There are studies of mouse epidermal histology that display the structure of a hexagonal surface area lying on a bed of ten basal cells (15-17). It was hypothesized as this structure is an epidermal proliferative unit (EPU) with one putative SC per unit. The above studies support the EPUs existence in the basal IFE.

2.2.3 The epidermal proliferative unit (EPU) concept: organization and stem cells location

Epidermal stem cells are within the basal layer(18), and there are epidermal proliferative unit (EPU) which is a three-dimensional organized microenvironment for a single-stem cell.

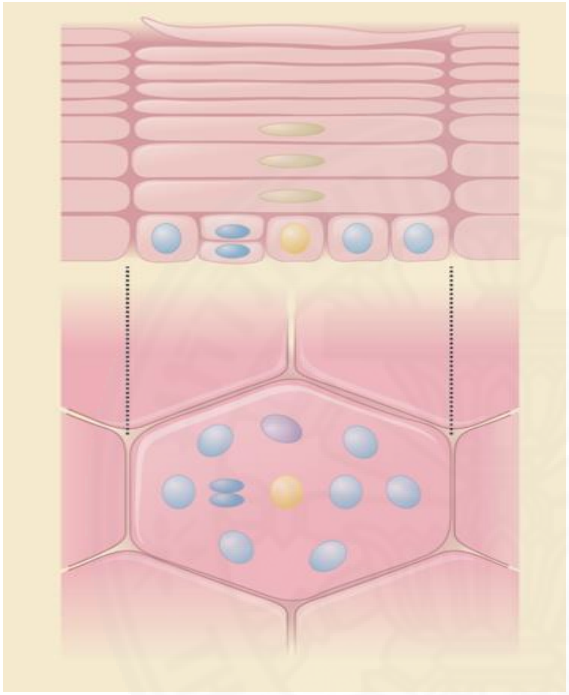

Figure 2.5 Epidermal Proliferative Unit (12)

Organization of the epidermal proliferative unit(12)

Each EPU contains close to 10 basal cells, an epidermal stem cell (yellow), immediate transit-amplifying progenitor cells (blue) and early-differentiating cells (purple).

From EPU, there is the division that which the cells differentiate to more differentiated keratinocytes (green) and mature squamous cells which enucleated.

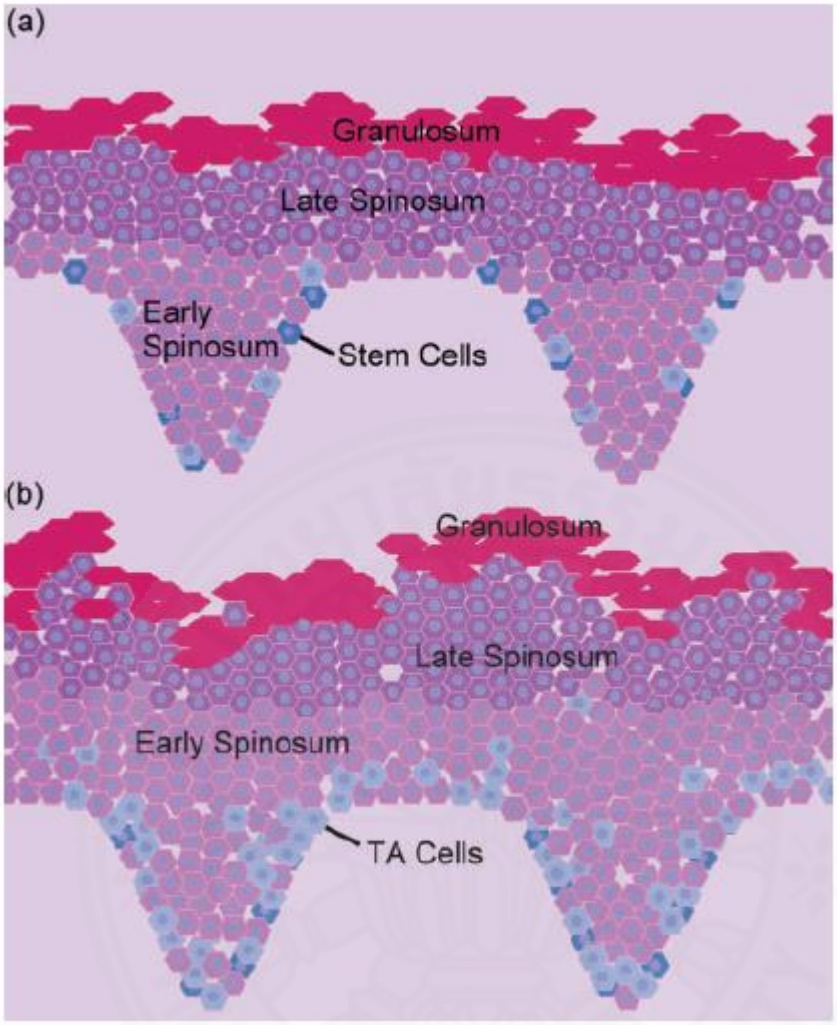

Figure 2.6 Diagram to demonstrate location and distribution of epidermal stem cells(19)

2.2.4 Relationship between compartment of epidermal stem cells

Lately emerged evidences support the existence of many stem and progenitor compartments within the cutaneous epithelium. These compartments would have no interaction if they are in balance situations (20). In hair follicle, there are various proliferative units e.g. the infundibulum of the follicle, the bulge, the upper isthmus, and the sebaceous gland, which have the capacities to assist in injured epidermis healing (12).

2.2.5 Epidermal keratinocytes morphogenesis sequence

In the process of maintaining unremitting tissue self-renewal, the epidermal keratinocytes must gradually undergo morphogenesis (19). Consistent with proliferation in the basal layer, epidermal keratinocytes move vertically through the suprabasal layers. Thereupon, the loss of terminally differentiated squames from the skin surface are compensated (19). These sequences are under the complex and tightly regulated gene expression.

Morphology of keratinocytes in each layer of epidermis are their unique characteristics. Morphology of a keratinocyte could help us to define its stage in differentiation. In epidermis, there are composed of 4 main layers, stratum basale, stratum spinosum, stratum granulosum and stratum corneum, from the deepest layer to the uppermost layer consequently, which have their own shape like this figure (21, 22).

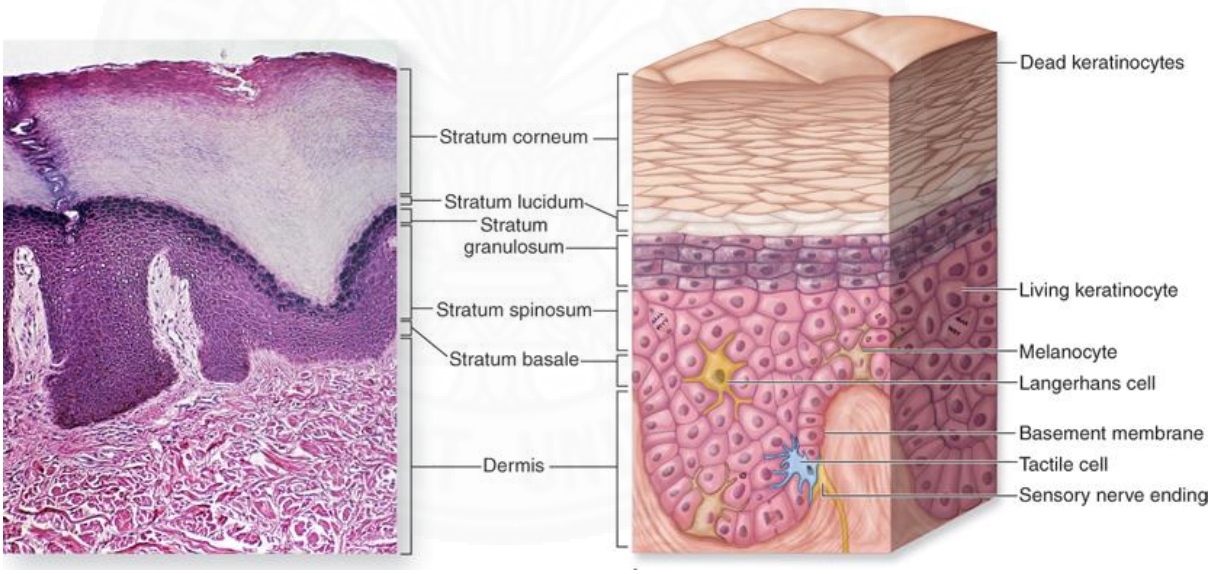

Figure 2.7 Epidermal layer (21) (Left) Micrograph of thick skin (X100; H&E) (Right) Illustrated diagram

Stratum basale: A uni-layer of columnar or cuboidal cells with oval shape nucleus. Basophilic cytoplasm and tonofibrils are found. The cells line on the basal lamina at the dermal-epidermal junction, which hemidesmosomes help tightening them together. Desmosomes also join the cells with other cells in lateral and upper sides. Epidermal stem cells and their niches reside in this layer. Intense mitotic activity cells

and progenitor cells are found at the area which joining with the deepest part of the next layer. These cells go upward when differentiation happens. Meanwhile, both amount and kind of keratin filaments increase until they reach the half of the amount that found in total protein of the superficial keratinocytes (21).

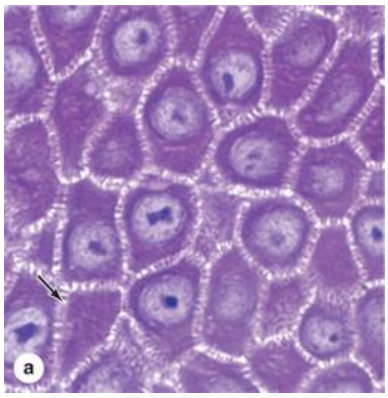

Figure 2.8 Stratum spinosum (21)

Stratum spinosum: Irregular, polyhedral-shaped cells which have central, large spheroid nucleus with nucleoli and basophilic cytoplasm. In addition, more tonofibrils was found comparing with stratum basale.

Tonofibrils are end at desmosomes. At both endings of each desmosome, there are little elongation of cells around the tonofibrils. When the cells shrink, the elongation would be distinct and would be called as “spines”, which are the hallmark characteristics of the keratinocytes in this layer (21).

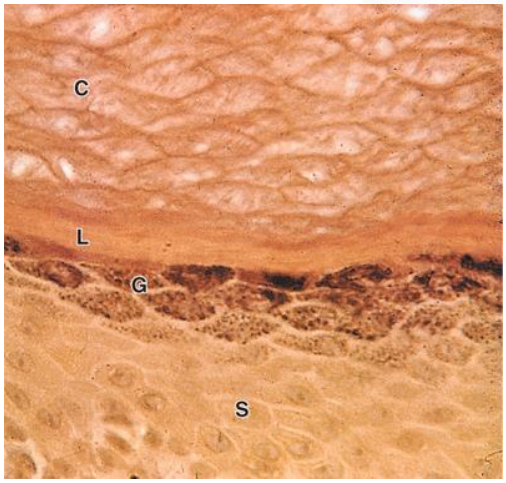

Figure 2.9 Stratum granulosum (21) X560, H&E staining

Stratum granulosum: Keratinocytes in this layer is in flat-shaped. The hallmark of this layer is intense basophilic masses in cytoplasm, named "Keratohyaline granules". Tonofibril keratin filament, filaggrin and other keratohyaline granule's protein are cross-linked together, forming tight bundles which help flattening the cells. Another feature of this layer is Golgi-derived lamellar granules, which contain diverse lipids and glycolipids (21).

Stratum corneum: The cell shape is flatter than cell in stratum granulosum. Cells lost their nuclei and cytoplasm (21). The keratin, which derived from keratohyalin granules, would replace the cytoplasm. These filaments are opaque and structureless. Thicker cytoplasmic membranes also be found (22).

2.2.6 Skin Diseases Arising from Proliferative Dysfunction(12)

Psoriasis is thought to be an example of hyperproliferation of transit amplifying cells, which among inflammatory and dermal changes, is characterized by increased numbers of β-1 integrin dim cells in the suprabasal layers (19). Additionally, markers of proliferation such as Ki67 and C-myc are upregulated.

2.3 Age-associated inflammation inhibits epidermal stem cell function

As we aged, it is hard to avoid the condition of age-associated inflammation skin. In that situation, activities and amounts of epidermal stem cells were found to be dramatically reduced.

As evidence indicating that the stem cells not only act as a new cell reservoir for regeneration, but they also produce several cytokines and growth factors supporting skin functions. Thus, the decrease of stem cells function and population from age-associated inflammation skin would deteriorate skin function.

2.3.1 Alterations in stem cells during skin aging (1)

- Increased numbers
- Decreased function
- An inability to tolerate stress

2.3.2 Phenotypical & functional changes (1)

- Decreased hair cycling
- Epidermal thinning
- Diminished sebaceous gland function
- An impaired wound response

2.4. HaCaT cell lines

HaCaT is a human epithelial immortalized cell line which is developed from an adult skin by Boukamp et al. in 1988. Boukamp et al. created HaCaT from epithelial cells that got from the distant periphery of a melanoma located on the upper half of the back of a 62-yr-old male patient (not extensively sun-exposed). Then these cells were infected by Simian virus 40 (SV40). Regardless of the ability to reproduced itself endlessly, HaCaT is almost identical to normal keratinocytes. When HaCaT was transplanted onto nude mice, orderly arrangement of the structure and same reformation of the tissue was found. K1, K10, involucrin and filaggrin, which are the differentiation markers are expressed and regularly located in this new tissue. Thus, these make HaCaT widely accepted in the use of regulation of human keratinization experiments.

Another identical confirmation test between HaCaT cell line and keratinocytes at the original tissue is DNA fingerprinting by hypervariable minisatellite probes. Both DNA are the same, which shows that even long-term cultivation, transformation, and multiple chromosomal alterations, DNA fingerprint pattern is still not affected. Gathering together, all of these results provide HaCaT as a promising tool that can be substituted for human keratinocytes in nowadays studies (23).

2.5 Triad of master transcription factors in pluripotency

Transcription factors control cell fate through development, by being like molecular switches to trigger or suppress particular gene expression programs (24).

Nowadays, transcription factors that are recognized as “master” in self-renewal and pluripotency of human and mouse embryonic stem cells and blastocyst inner cell mass cells consist of Oct-4, Nanog and Sox2 (24-26) (27, 28). These factors are established as critical triad of master transcription factors in pluripotency. Additionally, these factors drive each other expression level by both positive and negative feedback loops, as a gene regulatory network, to control the pluripotency properties (29).

2.5.1 Oct-4

2.5.1.1 Oct-4

Among regulators in pluripotent and germline cells, Octamer-binding transcription factor-4 (Oct-4) is a master which crucially helps forming a pluripotent founder cell population at the initial stage in the mammalian embryo (30). Oct-4 protein consists of a POU domain (POU5F1) which is a homeodomain (26). Due to its capacity to bind to and activate the Octamer DNA sequence 5´-ATGCAAAT-3´, it was named ‘Oct’ (28). All OCT’s family members have a same DNA-binding domain, the POU domain, which comprises 2 subdomains - the POU-specific domain (POUS) and the homeodomain (POUH), articulated together by a flexible joiner.

Oct-4 is a pioneer transcription factor which bind to chromatin, being as chromatin remodelers and is attached transcriptional co-activators (31) (32). Roles of Oct-4 in cell reprogramming is unique and varied. Oct-4 is not only important for lineage specification, but also controls multiple contrasting processes of cell identity change. However, cellular context and environment might affect Oct-4 function (32), e.g. depending on the flanking sequences123 (26).

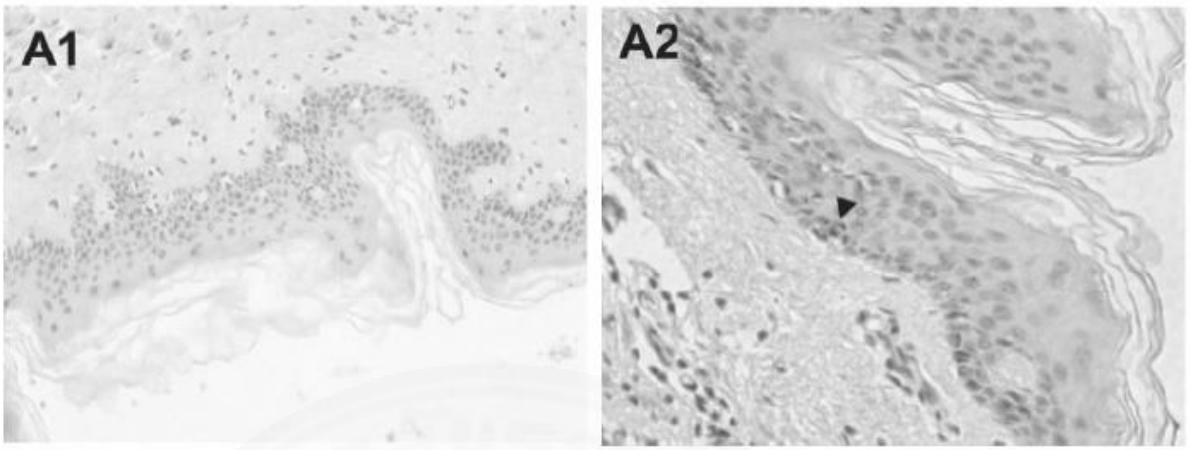

Figure 2.10 Expression of Oct-4 in human skin stem cells stem cells residing in the basal layer: After deparaffinized, Oct-4 primary antibody, avidin-HRP and DAB (dark brown color) was stained on the skin tissue consequently. Oct-4 (brown points at arrowheads) is in the nucleus of few cells at basal layer, indicated that these cells are stem cells (33).

Oct-4 expression was proved to be found in adult human stem cells and in epidermis. In Tai, et al. study (33),they immunostained Oct-4 on a human normal tissue MaxArray and found Oct4-positive cells in a small amount, scattered in the basal layer of epidermis, where is previously shown as location of skin stem cells (Figure 2.10).

2.5.1.2 Phosphorylated Oct-4

(1) Phosphorylation reaction

Among various post-translational modifications, the most common and the most garnered intense interest one is protein phosphorylation. It regulates a variety of pathways, such as pathways of cell cycle, growth, signal transduction and apoptosis.

In eukaryotes, phosphorylation happens at the side chains of only 3 acids, serine, threonine and tyrosine A hydroxy (-OH) group of the amino acids attacks the terminal phosphate group ($-PO_3$) on adenosine triphosphate (ATP). Then the phosphate group are transferred to the amino acid side chain.

Since in this reaction, PO_3 is got from changing ATP to adenosine diphosphate (ADP). During this step, the high quantity of free energy must be released. Thus, it makes the reaction unidirectional (34, 35).

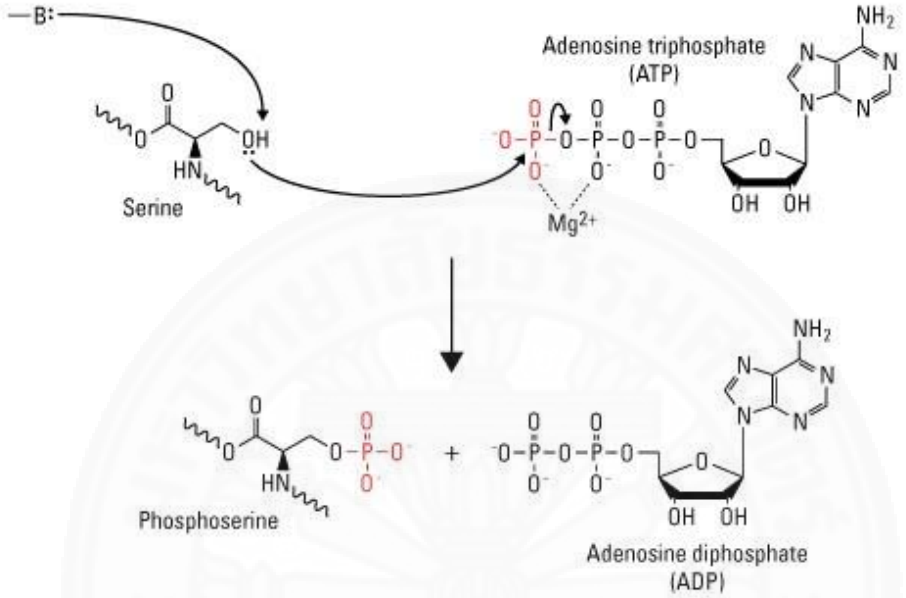

Figure 2.11 Diagram of serine phosphorylation (34)

(2) Phosphorylated Oct-4

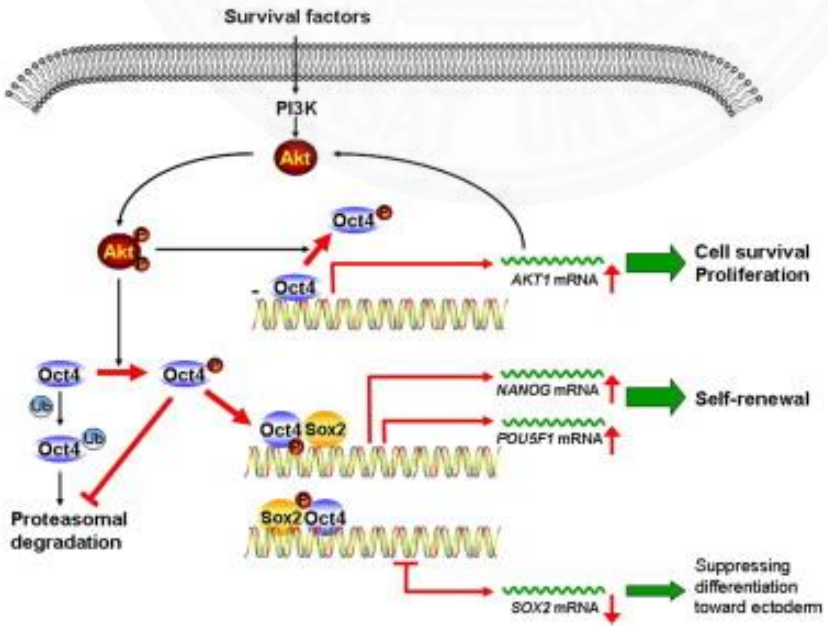

Figure 2.12 Action of phosphorylated Oct-4 (36)

Phosphorylated Oct-4 (p-Oct-4) is Oct-4 which has been phosphorylated. phosphorylated Oct-4 is involved in process of stem-like cells proliferation (37). p-Oct-4 has multiple functions. First, p-Oct-4 activates the transcription of a serine/threonine-specific protein kinase named Akt, results as increasing cell survival and proliferation. Second, after being phosphorylated, p-Oct-4 becomes more stable and regulates stemness genes transcription. Third, p-Oct-4 interact with Sox-2, increasing Oct-4 and Nanog mRNA transcription. Therefore, self-renewal is promoted (36).

In 2009, Saxe et al. demonstrated a phosphorylation-based mechanism that regulates specific Oct4 homodimer conformations. After a putative phosphorylation site was point mutated, the revoking of transcriptional activity of a particular homodimer assembly happened (38). Additionally, they could determine diverse signaling pathways that may mediate this phosphorylated process.

Nowadays, there are multiple well-known phosphorylation site. For instance, T235 which is Akt-phosphorylation site (36) and S236 from the study of Swaney et al., 2009 (39), which referred as S235 in Brumbaugh et al.,2012 (40) which located directly next to the T235 site. S235/236 is phosphorylated by protein kinase A (38).

2.5.2 Nanog

Nanog is discovered in 2003 and named by Professor Ian Chambers who isolated this gene from mouse. He thought that Nanog seems to be a master gene embryonic stem cell which makes stem cells can live perpetually, so he named this protein after the mythological Celtic land of the ever young “Tir nan Og” (41).

Nanog is a notable homeobox transcription factor (29). Nanog is an essential downstream signal of extrinsic signals such as leukemia inhibitory factor (LIF), Bone morphogenetic protein (BMP) and Wingless-type MMTV integration site family member (Wnt), which these extrinsic signals help maintaining pluripotency of the embryonic stem cells. Nanog is specific in pluripotent cells and indispensable in the maintenance of the undifferentiated state of early postimplantation embryos and embryonic stem cells in mouse and human. In 2005, Hatano.et al.(41) has discovered Nanog’s expression pattern by doing immunocytochemical analysis in vivo. No Nanog

was found in unfertilized oocytes. On the contrary, Nanog appeared in cells of morula-stage embryos, the inner cell mass of blastocysts and the epiblast of E6.5 and E7.5 embryos. In monkey and human ES cells, presentation of Nanog specified to undifferentiated cells.

Increased Nanog amount could keep up self-renewal of the mouse embryonic stem cells in spite of LIF withdrawal. Nanog also could enable human embryonic stem cells growth despite feeder cells withdrawal. As embryonic stem cells undergo its differentiation, Nanog is downregulated.

Other than these external signals, Nanog expression is regulated by intrinsic signals like FoxD3, p53 and Oct4. In maintaining stemness of cells, Nanog collaborate with Oct-4 and Sox2, which are also key signals to rule essential genes that regulate embryonic stem cells pluripotency. Nanog, Oct-4 and Sox-2 govern each other expression level as a gene regulatory network, to control the pluripotency properties.

2.5.3 Sox-2

Scientists has spent three decades to genotype sex-reversed XX men and XY women, to discover the Y-linked gene. In 1990 Sinclair et al. found it, and termed it as SRY (sex-determining region Y) (42).

In mammals, SRY, the Sox family has about 20 members (43). Every protein in this family has the same highly conserved DNA binding domains which is called as HMG box domains, due to this sequence and the DNA binding domains which found in the superfamily of High Mobility Group DNA binding proteins is alike (44). The Sox family members are involved in critical roles along every stages of mammalian development (43). Obvious aspects that are controlled by Sox gene subgroups are testis development, central nervous system neurogenesis, oligodendrocyte development, chondrogenesis, and neural crest cell development (43).

SRY-related HMG-box-2 (Sox-2) protein is a member of the SRY-related HMG-box (SOX) family. Sox-2 is a transcriptional factor which is produced from, Sox-2 gene, an intronless gene (45). Sox-2 plays key roles during in the regulation of embryonic development and in the determination of cell fate. Besides this, Sox-2 controls gene expression in the stomach. Sox-2 mutation is related with optic nerve hypoplasia and syndromic microphthalmia.

Sox-2 is also significant for induced pluripotent stem cells generation. Embryonic lethality during the peri-implantation stage of development could happened if Sox2 gene or the Oct-3/4 gene are deactivated. In embryonic stem cells, knocking down Oct-3/4 or Sox2 stimulate their differentiation into trophectoderm-like cells (46). Likewise, Sox-2 expression is put down as an expanding list of cancers (47, 48).

2.6 From pluripotency to differentiation: Cell Regulation in pluripotent and differentiated states(49)

In pluripotent state, pluripotency factors and epigenetic factors work cooperatively, causing transcription of pluripotency-associated genes, while holding differentiation-related genes in quiescence. At differentiation state, micro RNAs (length about 22 nt) support downregulation of pluripotency factors, influencing the fast shifting of the transcriptional profile. Holding pluripotency-associated genes in quiescence, differentially regulating development-related genes and shifting chromatin from a hyperdynamic state into a denser state.

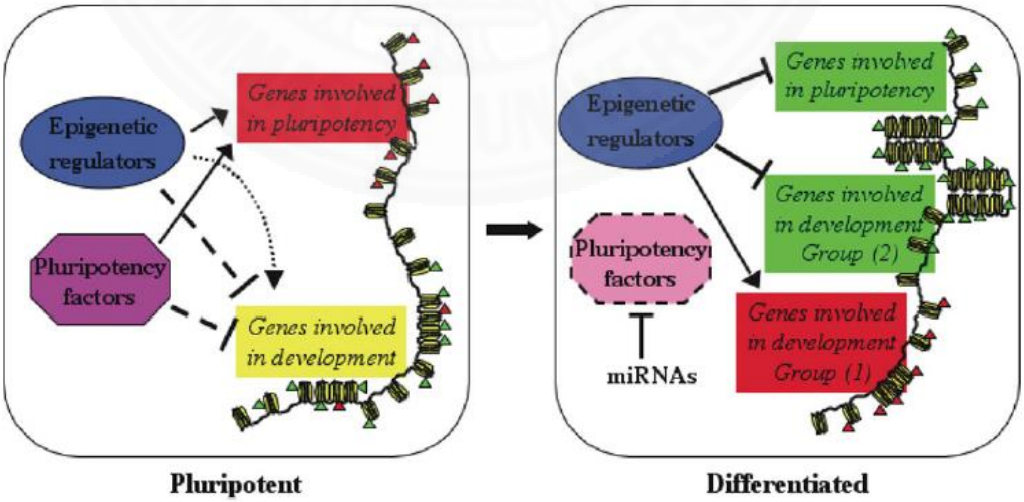

Figure 2.13 Cell Regulation in pluripotent and differentiated states(49)

2.7 Epithelial to mesenchymal transition (EMT)

Epithelial-to-mesenchymal transition or EMT referred to a stepwise process which let epithelial cells which usually attach to the basement membrane changing to mesenchymal cell phenotype. The higher migratory potency, invasiveness, obviously raised ECM components production and higher apoptosis resistance are found in the mesenchymal cell phenotype comparing to the epithelial cell phenotype (50). Addition of role in embryonic development and tumor progression, EMT still function in fibrogenic and chronic inflammatory disease (51).

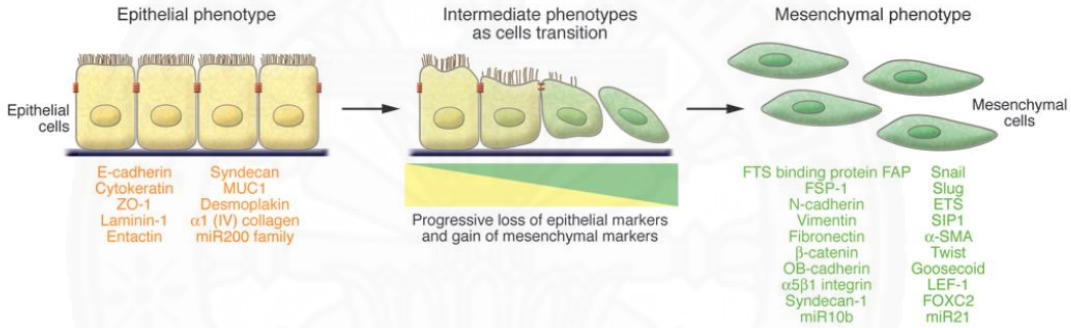

Figure 2.14 Epithelial to mesenchymal transition process (50)

Based on Kalluri's works(50), EMT are classified into 3 biological settings; setting 1, EMT during implantation, embryogenesis and organ development, setting 2, EMT associated with tissue regeneration and organ fibrosis, and setting 3, EMT associated with cancer progression and metastasis.

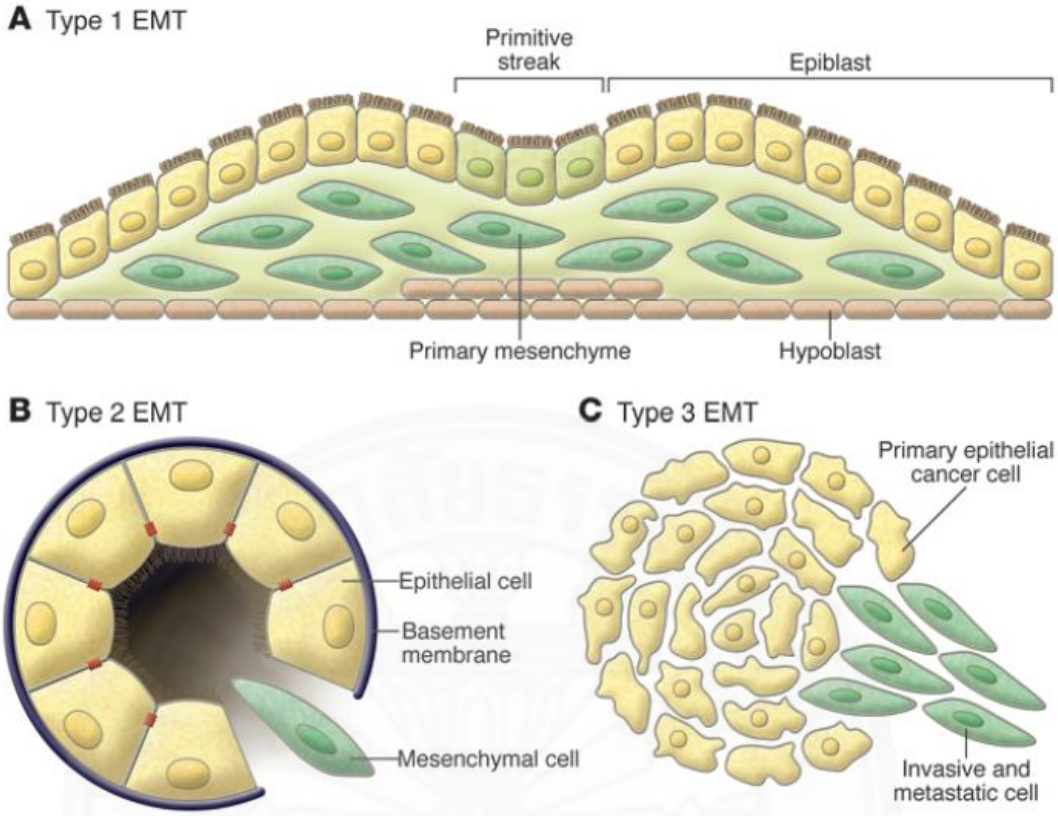

Figure 2.15 Types of epithelial to mesenchymal transition(50)

The basis hallmark of EMT is losing E-cadherin. To repress E-cadherin, a variety of transcription factors involve in this process, such as Snail/Slug, ZEB1, SIP1, Twist, and E12/E47 (51). Moreover, various markers have been involved in this process, as listed in table 2.1. And their interaction is showed in the below diagram.

E-cadherin and Snail/Slug family are of interest the EMT signals in post-development epidermis recently years study (51).

Table 2.1 Lists of general EMT markers (52)

EMT markers
Proteins that increase in abundance
N-cadherin
Vimentin
Fibronectin
Snail1 (Snail)
Snail2 (Slug)
Twist
Goosecoid
FOXC2
Sox10
MMP-2
MMP-3
MMP-9
Integrin αvβ6
Proteins that decrease in abundance
E-cadherin
Desmoplakin
Cytokeratin
Occludin
Proteins whose activity increases
ILK
GSK-3β
Rho
Proteins that accumulate in the nucleus
β-catenin
Smad-2/3
NF-κβ
Snail1 (Snail)
Snail2 (Slug)
Twist
In vitro functional markers
Increased migration
Increased invasion
Increased scattering
Elongation of cell shape
Resistance to anoikis

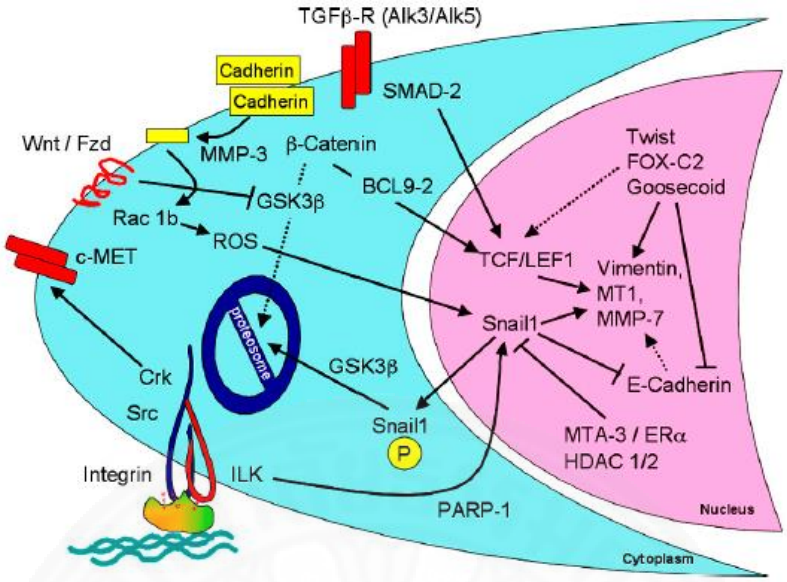

Figure 2.16 Diagram showing epithelial to mesenchymal transition pathway(52)

2.8 E-cadherin

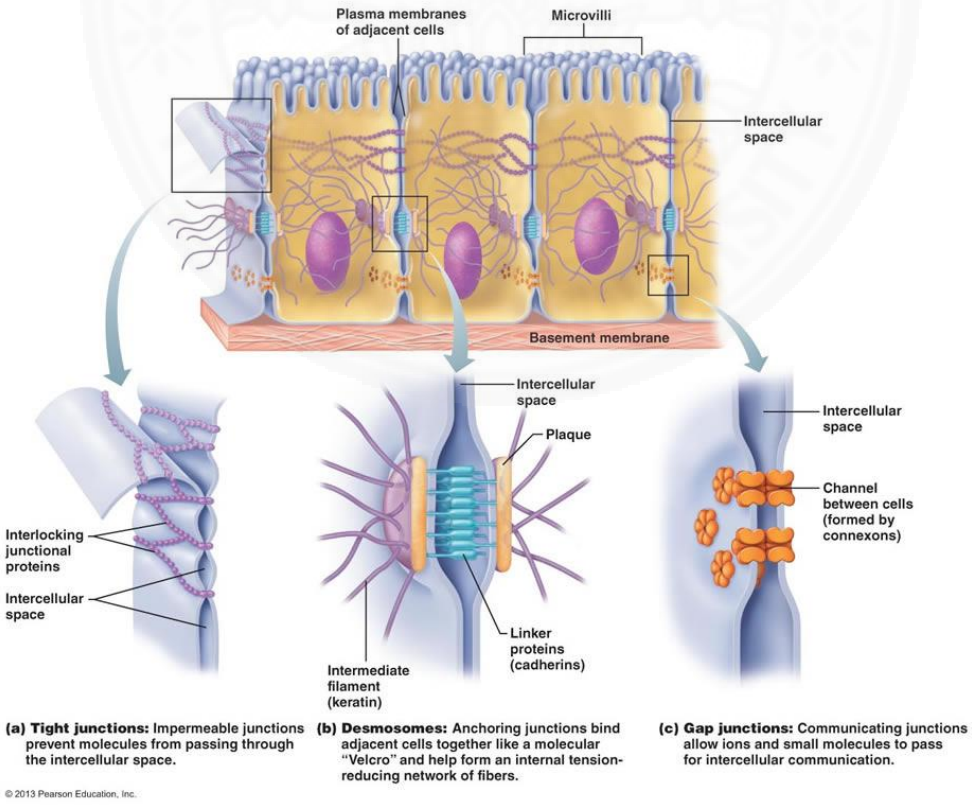

Figure 2.17 Cell junction(53)

Cadherins is a family of glycoproteins, function in mediating Ca^{2+}-dependent cell–cell adhesion. Additionally, cadherin influence keratinocyte cell-surface integrin levels (54).

Type of cadherin that expressed in adult human epidermis are

1. E-cadherin, which expressed in the epidermis thoroughly.
2. P-cadherin, which just expressed in the basal layer

Relationship of reduced E-cadherin expression and reduction of differentiation-specific protein expression has been found (55). E-cadherin and P-cadherin are composition of transmembrane proteins, which is in cell to cell junction.

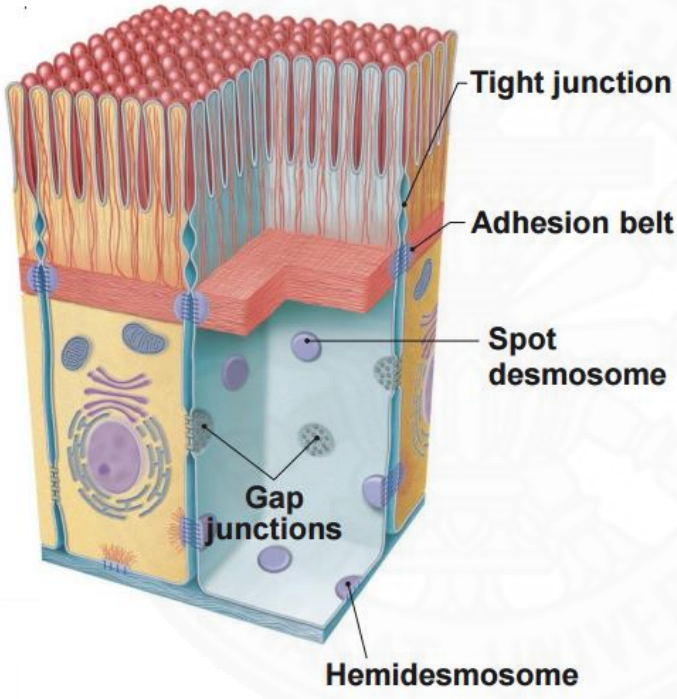

Figure 2.18 Cell junction in a three-dimensional view(53)

For E-cadherin roles in keratinocytes, *in vivo*, deficiency of E-cadherin is linked with reduced markers of differentiation. *In vitro* result was also going in the same way, if E-cadherin binding in human keratinocytes is impeded, the epidermal stratification would be disturbed, and localization of junction proteins would be postponed. Nevertheless, in case of E-cadherin is solely inhibited, some differentiation markers levels were elevated, suggested that the cell–cell contact on differentiation's effect is required multiple gene regulation.

A down-regulation of E-cadherin together with and up-regulation of N-cadherin expression indicates the classical conversion of epithelium into mesenchyme (50). E-cadherin is the major molecular component in establishing stable epithelial cell-cell adhesions including desmosome, adherens junction, and tight junction (56). These intercellular junctions allow communication between cells, restrict mobility of the epithelial tissue, and preserve the apico-basal polarization. The down-regulation of E-cadherin results in the dissemination of epithelial cell architecture by disrupting the apico-basal polarization and promoting the front-rear polarization supporting the migratory phenotype (57). In addition, the loss of E-cadherin also contributes to liberating of protein complex attaching to the cytoplasmic domain of E-cadherin including p120 catenin. Free p120 catenin accumulation increases cell survival and migrative behaviors (58).

By invented skin-specific RNAi technology (59), it was proved that when only E-cadherin is suppressed *in vitro*, deceleration of epidermal sheet formation was found. When E- and P-cadherins missed, expanding of defects to adherens junctions, desmosomes, tight junctions and cortical actin dynamics was exhibited. *In vivo*, E-cadherin inhibition caused impaired junction formation, deteriorated intercellular adhesion and raises apoptosis rate.

2.8.1 E-cadherin significance in epidermal stem cell

To sustain regulating stem cell function: retention, division and exit, stem cells need to get cell-cell and cell-matrix interaction. So, epidermal stem cells need niche anchoring via adhesion molecules (60-62). Therefore, stem cell-niche adhesion is crucial for stem cell self-renewal and is dynamically regulated.

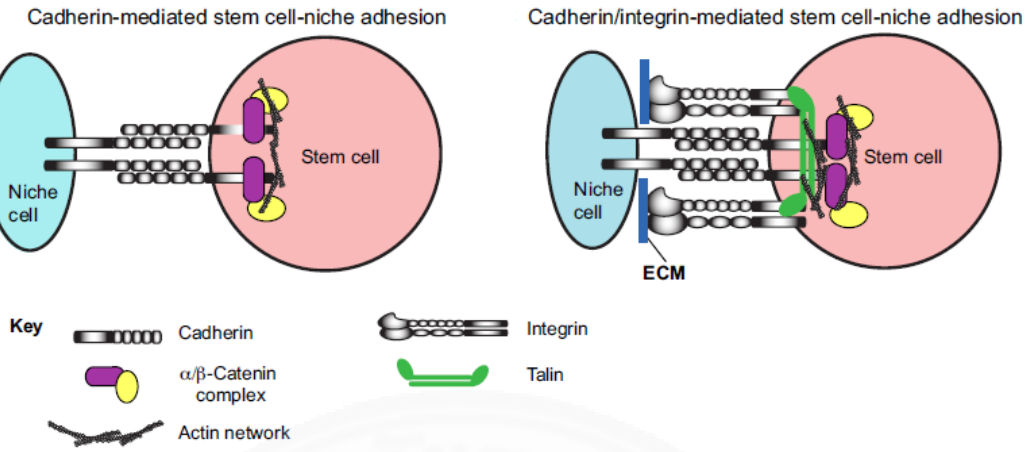

Figure 2.19 Cadherin- and integrin-mediated stem cell-niche adhesion (60)

Special adhesive characteristics of epidermal stem cells that distinct from their more differentiated progeny (63) are composed of 1) increased cell-cell cohesiveness and 2) increased extracellular matrix adhesiveness (54, 64-67). These characteristics are mainly regulated by cadherins for cell-cell interactions and integrins for regulate cell-matrix interactions.

E-cadherin still have other roles in stem cell regulation besides stem cell-niche anchorage (60). First, E-cadherin function as a signal, sending directly to stem cell or help receptor complex which mediate signal from the niche to stem cell, to regulate stem cell self-renewal, survival and proliferation, as shown in Figure 2.22A.

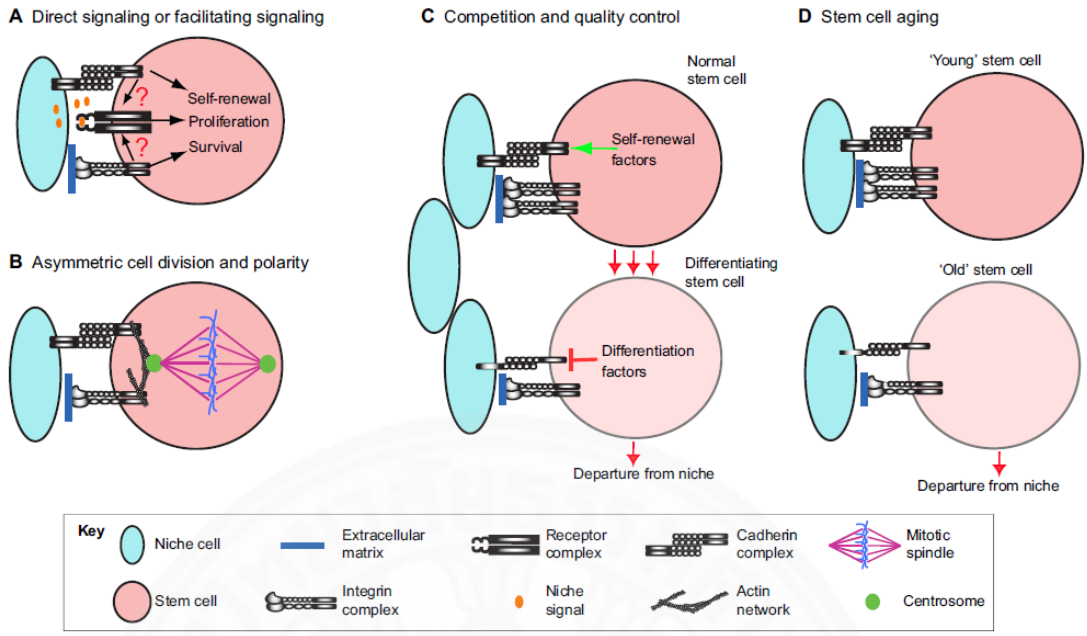

Figure 2.20 Other roles E-cadherin in stem cell regulation in addition to stem cell-niche anchorage (60)

Second, Cadherin obligate in asymmetric cell division and maintaining cell polarity. Cadherin or integrin collaborate with actin cytoskeleton network facilitate centrosome anchoring, restrict the direction of the mitotic spindle perpendicularly to the niche surface, as shown in Figure 2.22B.

Third, for niche occupancy, each stem cell within a niche must compete to anchored to the niche with each other (68). Hence, cell adhesion molecules play a pivotal role in this situation. Usually, regulation of adhesion molecules expression is controlled by self-renewal and differentiation factors. Thus, stem cell-niche adhesion is the way to govern stem cells to retain in the niches, while let the differentiating cells depart the niche, as shown in Figure 2.22C.

Forth, young stem cells would have more expression of adhesion molecule level more adhesion force to the niches than old stem cells. This mechanism let the old stem cells depart from the niches, as shown in Figure 2.22D.

2.9 Vimentin

There are three types of cytosolic fibers in the cytoskeleton, which are microfilaments (actin filaments), intermediate filaments and microtubules. Cell specific expression is indicated by its main types of intermediate filaments which are

1.Cytokeratins: main type intermediate filament of epithelial cells

2.Glial fibrillary acidic protein (GFAP): main type intermediate filament of glial cells

3.Desmin: main type intermediate filament of skeletal, visceral and certain vascular smooth muscle cells

4. Vimentin: main type intermediate filament of mesenchyme origin

5. Neurofilaments: main type intermediate filament of neurons

Addition of the major function, serving as a main composition of intermediate filaments of mesenchymal cells and helping cell migration (69, 70), vimentin also possess ability of spatial re-organization for extracellular stimuli affection. These characteristics assist in process of coordinating several signaling pathways (71). Vimentin is also a canonical cytoskeleton EMT marker and its correlation with oncogenic progression is widely accepted (72).

Besides this, during development, vimentin is found within non-mesenchymal cells, such as epithelial cells and neurons (73-75). Moreover, coexistence of vimentin and keratin was found in cultured keratinocytes (76, 77), in vitro outgrowths of epidermal explants (78). It was found that diploid keratinocytes exhibit vimentin in certain circumstances. In 2015, it was reported that a subgroup of diploid keratinocytes in human epidermal cultures, which showed vimentin positive, expressed the brightest α5β1 integrin and p63 (79). For α5β1 integrin and p63, there is considerable evidence that they are epidermal putative stem cell markers (80).

In the specific situation, diploid keratinocytes could show vimentin intermediate filaments. Additionally, expression of the brightest p63 and α5β1 integrin markers, which is believed as the epidermal putative stem cell markers, were found in the subpopulation of vimentin positive keratinocytes in the human epidermal culture colonies (81).

2.10 Snail family

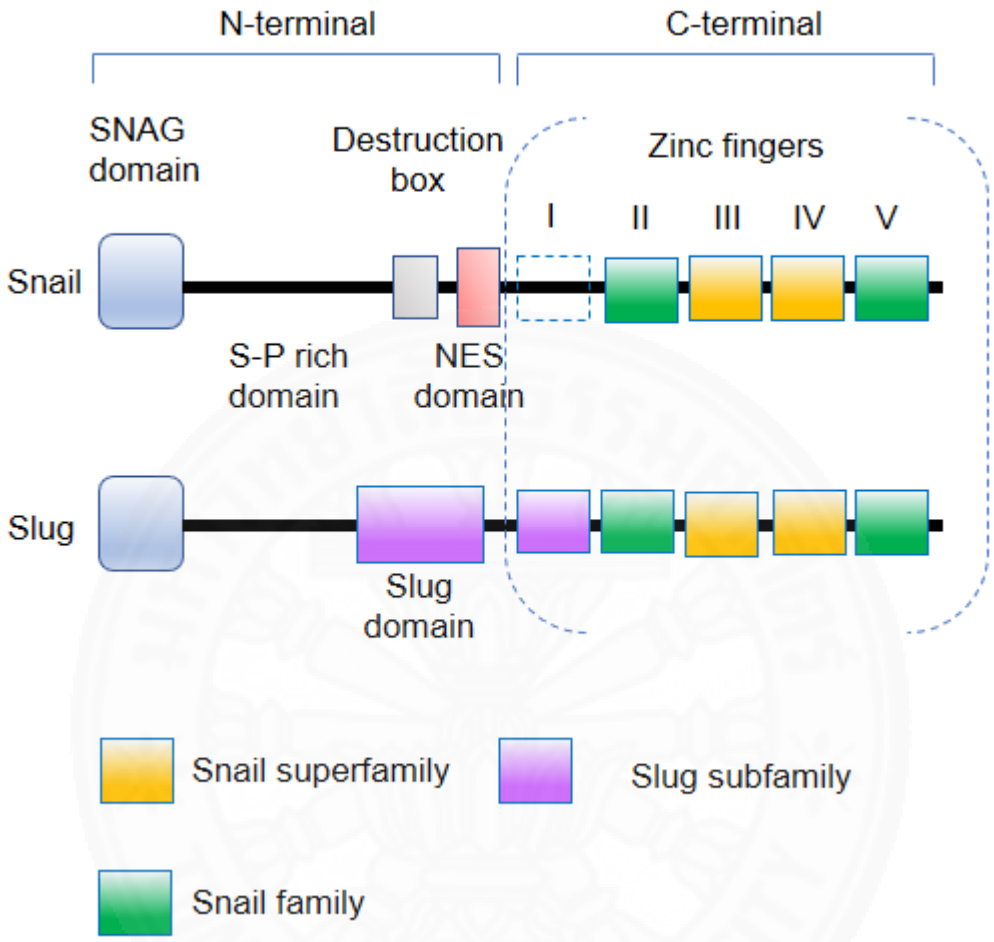

Figure 2.21 Structure of Snail family transcription factor, adapted from (82) and (83)

Snail family is a group of transcription factors, which composed of N-terminal and C-terminal. In vertebrate animals, they have developed the SNAG(Snail/Gfi) domain at N-terminal, which is useful for binding a myriad of transcriptional co-repressor complexes (84). In all creatures, they own C-terminal domain which composed of 4-6 C_2H_2 type zinc fingers and bind to the E-box motif (5′-CANNTG-3′) in target gene promoters (85). In vertebrates, three Snail family proteins are found, including Snail1 (Snail), Snail2 (Slug), and Snail3 (Smuc) (51, 84).

For Snail(Snail1), it contains a serine-rich domain (SRD) and a nuclear export sequence (NES) at the central region. SRD phosphorylation regulate exportation of Snail, and NES functions in cytosolic distribution (86).

2.10.1 Function of Snail family in keratinocytes

2.10.1.1 Snails helps re-epithelization by increase keratinocytes migration in wound healing process

To promote wound inflammation, reepithelialization and angiogenesis, wounds must use cytokines, TGF superfamily members and EGFR ligands in this process. Numerous of these cytokines and ligand signal via Snails or induce via Snails signaling.

Especially for Slug(Snail2), Slug is up-regulated by EGFR ligands and indispensable for keratinocytes migration. Snail1, which also stimulated EGFR ligands, could not replace this function of Slug (87, 88). In this pathway, lowering integrin expression, such as integrins α3, β1, and β4, is the downstream effect of Slug activation. These possibly increase keratinocytes detachment from the basement membrane before their migration (89).

Moreover, EMT-like morphological changing of keratinocytes could be happened if keratinocytes expose to EGFR ligands, TGF or interleukin-1 (90). At the margins of wounded skin, Slug are confirmed to be up-regulated in keratinocytes *in vivo* (88, 91).

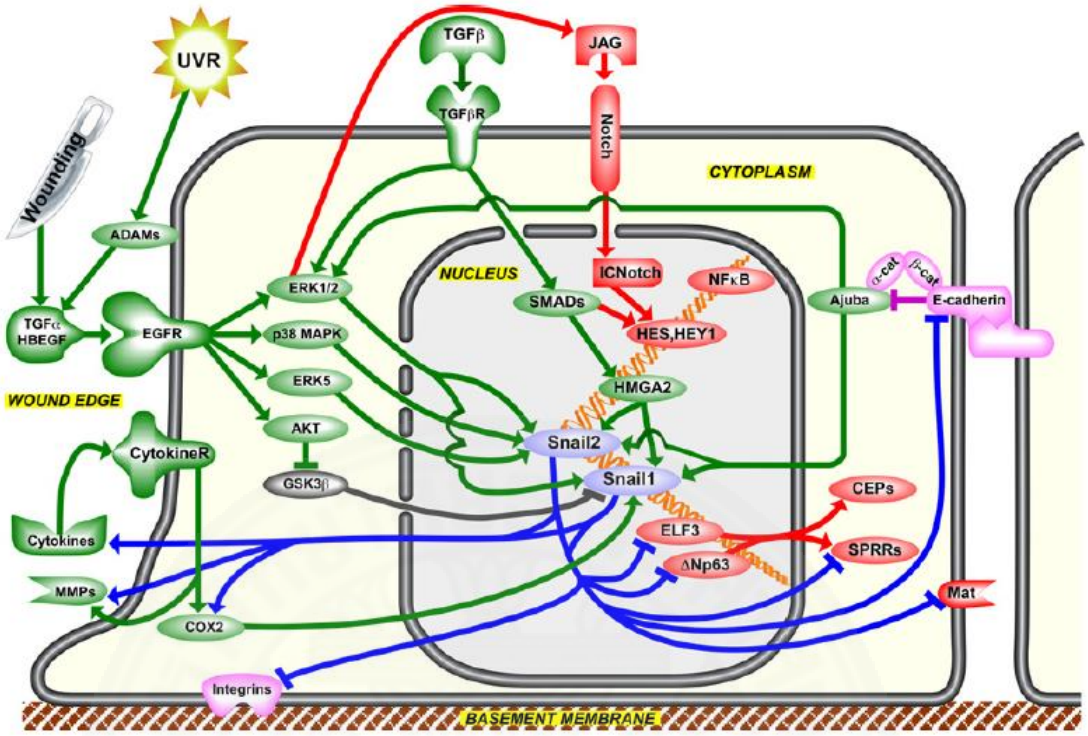

Figure 2.22 Snails is the nexus of wound healing in keratinocytes(92)

2.10.1.2 Snails increased inflammation in wound healing process

It is previously known that ultraviolet radiation exposure cause wound inflammation by upregulated ADAM17. ADAM17 stimulate EGFR ligands from the cell surface. Then the ligands signal EGFR in keratinocytes. The cascade goes on via ERK1/2 and p38 MAPK pathways, results as Snail and Slug expression, as explained in Figure 2.24 Snails expression would stimulate cyclooxygenase-2 (COX-2) and other cytokines. *In vivo*, Slug-deficient mice not only could not be found acute inflammatory effects of UVR, but also could not upregulated proinflammatory cytokines. Generally, slug expression would be raised at wound margins to promote wound inflammatory state and be decreased at wound closure to enable proceeding of wound resolution.

2.10.1.3 Snails abolish terminal differentiation in keratinocytes

Snails abolish terminal differentiation in keratinocytes via these pathways. First, Snails inhibit ELF3 and matriptase (Mat) (93). Function of these 2 signals is to exert expression and processing of cornified envelope proteins (CEPs),

inclusive of small proline-rich protein (SPRRs) through terminal differentiation. Second, Snails suppress the ΔN isoform of p63 which required for stratification. Therefore, this provides a means of the TA isoform of p63 expression (94). The study in mouse skin show epidermal thickening and expression of basal markers in the suprabasal layer, in addition to delay terminal differentiation (95).

2.10.2 Snail regulation at the transcription level

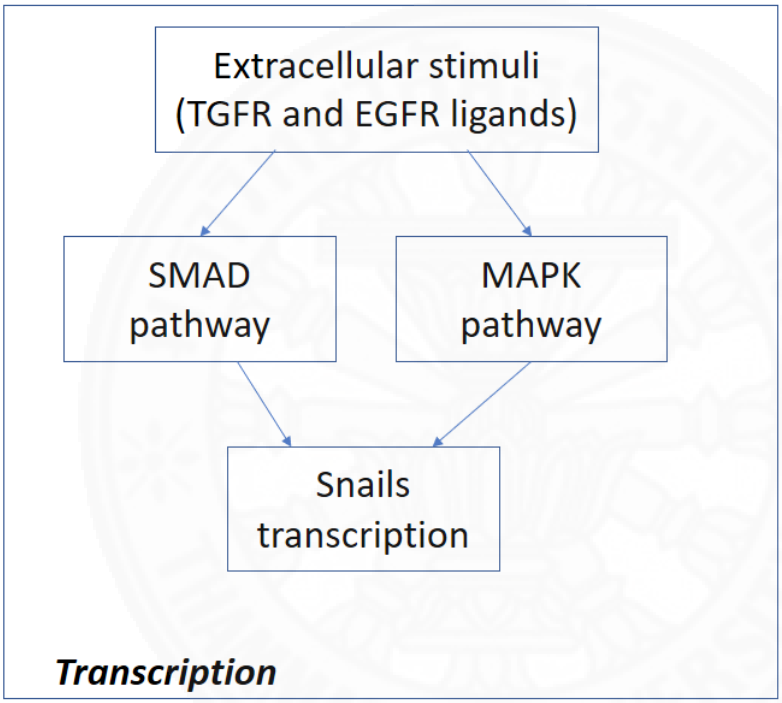

Figure 2.23 Snail regulation at the transcription level

2.10.2.1 SMADs

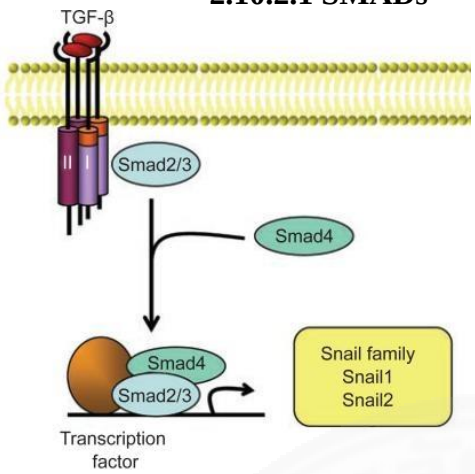

Figure 2.24 SMADs regulate Snails transcription(96)

SMADs is the intracellular mediators. TGFβ receptors signaling activate Smad2 and 3 via phosphorylation (at cell-surface serine/threonine kinase receptors) to form complexes with Smad4. Then this complex bind to DNA (97). Potently suppression of both basal and TGF-induced Snail1 mRNA levels was found after Smad4 was knocked down. However, knocking down Smad4 did not affect both basal and TGF-induced Slug expression level (92, 95, 98).

2.10.2.2 MAPK

(1) TGFβ In HaCaT cells, TGFβ receptors signaling stimulate Slug via SMAD pathway. However, TGFβ receptors signaling up-regulates Slug via ERK1/2 in HNSCC cells (99). A study in well-differentiated, Ras-mutant mouse keratinocytes demonstrated that TGFβ receptors signaling could raise Rac1 activity in that cells swiftly (100). Moreover, this activity, collaborate with ERK1/2, could induce expression of Snail1 (92, 100).

(2) EGFR In keratinocytes, EGFR signaling raise Slug transcription and expression of protein level via MEK5 phosphorylation of ERK5 (87, 88, 92).

2.11 Vanillin

2.11.1 General characteristics of vanillin

Vanillin is the primary aroma agents, about 2%, in the vanilla cured beans (101). It can be made synthetically from eugenol or guaiacol and from lignin. In analytical chemistry, a flavoring agent process, and in perfumery have used vanillin as a reagent (102). The worldwide authorities approved vanillin as a food additive, moreover, the vanillin GRAS status was granted by FDA (103). Concentrations of vanillin from 0.3 to 33 mM were reported from food and beverage products (104).

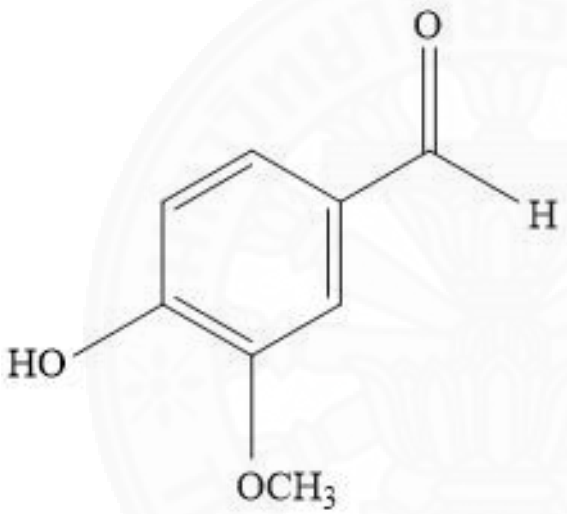

Figure 2.25 Vanillin structure

Vanillin structure is $C_8H_8O_3$. It is a phenolic aldehyde with molecular weight 152.14732 g/mol (105). It is oxidized easily on moist air exposure. About the soluble ability, vanillin 1 gram could dissolve in 100 ml water. At 80 °C vanillin 1 gram could dissolve in 16 ml water, or 20 ml glycerol. Vanillin is freely soluble in chloroform, ether, carbon disulfide, glacial acetic acid, pyridine. And also, vanillin is soluble in oils & in aqueous solution of alkali hydroxides (102).

2.11.2 Vanillin toxicity

About the toxicity, in vitro mutagenicity testing in mammalian cells, for human lymphocytes without metabolic activation, cytotoxicity was found at 612 mg/L. While in mouse fibroblast without metabolic activation, cytotoxicity was found

at 1224 mg/L. In addition, vanillin also induced multinuclear mutations in the fibroblasts at 153-918 mg/L (103).

2.11.3 Vanillin characteristics

2.11.3.1 Vanillin anticlastogen characteristics

Since 1990(3) and later studies(4-6) established vanillin as an anticlastogen, from its properties to suppress gene mutations in both bacterial and mammalian cells. In 2002, seven phenolic compounds were investigated their protective capacities against H_2O_2-induced DNA damage in human peripheral blood lymphocytes, vanillin was one of those compounds. This experiment was done by using single cell micro-gel electrophoresis. H_2O_2 increase the single cell DNA strand breakage in human peripheral blood lymphocytes by the concentration-dependent manner. At the induction of 50 μMol H_2O_2, vanillin showed its protective capacity at the concentration range of 6.25 - 25 μMol. So, it was concluded that vanillin has the ability in protection of human single cell DNA from oxidative attack (7).

In 2014, Lee et. al(8) found that vanillin could protect human keratinocyte stem cells against ultraviolet B irradiation. Vanillin's effect of decreasing UVB irradiation-induced cytotoxicity is remarkable. They used the results of the senescence-associated β-galactosidase and alkaline comet assays to exhibit the vanillin effects. Production of pro-inflammatory cytokines were also stimulated by vanillin. Moreover, they found that UVB-induced phosphorylation of ataxia telangiectasia mutated (ATM), serine threonine kinase checkpoint kinase 2 (Chk2), tumor suppressor protein 53 (p53), p38/mitogen-activated protein kinase (p38), c-Jun N-terminal kinase/stress-activated protein kinase (JNK), S6 ribosomal protein (S6RP), and histone 2A family member X (H2A.X) are also notably attenuated by vanillin. Vanillin also substantially inhibited UVB-induced activation of p53 luciferase reporter. Meanwhile vanillin has no effect on ATM inhibitor, mouse double minute 2 homolog (MDM2) inhibitor dramatically reduced the suppressive effects of vanillin on UVB-induced activation of p53 reporter in keratinocyte stem cell.

2.11.3.2 Vanillin anti-oxidative characteristics

In 2011, Tai, *et.al* (106)used multiple assay to systematically determine the antioxidant activity of vanillin. In the ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)) (+) scavenging assay, vanillin exhibited more powerful antioxidant activity than ascorbic acid and Trolox. In addition of the ORAC assay and the oxidative hemolysis inhibition assay (OxHLIA), vanillin exhibited much stronger antioxidant activity than ascorbic acid and Trolox. In these three assays, vanillin utilizes a self-dimerization mechanism to react with radicals. The dimerization results in the high reaction stoichiometry against ABTS(+) and AAPH-derived radicals, cause the strong effect of vanillin. Next, an in vivo study in mice was done by given a single dose of vanillin orally (100 mg/kg), then measure vanillin and its metabolites from mice plasma sample by HPLC and ORAC assay. Raised vanillin concentration and its antioxidant activity was found in plasma.

2.11.3.3 Vanillin anti-cancer characteristics

(1) Vanillin increases TRAIL-induced apoptosis in tumorigenic cells via inhibition of NF-KB activation

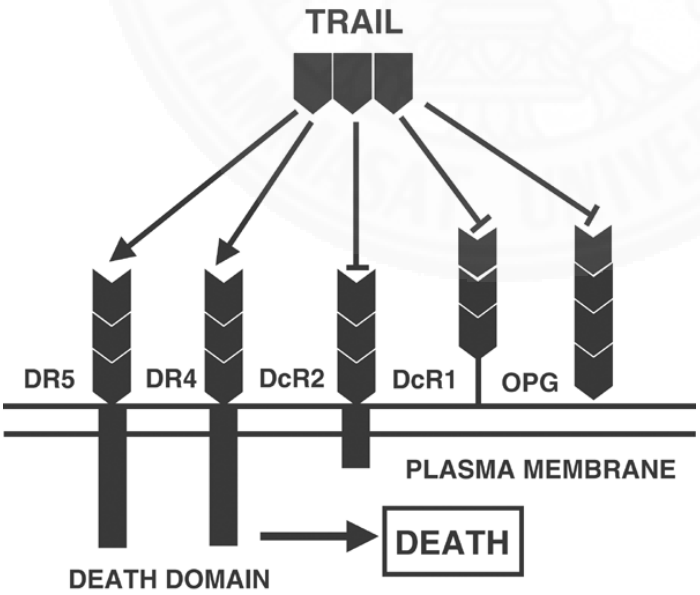

Figure 2.26 TRAIL and its receptors (84)

The knowledge about Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a member of the tumor necrosis factor family of ligands has emerged in 2003. TRAIL has been expected as anticancer molecule due to its ability to selectively induce cancer cells and transformed cells apoptosis (107). Mechanism of TRAIL is to initiate apoptosis via linking with the death receptors. Nevertheless, tumor cells often develop resistance toward TRAIL. Hence, to find the agents which could sensitizes tumor cells to TRAIL-induced apoptosis attracts researchers' interests.

In 2010, Lirdprapamongkol *et al.* has determined the effect of vanillin on HeLa, which is a TRAIL-resistant human cervical cancer cell line (108). They used 2 mM of vanillin pretreated Hela for 30 min. Then, Hela was incubated in the presence or absence of 200 ng/ml TRAIL for 12 hours consequently. Increased TRAIL-induced cell death was found in the vanillin pretreated Hela group. And the mechanism of vanillin is to inhibit TRAIL-induced phosphorylation of p65 and transcriptional activity of NF-κB. Taken together, this suggested vanillin could sensitize HeLa cells to TRAIL-induced apoptosis via inhibiting NF-κB activation.

(2) Vanillin antimetastatic characteristics

After the potency to suppress cancer cell migration and metastasis in a mouse model of vanillin was found (109), there were attempts to reveal the mechanism behind that. By means of the Transwell assay, vanillin and its derivatives, apocynin was proved the ability to suppress hepatocyte growth factor (HGF)-induced pulmonary tumorigenic cell migration. Vanillin is capable of selectively blocking Akt phosphorylation of HGF signaling, without blocking Met and Erk phosphorylation. Moreover, ability to inhibit phosphoinositide 3-kinase (PI3K) of vanillin and apocynin were demonstrated by the lipid kinase assay. This indicated the mechanism of inhibition tumorigenic cell migration is PI3K activity inhibition. This study also suggested that a group of aldehyde or ketone in the vanillin structure had the crucial role for this inhibition (110).

2.11.3.4 Vanillin antiangiogenetic effects

Antiangiogenetic effect of vanillin is in the scientist's interests along this decade due to 2 main reasons. First, it was found that Gastrodiaelata Blume rhizome, a famous Chinese herb own the anti-angiogenic potency (111). Additionally, this herbal extract's active ingredients are vanillin, 4-hydroxybenzyl alcohol and 4-hydroxybenzaldehyde (112). Second, vanillin ability to decrease PI3K/Akt signaling was found (110).This signal is the key mediator in angiogenetic induction by HGF, VEGF, basic fibroblast growth factor, and angiopoietins (113). So, there were investigated antiangiogenetic potency of vanillin. By using chorioallantoic membrane (CAM) assay, significant decreased area of minor blood vessels of eggs in the areas that exposed to the vanillin was found. In study of Lim, *et al.*, they applied vanillin 0.3, 1.0 and 3.0 μg per each egg in CAM assay for 2 days. CAM angiogenesis decreased for 38.0%, 63.6% and 71.0%, consequently. As well as in study of Lirdprapamongkol, *et al.* (110), they applied agarose pellets with vanillin dose of 100-500 nM in CAM assay for 24 h, and significantly reduced angiogenesis was also found. Taken together, these results suggested that vanillin could inhibited angiogenesis (110).

2.11.3.5 Vanillin anti-analgesic effects

An experiment in mice indicated vanillin's anti-nociceptive potency. After induced nociception via injection of 0.7% acetic acid solution, 0.1 ml/10 g body weight to mice intraperitoneal, which is called "the acetic acid-induced writhing test". Normal saline or vanillin 25, 50, 100 mg/kg or a positive control were given to each group of ICR mice. After that, the researchers counted the number of writhes during the period. Inhibition of writhing response was found to decrease 47.7%, 52.8% and 64.7%, when vanillin 25, 50, 100 mg/kg was applied to mice respectively (114).

2.11.3.6 Vanillin suppresses matrix metalloproteinase-9 expression

MMP-9, an enzyme which influence tumor invasion and metastasis, could be reduced by vanillin. In 2008, Liang, *et al*. revealed that vanillin could decrease 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced MMP-9 gelatinolytic activity. Thus, the gene transcription of MMP-9 was down-regulated,

decreasing invasion of a tumorigenic hepatocellular cell line, HepG2 cells. Vanillin could decrease HepG2 cells invasive capacity about 6.6-fold. And this effect was dose dependent manner. Moreover, it was determined that transcription of MMP-9 was inhibit by vanillin via suppressing NF-κB. And by using western blot, the mechanism which vanillin inhibit NF-κB was unveiled, that it is by the means of inhibiting IκB-α phosphorylation and degradation (115).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 Research objectives

To evaluate the effect of vanillin on stem cell regulatory molecular signal in human keratinocytes.

3.2 Cell culture

Human keratinocyte HaCaT cells were obtained the Cell Lines Service (CLS, Heidelberg, Germany). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 100 U/ml penicillin and streptomycin. Cell cultures were maintained in a 37°C humidified incubator with a 5% CO_2 atmosphere. Cells were routinely passaged at preconfluent density using a 0.25% trypsin solution with 0.53 mM EDTA. DMEM medium, FBS, L-glutamine, phosphate-buffered saline (PBS), trypsin, and EDTA were purchased from GIBCO (Grand Island, NY).

3.3 Chemicals, antibodies and reagents

Table 3.1 Chemicals, antibodies and reagents

Cell culture	
Chemicals	**Sources**
Vanillin	Sigma (St. Louis, MO, USA)
Dulbecco's Modified Eagle Medium (DMEM)	GIBCO (Grand Island, NY)
100 U/ml penicillin and streptomycin	Invitrogen (Carlsbad, CA, USA)
Fetal bovine serum	GIBCO (Grand Island, NY)

Trypsin	GIBCO (Grand Island, NY)
Cell growth and cell viability assay	
MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrasolium bromide)	Life technologies (Oregon, USA)
Dimethyl sulphoxide (DMSO)	RCI Labscan (Bangkok, Thailand)
Western blotting	
Anti-Oct-4 antibody	Sigma-Aldrich (St. Louis, MO, USA)
Anti-Nanog antibody	Cell Signaling Technology (Danvers, MA, USA)
Anti-phospho-Oct-4 S236 antibody	Sigma-Aldrich (St. Louis, MO, USA)
Anti-E-cadherin antibody	Cell Signaling Technology (Danvers, MA, USA)
Anti-Slug antibody	Cell Signaling Technology (Danvers, MA, USA)
Anti-GAPDH antibody	Cell Signaling Technology (Danvers, MA, USA)
Anti-α-tubulin antibody	Cell Signaling Technology (Danvers, MA, USA)
HRP goat anti-mouse IgG antibody	Cell Signaling Technology (Danvers, MA, USA)
HRP goat anti-rabbit IgG antibody	Cell Signaling Technology (Danvers, MA, USA)
TEMED	Biorad (Hercules, CA, USA)
Biscinchoninic acid (BCA)	Pierce Biotechnology (Rockford, IL, USA)
Albumin, Bovine Serum, Fraction V, Low Heavy Metals	EMD Millipore (Billerica, MA, USA)
Bovine serum albumin (BSA) standard	Thermo Scientific (Rockford, MD, USA)
Protease inhibitor cocktail	Roche diagnostic GmbH (Mannheim, Germany)

Precision plus protein standard all blue	Biorad (Hercules, CA, USA)
Acrylamide	Biorad (Hercules, CA, USA)
Supersignal West Pico Chemiluminasencent	Thermo Scientific (Rockford, MD, USA)

3.4 Experiment procedures

3.4.1 Cell growth

Human keratinocyte HaCaT cells were obtained the Cell Lines Service (CLS, Heidelberg, Germany). The cells were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, and 100 U/ml penicillin and streptomycin. Cell cultures were maintained in a 37°C humidified incubator with a 5% CO_2 atmosphere. Cells were routinely passaged at preconfluent density using a 0.25% trypsin solution with 0.53 mM EDTA. DMEM medium, FBS, L-glutamine, phosphate-buffered saline (PBS), trypsin, and EDTA were purchased from GIBCO (Grand Island, NY).

3.4.2 Cell Viability Assay

Evaluation of cell viability is done by MTT assay, which measures cellular capacity in reducing 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (yellow) to purple formazan crystal by mitochondria dehydrogenase enzyme.

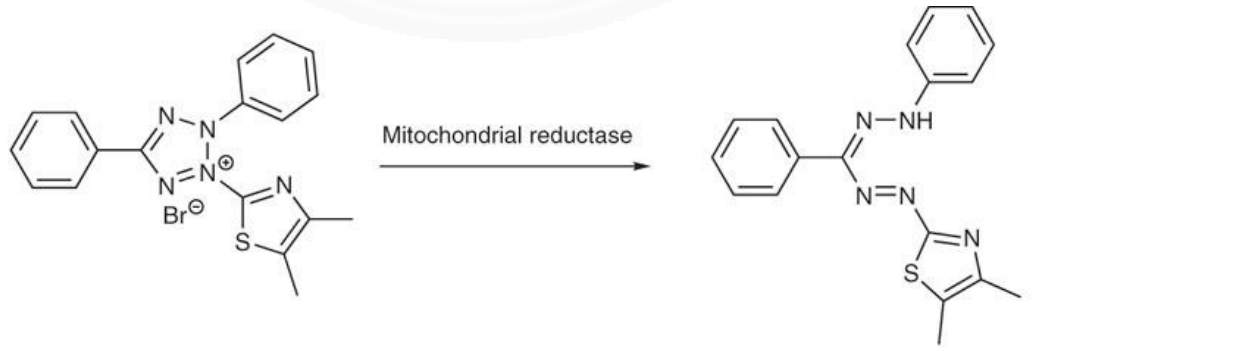

Figure 3.1 Structure of MTT and formazan crystal(116)

After specific treatments, the cells were incubated with 100 µl of MTT (500 µg/ml) (Invitrogen, Carlsbad, CA) for 4 h at 37°C.

Figure 3.2 Workflow of MTT assay, adapted from (117-121)

The intensity of MTT product was measured at 570 nm using a microplate reader (Anthos, Durham, NC). Cell viability was calculated by the following equation to the relative cell viability of the different treated dose.

$$\text{Cell viability (\%)} = \frac{\text{A570 of treatment}}{\text{A570 of control}} \times 100$$

3.4.3 Western blot Analysis

3.4.3.1 Vanillin treatment

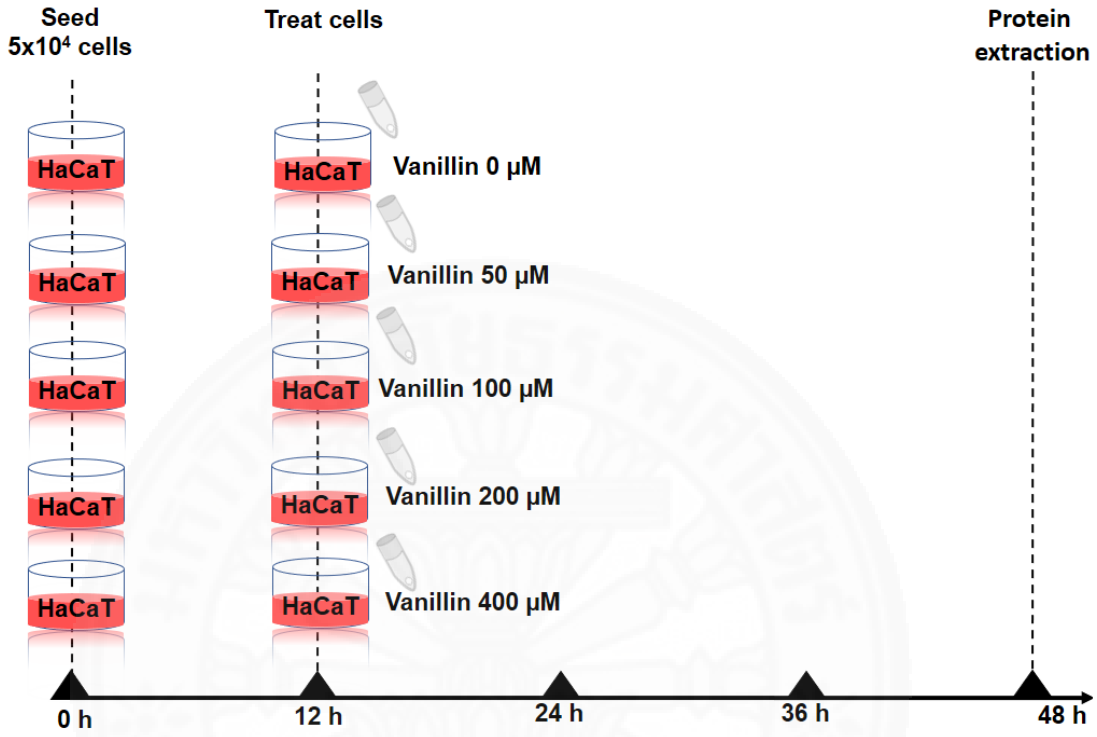

Figure 3.3 Vanillin treatment on HaCaT cells

HaCaT Cells were seeded at a density of 5 x 10^4 cells/well onto 6-well plate for 12 h and cultured in the presence of various concentrations of vanillin (0,50,100,200,400 µM) for 48 h.

3.4.3.2 Sample preparation

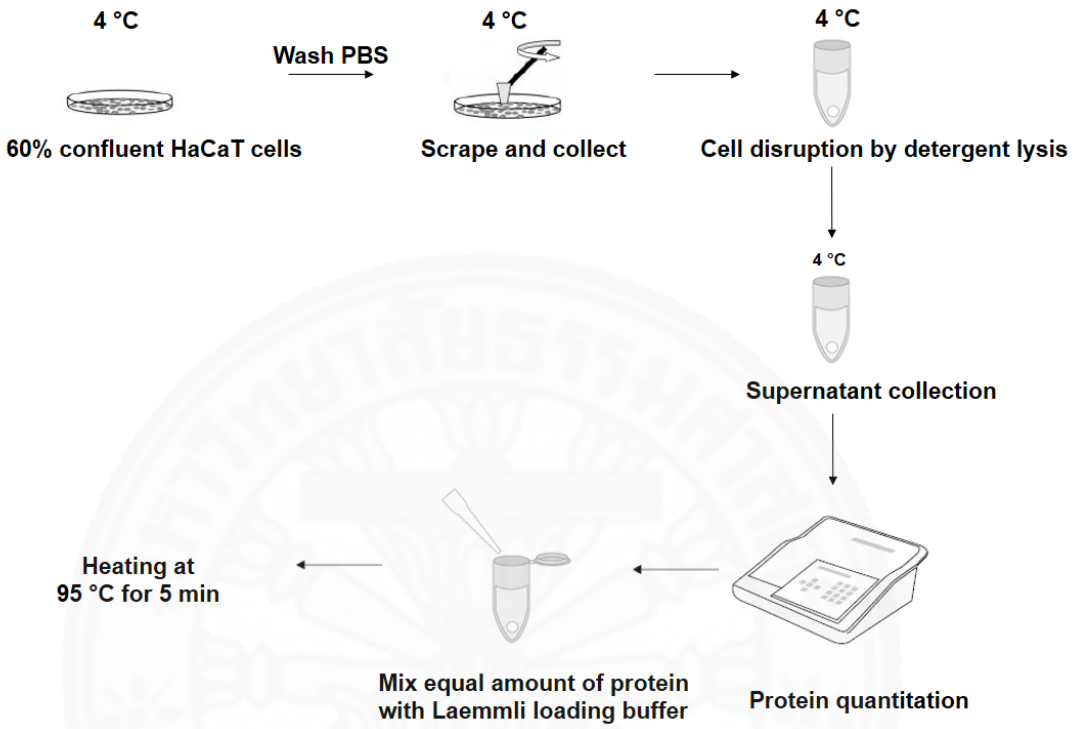

Figure 3.4 Sample preparation, adapted from (122, 123)

(1) Cell Disruption Cell disruption is the way to get the intracellular protein. Due to our sample cells are tissue culture cells, which could be lysed easily. Gentle protocol is selected. In this experiment, we use detergent lysis technique. Our lysis buffer contains 20 mM TrisHCl (pH7.5), 0.5% Triton X-100, 50 mM sodium fluoride, 150 mM sodium chloride, 1mM sodium orthovanadate, 1mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche Molecular Biochemicals).

(2) Supernatant Collection The lysates were centrifuged at high speed. Only supernatant lysates were collected.

(3) Protein Quantitation Protein content was then evaluated using BCA protein assay kit from Pierce Biotechnology (Rockford, IL). Equal amounts of proteins (100 µg) were heated at 95°C for 5 min with Laemmli loading buffer.

3.4.3.3 Polyacrylamide gel electrophoresis (PAGE)

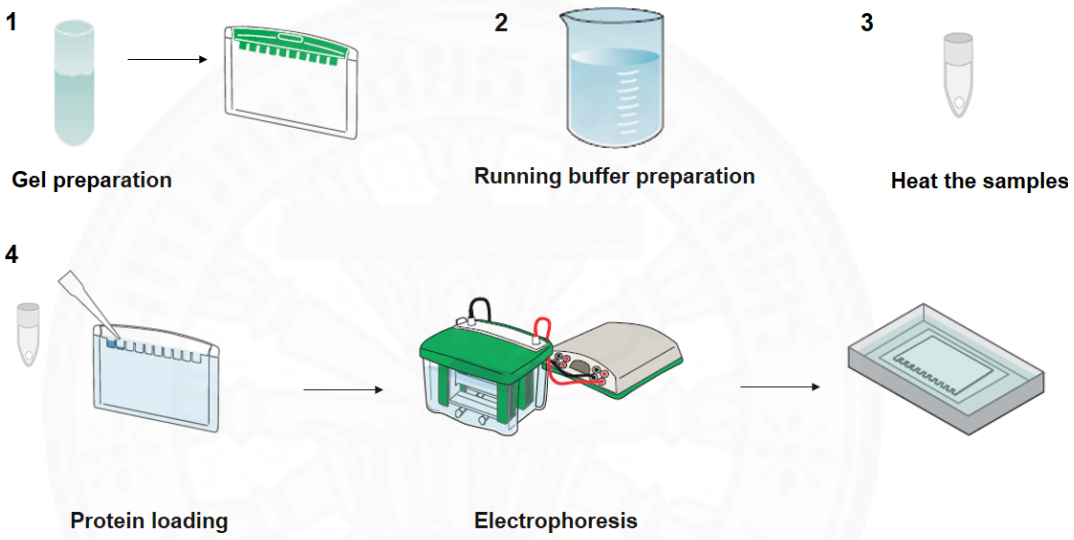

Figure 3.5 Polyacrylamide gel electrophoresis, adapted from (123)

(1) Gel preparation In this experiment, we selected 10% SDS-polyacrylamide gel.

(2) Running buffer preparation Prepare 1X running buffer, which in 1000 mL composed of 100 mL of 10X running buffer and 900 mL of distilled water.

(3) Heat the samples Heat the samples at 95°C for 5 min.

(4) Protein loading Assembly the cell into the tank. Fill the running buffer into the inner and outer chamber until the tank is full. Remove the comb from the gel. Then the proteins were then loaded on 10% SDS-polyacrylamide gel carefully.

(5) Electrophoresis Apply an electric field in the proper amount of power and duration of time.

3.4.3.4 Protein Electro-transfer

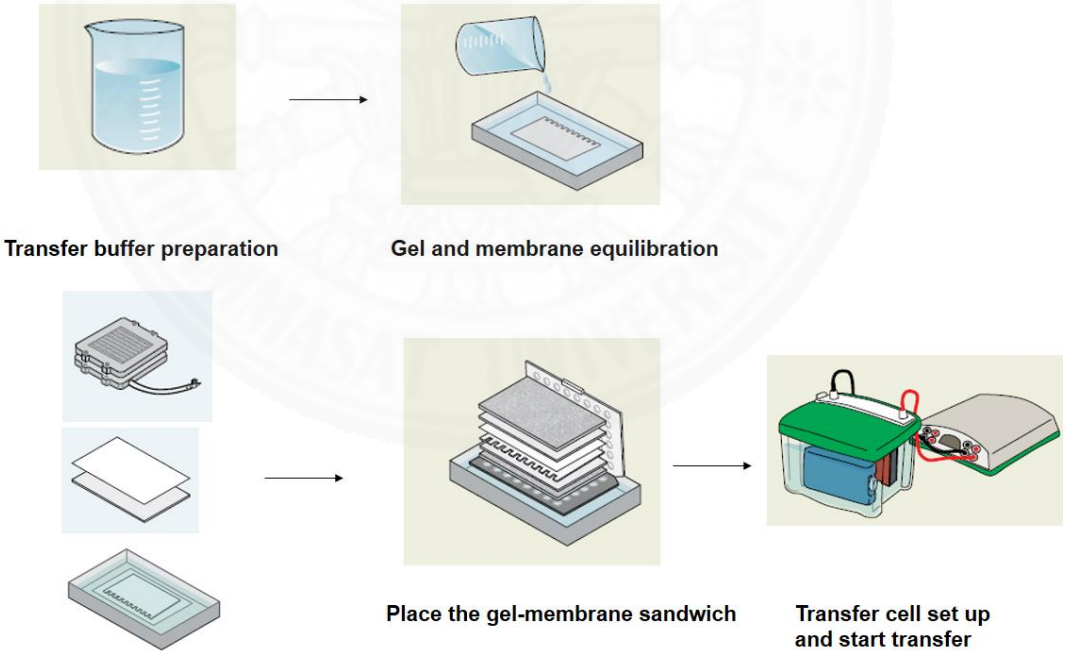

Figure 3.6 Electro-transfer workflow, adapted from (124)

After gel separation, proteins were subsequently transferred onto 0.45 µm nitrocellulose membranes.

(1) Transfer buffer preparation Prepare 1X transfer buffer, which in 1000 mL composed of 100 mL of 10X transfer buffer, 200 mL of analytical grade methanol and 700 mL of distilled water. Make sure that it is sufficient for transfer and gel-membrane equilibration.

(2) Gel and membrane equilibration Equilibrate a piece of gel and a piece of membrane in the transfer buffer. Immerse filter papers and foam pads into transfer buffer until they are soaked.

(3) Gel-membrane sandwiching Place a piece of nitrocellulose in direct contact with a piece of polyacrylamide gel. Sandwich them between buffer soaked filter papers and foam pads. two electrodes. Assembly them between two electrodes.

(4) Transfer cell set up and start transfer Assembly the sandwich into transfer tank. Fill the transfer buffer into the tank until the tank is full. Apply an electric field in the proper amount of power and duration of time.

3.4.3.5 Detection and imaging

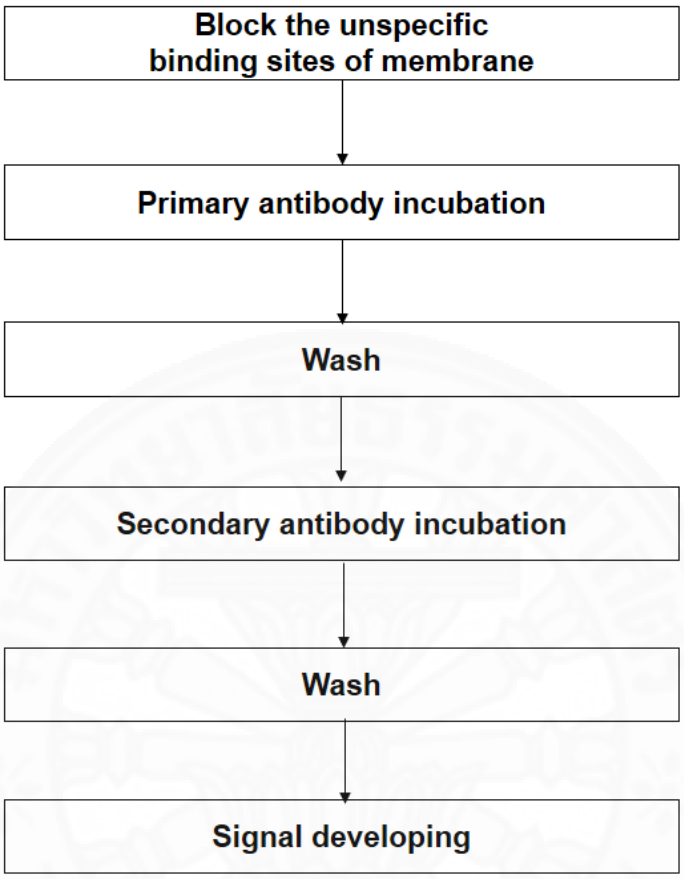

Figure 3.7 Immunodetection workflow

(1) Blocking To decrease the amount of nonspecific binding, the membranes were blocked with 5% skim milk in TBST (25 mM Tris-HCl (pH 7.5), 125 mM NaCl, 0.05% Tween-20) at room temperature for 1 hour. Thereafter, membranes were washed 3 times in TBST for 5 min.

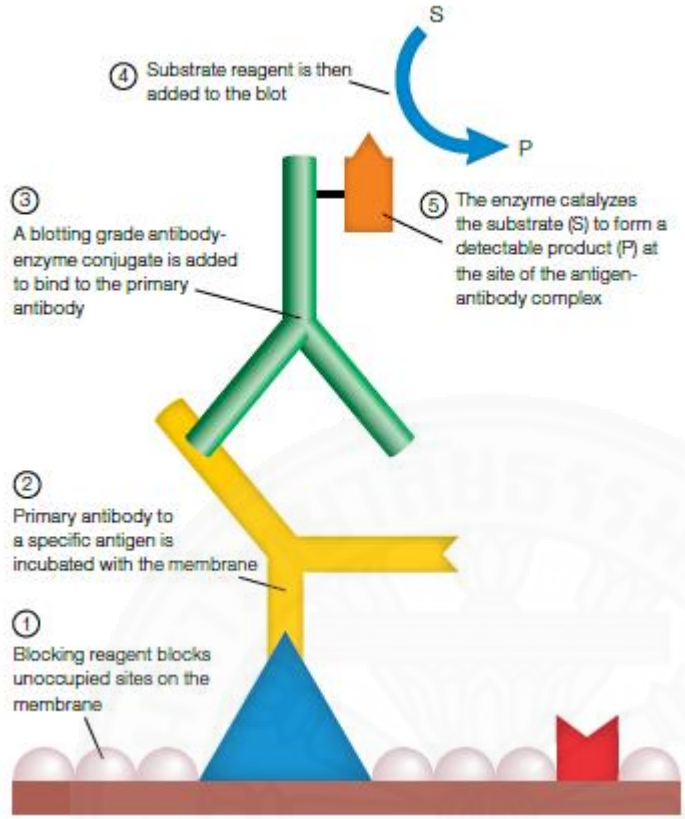

Figure 3.8 Indirect antibody detection(124)

(2) Primary antibody incubation The membranes were incubated with specific primary antibodies against Oct-4, Nanog, p-Oct, E-cadherin, α-tubulin and GAPDH (Cell Signaling, Danvers, MA, USA) at 4°C overnight. Membranes were washed in TBST for 5 min 3 times

(3) Secondary antibody incubation The membranes incubated with horseradish peroxidase-coupled secondary antibodies for 2 h at room temperature. Membranes were washed in TBST for 5 min 3 times.

(4) Signal developing The immune complexs were detected with chemiluminescence substrate (Super signal West Pico; Pierrce, Rockford, IL).

3.5 Outcome measurement

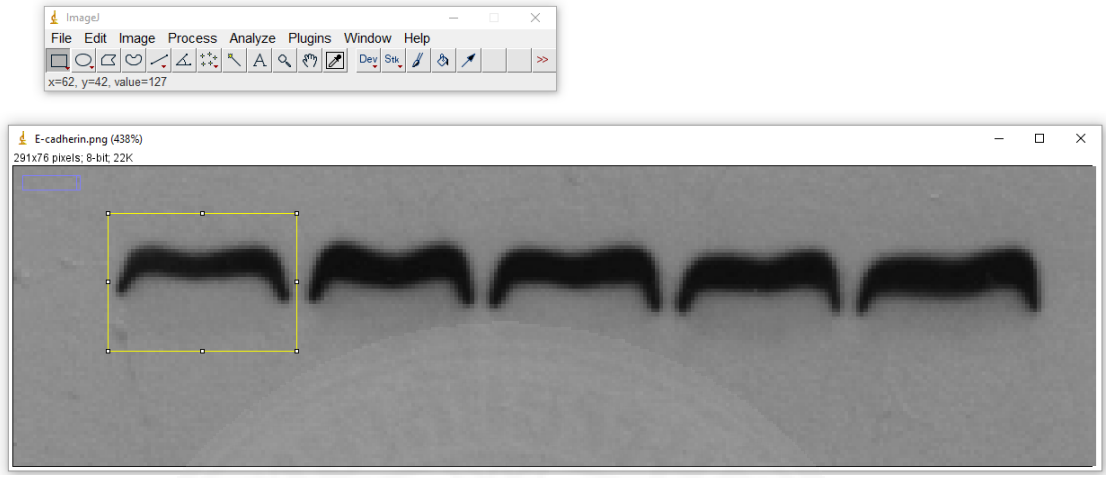

Figure 3.9 Process in ImageJ

The signal from each band is quantified by using analyst/PC densitometry software (ImageJ; National Institutes of Health, USA).

3.6 Data Analysis

Data were obtained from at least three independent experiments, and presented as means ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA followed by post hoc test at a significance level (α) of $p < 0.05$. These analyses were done by using SPSS Version 17(SPSS Inc., Chicago, IL).

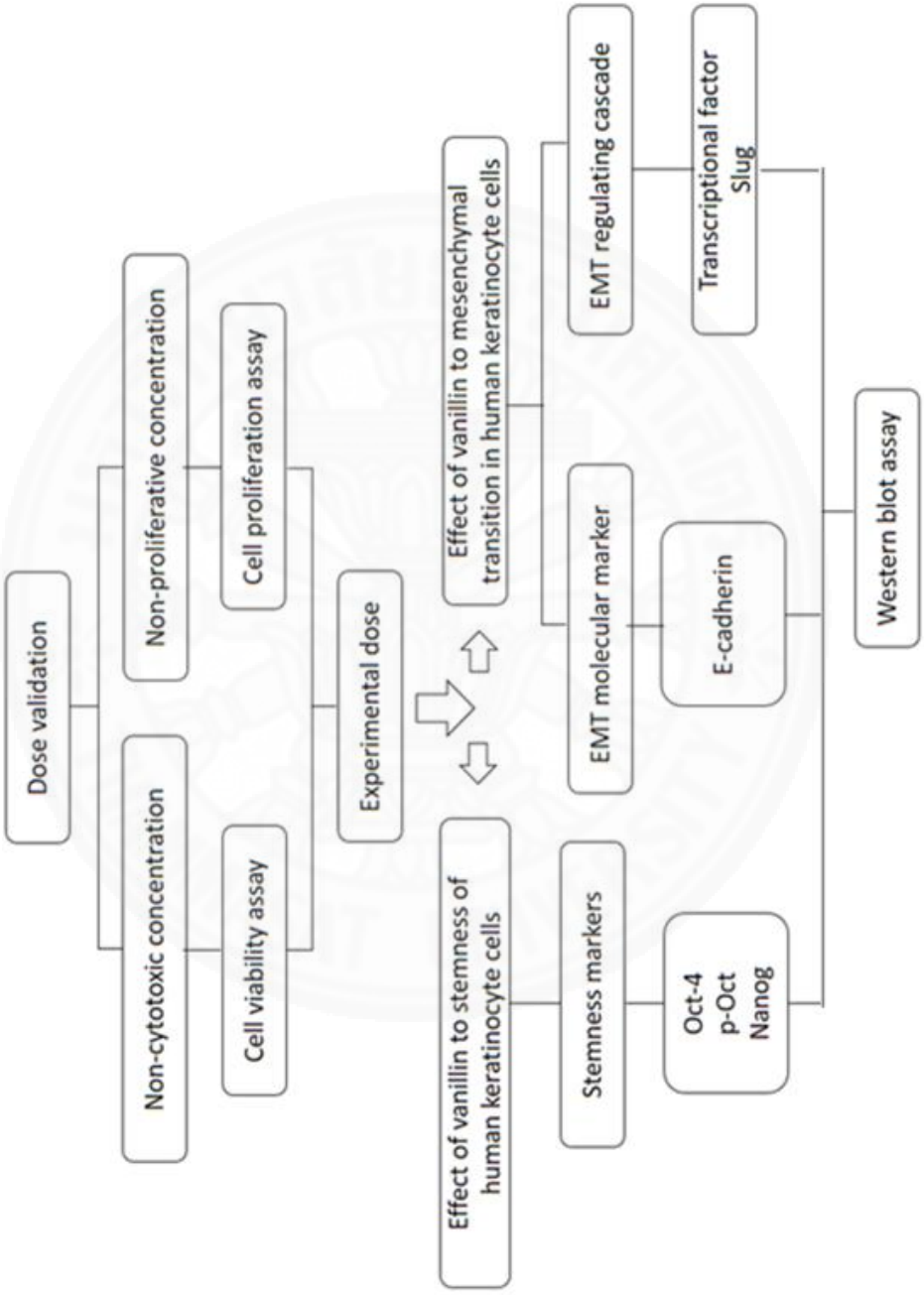

Figure 3.10 Experimental design

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Results

4.1.1 Effect of vanillin on viability of HaCaT cells

To evaluate the effect of vanillin on stemness, the effect of vanillin on HaCaT cells viability was first evaluated. The non-cytotoxic concentrations of extract were determined by treatment of the human keratinocyte HaCaT cells with vanillin at the concentrations of 0-400 μM and cell viability was determined after 24 h by MTT viability assay. Results indicated that treatment of the cells with vanillin at the concentrations of 0-400 μM caused no significant change in cell viability compared with non-treated control (p>0.05) (Figure 4.1). This information may help to clarify that the following effects of vanillin on HaCaTs were not a consequence of cytotoxic effect or cell stress.

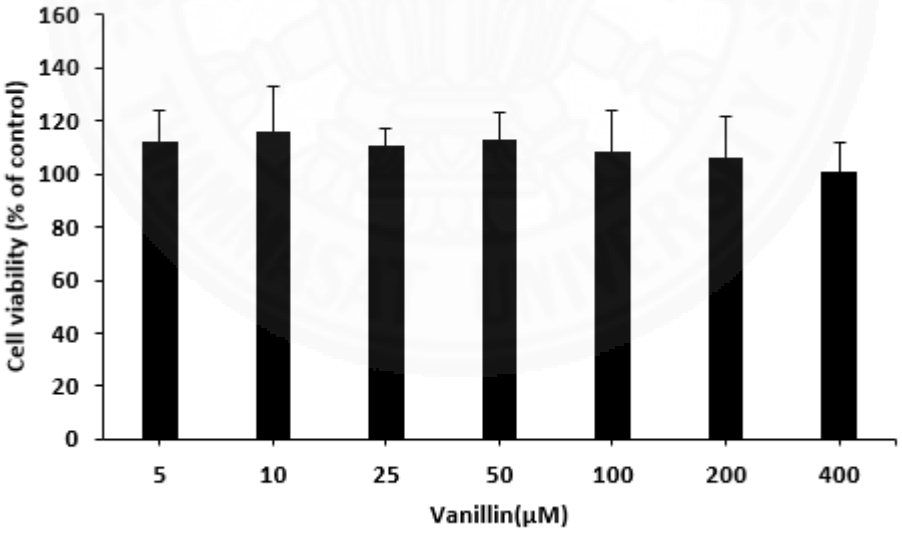

Figure 4.1 Effect of vanillin on viability of HaCaT cells by MTT assay. Cell viability of HaCaTs were determined after treated with vanillin (0-400 μM) for 24 h. The data represent the means of three independent experiments ± SD. *p<0.05 versus non-treated control.

4.1.2 Effect of vanillin on HaCaT cell proliferation

The effect of vanillin on the proliferation of HaCaT cells is shown in Figure 4.2. Cells were exposed to various concentrations of vanillin (0-400 μM) for 72 h, and cells were cultured in normal growth condition for 72 h. Results indicated that all tested concentrations of vanillin did not affect the proliferative activity of the cells (p>0.05). This information further informed that vanillin used in these experiments had no proliferative effect.

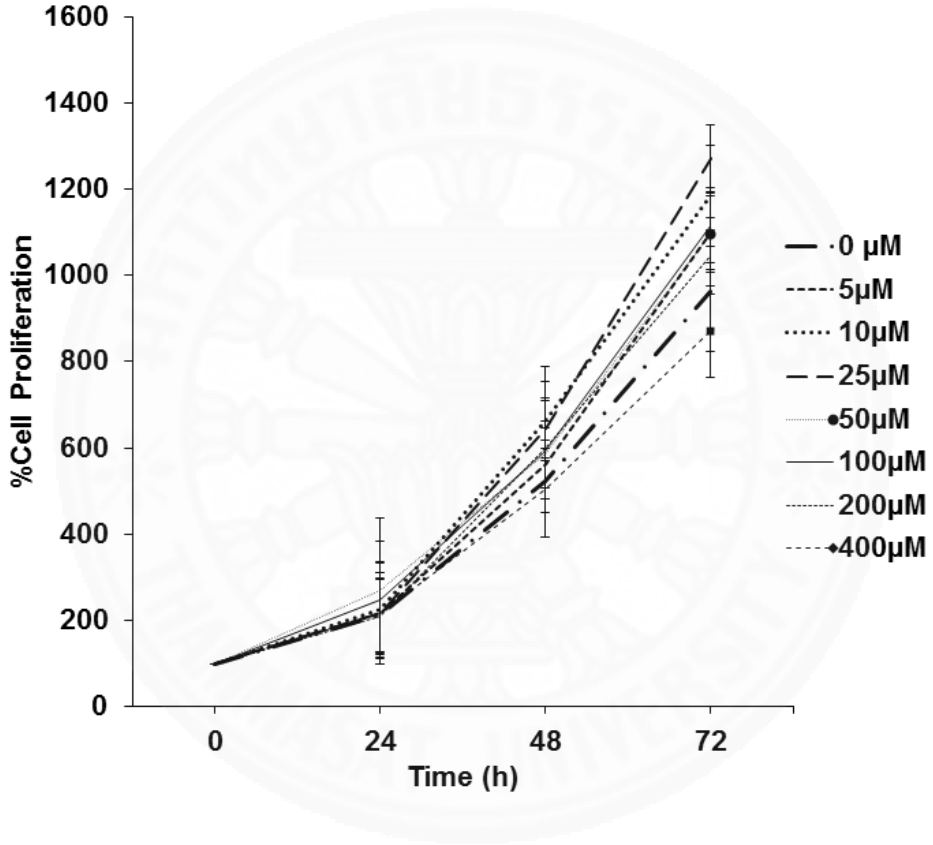

Figure 4.2 Effect of vanillin on HaCaT cells proliferation. Cells were treated with vanillin (0-400 μM) for 72 h, after that cells were replated. HaCaT cells proliferation at 0, 24, 48, 72 h was determined by MTT assay. The data represent the means of three independent experiments ± SD. *p<0.05 versus non-treated control.

4.1.3 Vanillin increases expression of stem cell markers

To prove the effect of vanillin to increase stemness of keratinocytes, the expression of stem cell key molecular markers was investigated. The Oct-4, Nanog and p-Oct-4 expressions have been recognized as the indicators for stemness of cells (27, 125). Cells were treated with vanillin in dose-dependent manners and the expression level of Oct-4, Nanog and p-Oct-4 was determined by Western blot analysis. GAPDH is a housekeeping gene, which is used to ensure equal sample loading (126). Results indicated that vanillin increased the cellular level of p-Oct-4 in keratinocyte at the concentration of 200-400 μM (Figures 4.3A and C). Also, the level of Oct-4 and Nanog was significantly increase in the 400 μM vanillin-treated cells (Figures 4.3A, B and D). These data indicated that vanillin increased the stemness of keratinocyte cells.

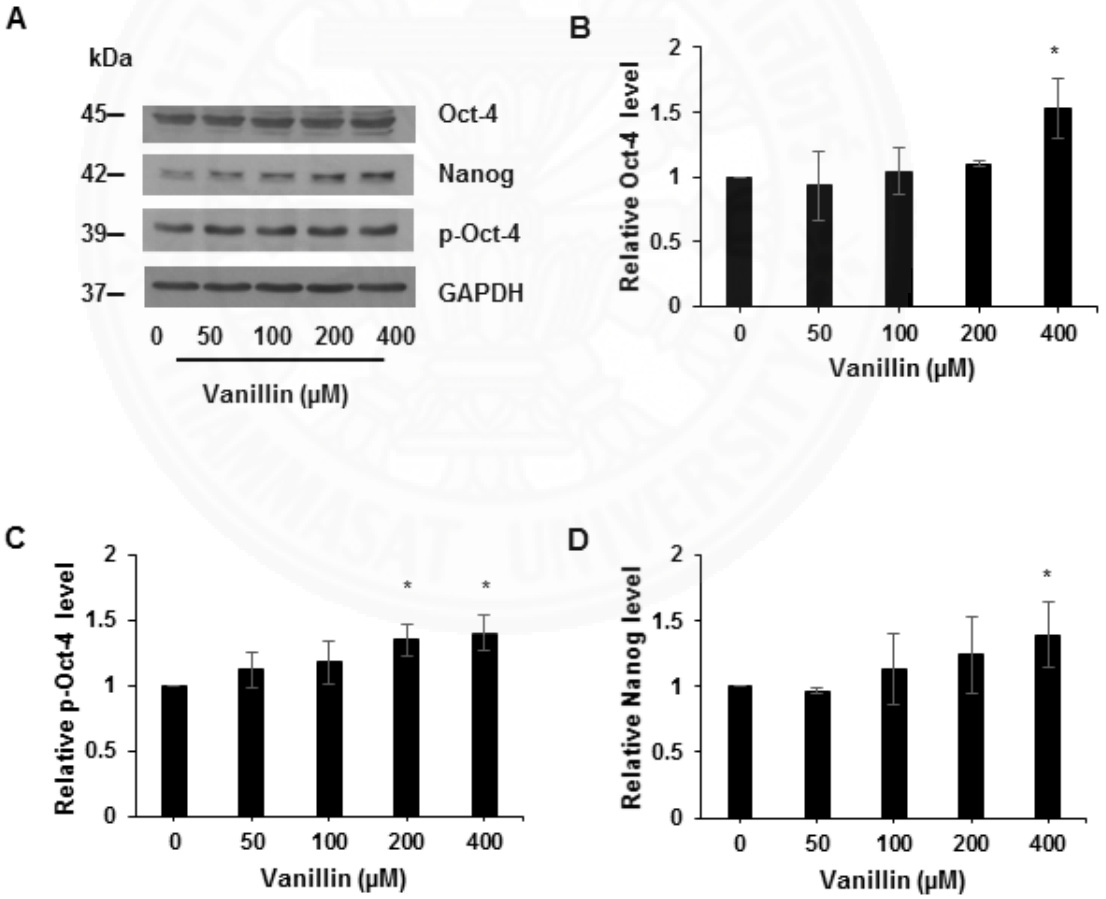

Figure 4.3 Evaluation of stemness markers. HaCaT cells were treated with vanillin for 48 h. (A) The expression levels of stemness markers in HaCaT cells were determined by western blotting. (B) Relative Oct-4 levels, (C) relative p-Oct-4 levels and (D)

relative Nanog levels were quantified by densitometry. Data represent mean±SD (n=3). *p<0.05 versus non-treated control.

4.1.4 Vanillin increases expression of cell adhesion E-cadherin

To further provide the additive information about vanillin in regulation of epithelial to mesenchymal transition (EMT) and cell adhesion, the cells were treated with non-toxic concentrations of vanillin as previously described and the expression of EMT and adhesion was investigated by western blot analysis. E-cadherin expressions have been recognized as the indicators for adhesion strength (60). Results indicated that vanillin increased E-cadherin in a concentration dependent manner at 48 h of treatment compared with non-treated control (Figure 4.4). However, there was no significant change in the level of EMT marker Slug. These data indicated that vanillin increased the adhesion strength of keratinocyte cells. α-tubulin is a housekeeping gene, which is used to ensure equal sample loading (127).

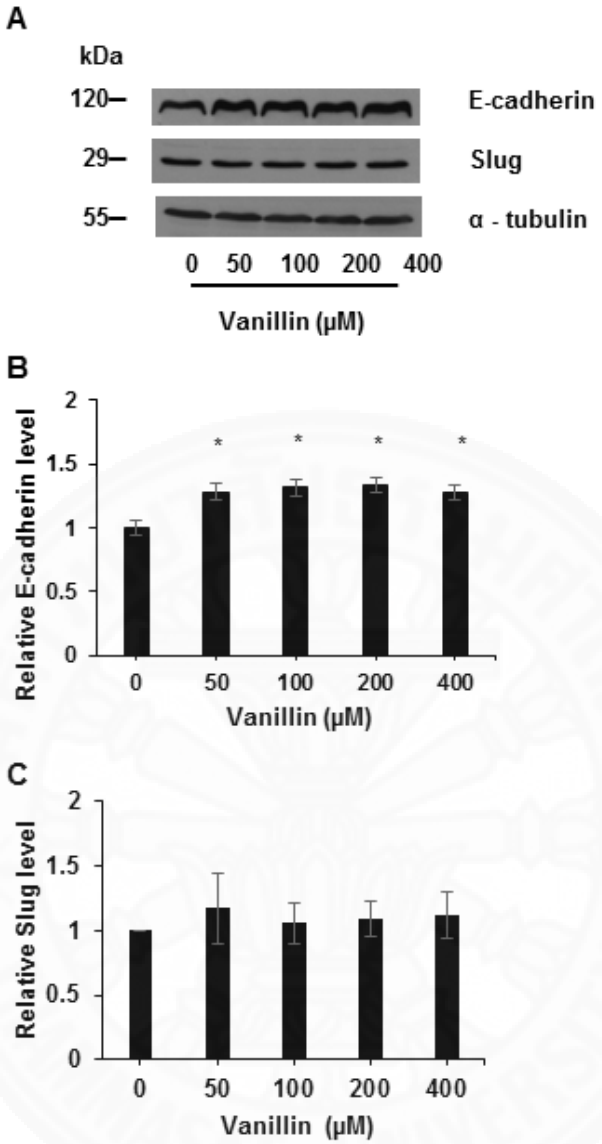

Figure 4.4 Evaluation of epithelial to mesenchymal transition markers. HaCaT cells were treated with vanillin for 48 h. (A) The expression levels of epithelial to mesenchymal transition markers in HaCaT cells were determined by western blotting. (B) Relative E-cadherin levels and (C) relative Slug levels were quantified by densitometry. Data represent mean±SD (n=3). *p<0.05 versus non-treated control.

4.2 Discussion

Epidermal stem cells residing in the basal layer (18), corporate in the epidermal proliferative unit (EPU) which is a three-dimensional organized microenvironment surrounding one single-stem cell. Epidermal stem cells are the key factor in maintaining epidermal homeostasis via the cell renewal process and cytokine production (1). Such stem cells have the self-renewal ability along human lifespan. Here we found the effect of the safety compound vanillin in augmenting the stem cell signals in human keratinocyte cells.

In stem cell research, transcription factors namely Oct-4, p-Oct-4 and Nanog have been accepted as a dominant stemness mediator, and were widely used as biomarkers for human stem cells detection (21). Oct-4 is the essential signal which governs the pluripotency of stem cells (31). In pluripotent cells, Oct-4 is normally found numerously, while it is dramatically depressed upon the process of cell differentiation (27, 128). Moreover, Oct-4 collaborates with Nanog, Sox-2 and other core transcriptional factors, to activate essential RNAs for pluripotency of the stem cells (22). Our study found that vanillin has risen up the cellular levels of Oct-4, p-Oct and Nanog in HaCaT keratinocytes, indicating the augmentation effect of the compound on keratinocyte stemness. Hence, to sustain self-renewal, stem cells must have niche anchoring via adhesion molecules (60). Moreover, other important role of adhesion molecules is to regulate stem cell function - retention, division and exit (62). Therefore, stem cell-niche adhesion is crucial for stem cell self-renewal and is dynamically regulated.

Special adhesive characteristics of epidermal stem cells that distinct from their more differentiated progeny (63) are composed of 1) increased cell-cell cohesiveness and 2) increased extracellular matrix adhesiveness (54, 64-67), These characteristics are mainly regulated by cadherins for cell-cell interactions and integrins for regulate cell-matrix interactions.

By invented skin-specific RNAi technology (59), it was proved that when only E-cadherin is suppressed *in vitro*, deceleration of epidermal sheet formation was found. When E- and P-cadherins missed, expanding of defects to adherens junctions, desmosomes, tight junctions and cortical actin dynamics was exhibited. *In vivo*, E-

cadherin inhibition caused impaired junction formation, deteriorated intercellular adhesion and raises apoptosis rate. Therefore, the active compound like vanillin could benefit the skin barrier function via the positive regulation on E-cadherin protein.

As we found that vanillin increased E-cadherin expression in human keratinocytes, through this manner, the strength of cohesion between cells are increased, consequently. Besides, the stem cells could tightly incorporate to the surrounding cells and receive signals from their niches more effectively. As stem cell-niche adhesion is crucial for stem cell self-renewal, increase expression of adhesion molecule in the niche might affect stemness marker to raise in the same way. As we aged, it is hard to avoid the condition of age-associated inflammation skin. In such a situation, activities and amounts of epidermal stem cells were found to be dramatically reduced (1). Decrease stem cells function and population from age-associated inflammation skin would deteriorate skin function (1); therefore, enhancement of cell adhesion and stemness by vanillin may attenuated the defect skin in such a process of inflammation and ageing.

Next, to know whether vanillin affect the epithelial to mesenchymal marker level of HaCaT cells. Two significant EMT markers was selected, slug and vimentin. Slug is a transcriptional factor which could be found in all layer of epidermis (89). Slug is essentially involved in migration, inflammation and terminal differentiation process of keratinocytes (92). Our study found that vanillin did not alter the level of Slug in HaCaT cells significantly. Another EMT marker that we probed in this study is vimentin. It is an intermediate filament which found numerously in cell that developed from mesenchymal origin or in tumorigenic cells (129). However, in non-disease keratinocytes which is developed from ectoderm, vimentin is found in a minute amount, while it still has the essential function in keratinocytes. Vimentin interaction with keratin intermediate filaments is indispensable for epithelial migration, which is the critical process in development and wound repair (81). Additionally, increasing vimentin is found in psoriatic epidermal keratinocytes (130). So, this study tried to investigate whether vanillin affect the vimentin level of HaCaT cells. Unfortunately, level of vimentin in non-disease and non-tumorigenic keratinocytes lysate is too low to appear on the western blot band, even the 120 μg of protein per each band was loaded (The result is not showed). The outcome of this study is consistent with immunostaining

and confocal image of human keratinocyte colony which found vimentin expression just only the peripheral cells of keratinocyte colony (81). Thus, it might be concluded that vanillin has no significant effect in changing EMT markers in HaCaT keratinocytes.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

We demonstrated that vanillin has a potential to increase cell-cell cohesion and stemness in human keratinocyte cells. Cell-cell cohesion is essential for skin barrier function, being strength niches and benefit stem cell property. As the stemness of keratinocytes is an important factor determining the function of epidermis as well as it regeneration processes, vanillin may be a potential candidate for enhancement of the skin protective and repair functions. This study provides for the first time the interesting effects of vanillin on cell stemness enhancement and cell adhesion

5.2 Limitation

Using HaCaT cells in this study make convenient, because of HaCaT cell lines advantage, the ability to reproduced itself endlessly while its other characteristics e.g. DNA fingerprint almost identical to normal keratinocytes. HaCaT was taken from epidermal basal layer. So, in HaCaT cell pool belongs stem cells and the western blot results also confirmed this by showing stemness marker expression (Oct-4, p-Oct-4 and Nanog). However, every cell in HaCaT cell pool is not stem cells. Therefore, the result could not show purity of stem cell-only reaction to this compound.

5.3 Recommendation

5.3.1 Further studies

5.3.1.1 *In vitro* studies

(1) Do the experiment in epidermal stem cells from primary epidermal cells We might select epidermal stem cells by using the collagen type IV-coated dish. Collagen type IV is the ligand integrin β-1 (CD29), which is highly express in epidermal stem cells. These might show more obvious and more precise result of stemness markers.

(2) Use the three-dimensional cellular system of human epidermal keratinocytes (3D skin model) Since 3D skin model provide physiological atmosphere. We could formulate the vanillin serum and apply the experiment on 3D skin model to study percutaneous absorption, penetration and efficacy of the vanillin serum in the similar atmosphere to real skin before testing in human. Owing to moral concern, in cosmetic section of some developed countries no longer allow the use of animal experiments for confirming the effect of compounds. Thus, 3D skin model is an excellent choice.

(3) Finding the underlying mechanism The underlying mechanism which increase these markers should investigated to corroborate this conclusion.

5.3.1.2 *In vivo* studies

(1) Study in human being This experiment should be achieved to confirm the clinical efficiency before developing this compound for therapeutic and cosmeceutical applications.

5.3.2 Clinical applications

Topical vanillin serum could be developed to increase cohesion strength between keratinocyte stem cells and their niches, and improve stemness of keratinocyte stem cells.

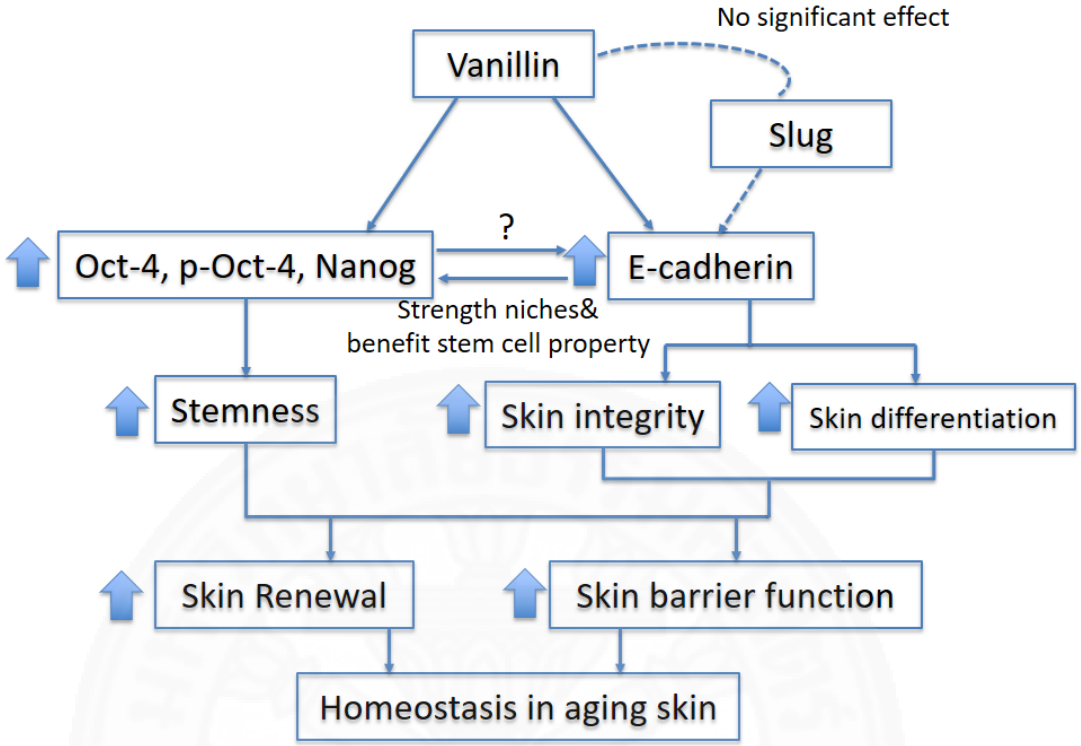

Figure 5.1 Conclusion diagram

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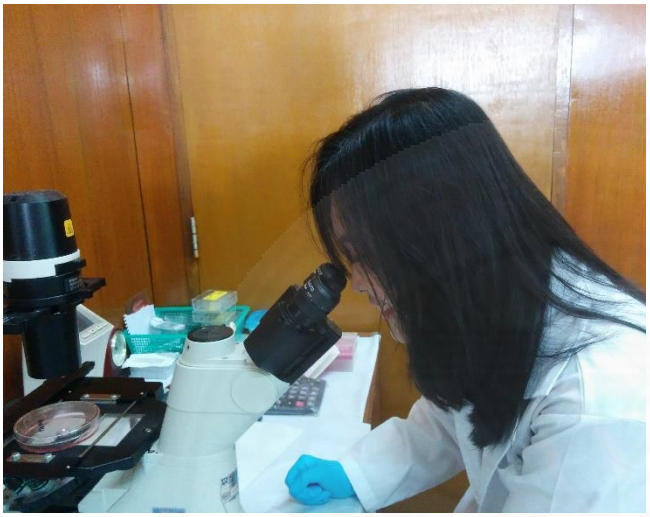
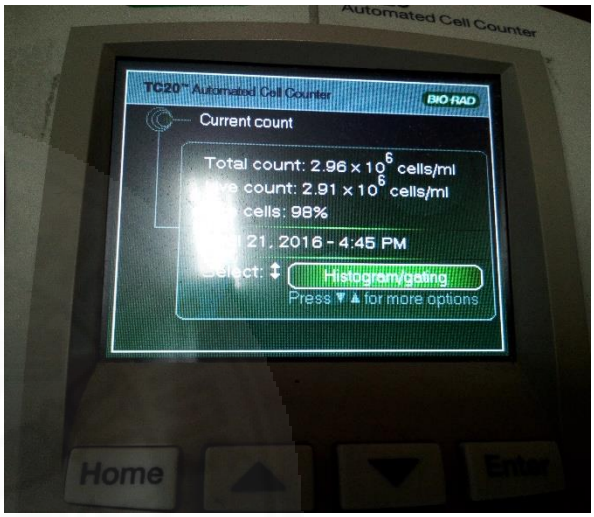
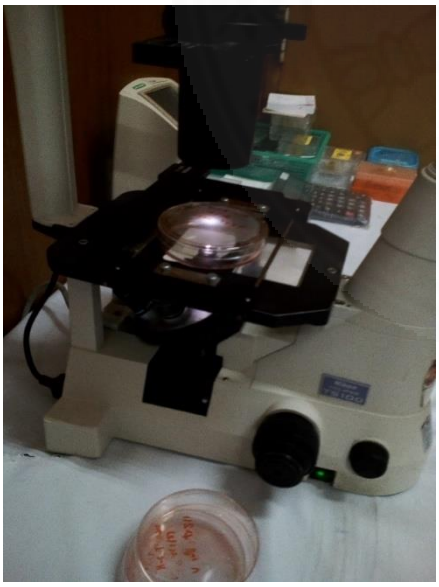
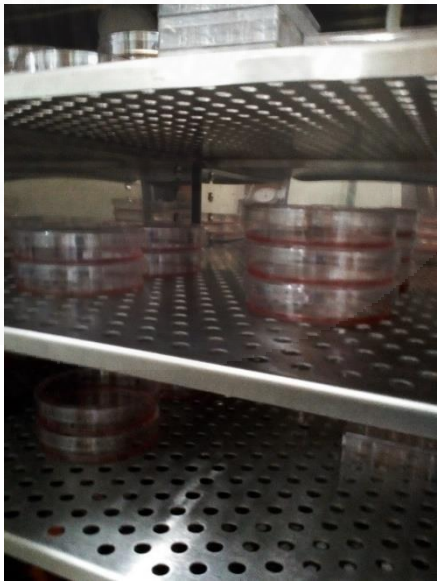
APPENDICES

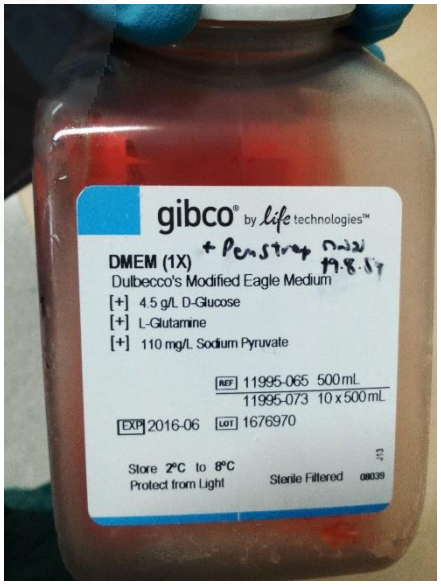
APPENDIX A
LABORATORY PROCEDURES









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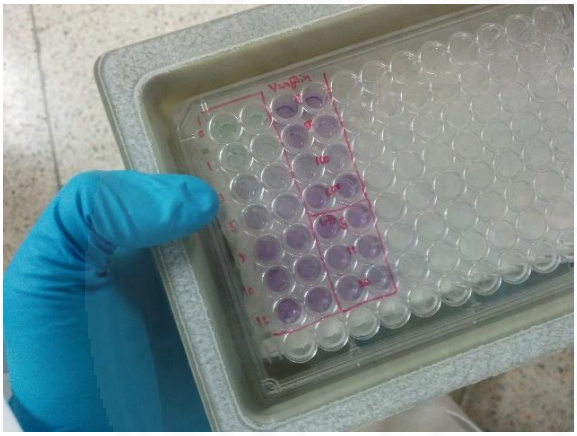

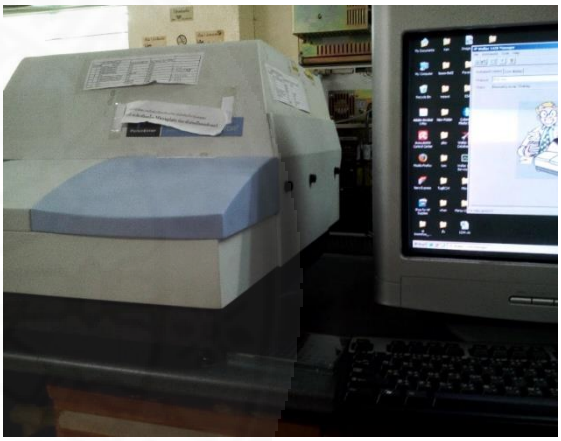

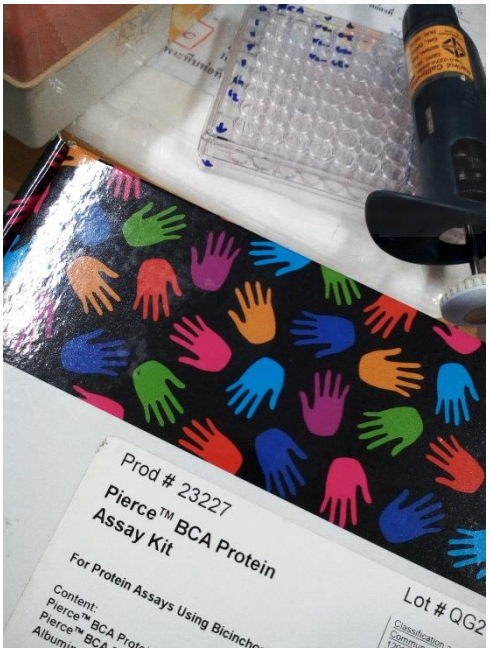
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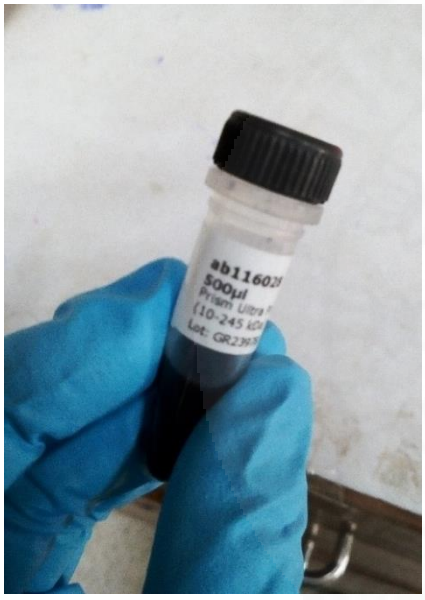
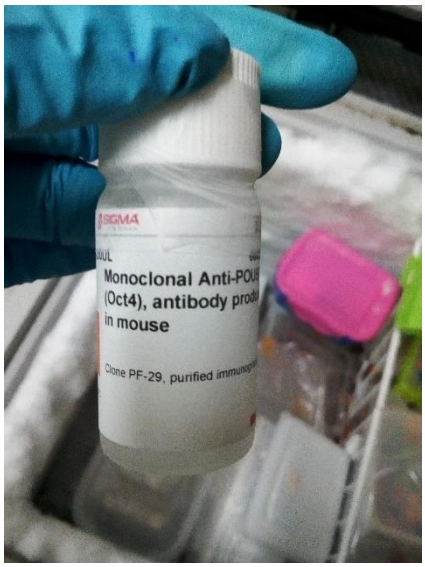
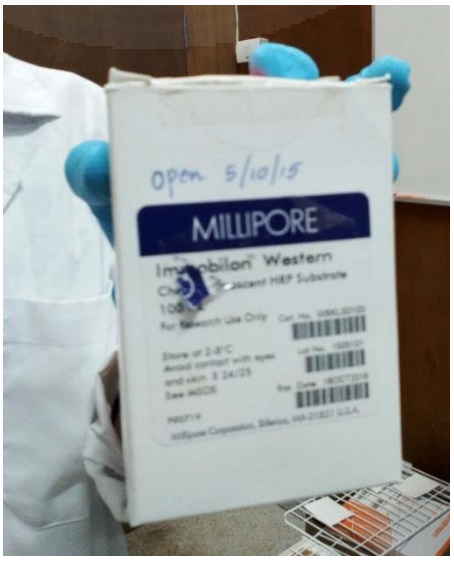



SIGMA
Monoclonal Anti-POU
(Oct4), antibody prod
in mouse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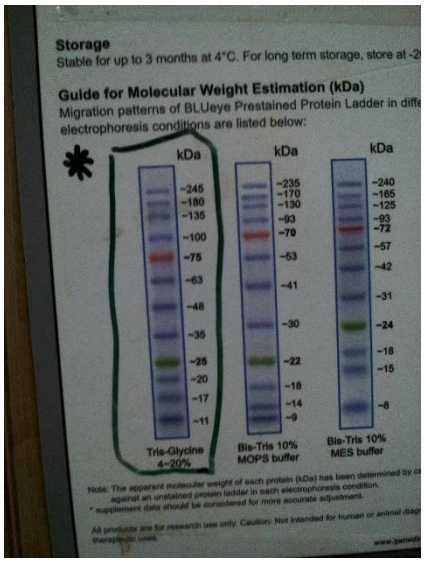
MILLIPORE


Storage
Stable for up to 3 months at 4°C. For long term storage, store at -2
Guide for Molecular Weight Estimation (kDa)
Migration patterns of BLUeye Prestained Protein Ladder in diff
electrophoresis conditions are listed below:
kDa
kDa
kDa
Tris-Glycine 4–20%
Bis-Tris 10% MOPS buffer
Bis-Tris 10% MES buffer

APPENDIX B

THE ACCEPTANCE LETTER FROM THE THAI JOURNAL OF PHARMACEUTICAL SCIENCES (TJPS)

Subject: [TJPS 368] Editor Decision

From: TJPS (tjpsmanage@gmail.com)

To: pithi_chan@yahoo.com;

Date: Sunday, April 23, 2017 11:22 AM

The following message is being delivered on behalf of The Thai Journal of Pharmaceutical Sciences (TJPS).

Dear Prof. Dr. Pithi Chanvorachote.

I am pleased to inform that your revised manuscript entitled "Vanillin increases stem cell signal and cell adhesion in keratinocytes" has been thoroughly reviewed and considered "accepted" for publication in TJPS.

The manuscript has been sent to our production unit for formating. Its galley proof version will be sent to you soon for a final check before being published in TJPS.

I would like to thank you for submission your interesting research work in TJPS.

Best regards,

Wanchai De-Eknamkul
Chief Editor
Wanchai.D@Chula.ac.th

The Thai Journal of Pharmaceutical Sciences (TJPS)
http://www.tjps.pharm.chula.ac.th/ojs/index.php/tjps

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Work Experiences	2012-2016: Physicain