EFFECTS OF ALK-5 INHIBITOR AND TRANSFORMING GROWTH FACTOR-BETA1 IN THE DIFFERENTIATION OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED) INTO EPITHELIAL-LIKE CELLS

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

ADV	Average density value
ALK	Activin receptor-like kinase
β	Beta
BMP	Bone morphogenetic protein
bp	Base pair
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
Ca^{2+}	Calcium
Cat	Catalog
CD	Cluster of differentiation
Cdk1	Cyclin-dependant kinase 1
cDNA	Complementary deoxyribonucleic acid
cm^2	Centimeter square
CNS	Central nervous system
CO_2	Carbon dioxide
DEPC	Diethyl pyrocarbonate
DFPC	Dental follicle progenitor cell
DMEM	Dulbecco’s modified eagle’s medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide nucleoside triphosphate
DPPSC	Dental pulp pluripotent-like stem cell
DPSC	Dental pulp stem cell
DTT	Dithiothreitol

EB	Embryoid body
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FZD-7	Frizzled-7
g	Gram
h	Hour
HCl	Hydrochloric acid
HNSCC	Squamous cell carcinomas of the lung, head, neck
IF	Intermediate filament
IgG	Immunoglobulin G
KBM-CD	Keratinocyte basal medium-chemically defined
KGM	Keratinocyte growth medium
KGM-CD	Keratinocyte growth medium-chemically defined
Klf4	Kruppel-like factor 4
KRT	Keratinocyte cell
KRT5	Keratin 5
KRT14	Keratin 14
KSF	Keratinocyte-serum free
LAP	Latency associated protein

LB	Lithium boric buffer
LIF	Leukaemia inhibitor factor
LTBP	Latent TGF-β binding protein
M	Molar
mg	Miligram
MET	Mesenchymal to epithelial transition
ml	Mililiter
mM	MiliMolar
MMLV RT	Moloney murine leukemia virus reverse transcriptase
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
MSCBM	Mesenchymal stem cell basal medium
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
MW	Molecular weight
NaOH	Sodium hydroxide
ng	Nanogram
NGS	Normal goat serum
nm	Nanometer
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDLSC	Periodontal ligament stem cell
PDT	Population doubling time
pg	Picogram

r	Multiplication rate
RNA	Ribonucleic acid
rpm	Round per minute
RT-PCR	Reverse transcriptase – polymerase chain reaction
Oct4	Octamer-binding transcription factor 4
SB431542	(SB) 4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide
SC	Stem cell
SCAP	Stem cells from apical papilla
secs	Seconds
SHED	Stem cells from human exfoliated deciduous teeth
Sox2	(Sex determining region-Y)-box 2
SYBR Green I	N’,N’-dimethyl-N-[4-[E-(3-methyl-1,3-1,3-benzothiazol-2-ylidene)methyl]-1-phenylquinolin-1-ium-2-yl]-N-propylpropane-1,3-diamine
TGF-β	Transforming growth factor beta
TGF-β1	Transforming growth factor beta 1
TGF-β2	Transforming growth factor beta 2
TGF-β3	Transforming growth factor beta 3
TGF-βR1	Transforming growth factor beta receptor type 1
TGF-βR2	Transforming growth factor beta receptor type 2
TGF-βR3	Transforming growth factor beta receptor type 3
TP63	Transformation-related protein 63
V	Voltage
w/v	Weight per volume

XTT	(2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide)
Zfp42	Zink finger protein 42
α-MEM	Minimum Essential Medium Alpha
µg	Microgram
µl	Microliter
µm	Micrometer
µM	MicroMolar
°C	Degree celcius
%	Percentage

KESAN PERENCAT ALK-5 DAN FAKTOR TRANSFORMASI PERTUMBUHAN BETA1 DALAM PEMBEZAAN SEL STEM DARI GIGI SUSU MANUSIA YANG TERKELUPAS (SHED) KEPADA SEL BERSIFAT EPITELIAL

ABSTRAK

Sel stem daripada gigi susu manusia yang terkelupas (SHED) boleh membahagi, membeza dan matang kepada sel jenis tertentu dan berupaya membaikpulih diri sendiri untuk menghidupkan sel-sel yang lain. Kajian terdahulu menunjukkan SHED boleh membeza menjadi sel bersifat epitelial. Walaubagaimanapun, kesan-kesan Faktor Transformasi Pertumbuhan Beta1 (*TGF-β1*) atau perencat reseptor aktivin bersifat kinase 5 (*ALK-5*) ke atas SHED masih belum diterokai. Oleh itu, kajian ini bertujuan untuk mengkaji kesan TGF-β1 dan perencat ALK-5 ke atas SHED yang dikultur dalam Media Pertumbuhan Keratinocyte (*KGM*) ke atas potensinya untuk membeza kepada sel bersifat epitelial menggunakan asai MTT [*(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)*] dan asai biru alamar, analisis morfologi sel, teknik Tindakbalas Rantai Polimerase-Transkriptase Berbalik (*RT-PCR*) dan sitometri aliran (*flow cytometry*). Analisa sitotoksisiti menggunakan asai MTT dijalankan selama 72 jam melalui pencairan bersiri TGF-β1 (0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, dan 160.0 ng/ml) dan perencat ALK-5 (0.156, 0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, dan 160.0 μM). Kemudian, tiga kepekatan TGF-β1 (0.3125, 0.625, dan 1.25 ng/ml) dan perencat ALK-5 (0.156, 0.3125, dan 0.625 μM) yang terpilih digunakan bagi penentuan masa penggandaan populasi (*PDT*) sel melalui asai biru alamar pada hari 1, 3, 5, 7, dan 10,

dan pengkulturan SHED di dalam *α-MEM*. Kajian ini selanjutnya diteruskan dengan pemerhatian perubahan morfologi sel ke atas SHED yang dikultur dalam KGM sahaja dan dengan kepekatan TGF-β1 (1.25 ng/ml) atau perencat ALK-5 (0.625 μM) terpilih pada hari 1, 3, 7, 14, dan 21. Isolasi RNA SHED yang dikultur dalam tiga keadaan berbeza ini dilakukan pada hari 1, 3, 7, 14, dan 21. Kemudian, analisis ekspresi gen sel stem, sel epitelial, dan gen-gen tertentu yang terlibat dalam isyarat TGF-β dikenalpasti sewaktu proses pembezaan menggunakan RT-PCR Dua Langkah. Selain daripada itu, analisis ekspresi protein ke atas penanda epitelial juga ditentukan menggunakan sitometri aliran pada hari 1 dan 21. Berdasarkan asai MTT, didapati kepekatan TGF-β1 pada 0.3125, 0.625, dan 1.25 ng/ml dan perencat ALK-5 pada 0.156, 0.3125, dan 0.625 μM menunjukkan kesan sitotoksik yang paling minima (lebih daripada 50%) dan dipilih untuk asai proliferasi. PDT terpendek diwakili oleh 1.25 ng/ml TGF-β1 (75 jam) dan 0.625 μM perencat ALK-5 (68 jam) dan kepekatan ini termasuklah sel-sel hanya dalam KGM, menunjukkan terdapat perubahan morfologi berbanding kawalan. Profil ekspresi penanda epitelial menunjukkan ketiadaan pengekspresan gen *E-cadherin*, Δ*Np63*, dan *Keratin5* beserta protein E-cadherin dan pan-sitokeratin yang menunjukkan keadaan kultur yang tidak berupaya meransang proses pembezaan SHED kepada sel bersifat epitelial. Walaubagaimanapun, kehadiran penanda sel stem (*NANOG*, *nestin*, *Rex1*, dan *vimentin*) dan molekul spesifik yang terlibat dalam isyarat TGF-β (*TGFβR1*, *TGFβ1*, *Smad3*, dan *Smad4*) menunjukkan bahawa kultur sel dalam tiga keadaan berbeza menggalakkan proses peralihan epitelial kepada mesenkima (*EMT*) dengan kehadiran *TGFβ1* dan *Smad3* dalam isyarat TGF-β yang sudah dikaitkan dengan EMT. Oleh yang demikian, KGM tidak dapat membezakan SHED sepenuhnya kepada sel seperti sel epitelial dan seterusnya, SHED tidak dapat menjalani proses peralihan mesenkima kepada epithelial (MET).

EFFECTS OF ALK-5 INHIBITOR AND TRANSFORMING GROWTH FACTOR-BETA1 IN THE DIFFERENTIATION OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH (SHED) INTO EPITHELIAL-LIKE CELLS

ABSTRACT

Stem cells from human exfoliated deciduous teeth (SHED) are capable to divide, differentiate and mature to the specific types of cells as well as to replenish themselves to regenerate other living cells. Previous study has showed that SHEDs could differentiate into epithelial-like cells. Yet, the effects of Transforming Growth Factor-Beta1 (TGF-β1) or activin like kinase 5 (ALK-5) inhibitor on SHEDs remain unexplored. Thus, the present study was aimed to investigate the effects of TGF-β1 and ALK-5 inhibitor on SHEDs cultured in Keratinocyte Growth Medium (KGM) on its potential to differentiate into epithelial-like cells employing MTT [*(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)*] and alamar blue assays, cell morphology analysis, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) technique and flow cytometry. A serial dilution of TGF-β1 (0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, and 160.0 ng/ml) and ALK-5 inhibitor (0.156, 0.3125, 0.625, 1.25, 2.5, 5.0, 10.0, 20.0, 40.0, 80.0, and 160.0 μM) concentration was carried out to determine the cell cytotoxicity for each treatment using MTT assay for 72 hours. Afterwards, the three selected concentrations for TGF-β1 (0.3125, 0.625, and 1.25 ng/ml) and ALK-5 inhibitor (0.156, 0.3125, and 0.625 μM) were analysed using alamar blue assay on day 1, 3, 5, 7, and 10 and was done in alpha Minimum Essential Medium (α-MEM) to determine the population doubling time (PDT). The study was

further investigated with observation of the cell morphological changes on SHED cultured in KGM only, and with selected concentration of TGF-β1 or ALK-5 inhibitor on day 1, 3, 7, 14, and 21. RNA isolation of SHED culture in three different conditions were harvested at day 1, 3, 7, 14, and 21. Then, the gene expression analysis of stem cell, epithelial cell, and specific genes involved in TGF-β signalling were identified during the differentiation process using Two-step RT-PCR. Apart from that, protein expression analysis of epithelial markers was also determined using flow cytometer on day 1 and 21. Based on MTT assay, 0.3125, 0.625, and 1.25 ng/ml TGF-β1 and 0.156, 0.3125, and 0.625 µM ALK-5 inhibitor showed less cytotoxicity effects (more than 50%) and were selected for proliferation assay. The shortest PDT was represented by 1.25 ng/ml TGF-β1 (75 hours) and 0.625 µM ALK-5 inhibitor (68 hours) and these concentrations including cells in KGM only, showed there were cell morphological changes compared to control. The gene expression profile showed an absence of epithelial markers *E-cadherin*, Δ*Np63*, and *Keratin5* for gene, and E-cadherin and pan-cytokeratin for protein expression indicated that the culture conditions unable to induce the differentiation process into epithelial-like cells. However, the presence of stem cell markers (*NANOG*, *nestin*, *Rex1*, and *vimentin*) and specific molecules involved in TGF-β signalling (*TGFβR1*, *TGFβ1*, *Smad3*, and *Smad4*) indicated that the cell culture in three different condition induced epithelial to mesenchymal transition (EMT) since the presence of *TGFβ1* and *Smad3* in TGF-β signalling that have been associated with EMT. Thus, KGM was unable to fully differentiate SHED into epithelial-like cells and hence, SHED were incapable to undergo mesenchymal to epithelial transition (MET).

CHAPTER ONE

INTRODUCTION

1.1 Background of the study

Stem cell (SC) research has been recognised as one of the demanding fields in tissue regeneration, engineering, and therapeutic research. SCs are undifferentiated cells which are found in the embryonic, fetal, and adult organism. These SCs are capable of self-renewal through cell division, clonality, and differentiated into other types of cells for cell and tissues survival (Kolios and Moodley, 2013).

Based on their origin and differentiation capabilities, SCs can be categorised into two broad groups; embryonic stem cells (ESCs) which are derived from blastocyst and stem cells isolated from adult tissues. Both these SCs differ in their potential to differentiate into different cell types. ESCs can differentiate into three germ layers; endoderm, mesoderm, and ectoderm, besides can be maintained in undifferentiated state for a prolonged period in cell culture (Yao *et al.*, 2006). On the other hand, adult SCs have been known to have limitation in their differentiation capacity although these cells have differentiated into tissue from different germ cell layers *in vitro* (Ilancheran *et al.*, 2009; Moodley *et al.*, 2010).

Adult SCs can be isolated from diverse tissues, including bone marrow, muscle, fat, dermis, placenta, dental pulp, synovial membrane, peripheral blood, periodontal ligament, endometrium, umbilical cord, and umbilical cord blood

(Fukuchi *et al.*, 2004; Sarugaser *et al.*, 2005; Tondreau *et al.*, 2005; Baksh *et al.*, 2007; Crisan *et al.*, 2008; Martin-Rendon *et al.*, 2008; Schwab *et al.*, 2008; Huang *et al.*, 2009b; Hermida-Gómez *et al.*, 2011; Park *et al.*, 2011b; Singer and Caplan, 2011; Eirin *et al.*, 2012). Bobis *et al.* (2006) reported that mesenchymal stem cells (MSCs) were found to be the most abundant among adult SCs. These cells are defined as multipotent cells, where they have limited capabilities of specialisation, and they can form bone, cartilage, muscle, fat, and other connective tissues when were stimulated with cytokine or specific culture medium (Caplan, 2007). In 2009, Nam and Lee discovered that stem cells from human exfoliated deciduous teeth (SHED) can be induced to differentiate into epithelial-like cells when directly cultured in specific media, Keratinocyte Growth Medium (KGM) (Nam and Lee, 2009). Formation of epithelium was crucial in tissue regeneration and engineering to regenerate damaged human cells due to illness, developmental defects and accidents.

In view with the advancement in tissue engineering together with stem cell's regeneration potential, researchers started to explore stem cells from different sources and one of the promising stem cells are SHED which was first discovered by Miura and his colleagues in 2003 (Miura *et al.*, 2003). These stem cells have been considered as one of the potential sources since the deciduous tooth was easily accessible and extraction of tooth was less invasive compared to other types of stem cells.

Transforming Growth Factor-Beta (TGF-β) family consists of important secreted structurally related polypeptides such as TGF-βs, activins, bone morphogenetic proteins (BMPs), growth and differentiation factor, Müllerian inhibitory factor, and inhibin (Santibañez *et al.*, 2011). This family secreted wide range

of proteins involved in many physiological processes including embryonic development, immune responses, and wound healing, and cellular biological function such as cell proliferation, differentiation, apoptosis, migration, and extracellular matrix production (Roberts and Sporn, 1990; Inman *et al.*, 2002; Gordon and Blobe, 2008; Wu and Hill, 2009; Gui *et al.*, 2012). Interestingly, TGF-β signalling has been found to play an important regulatory function in epithelial proliferation and differentiation (Roberts, 1998; Ten Dijke *et al.*, 2002; Lee *et al.*, 2013). The TGF-β consists of three isoforms; TGF-β1, TGF-β2, and TGF-β3 and among the isoforms, TGF-β1 was found to be the most abundantly and universally expressed isoform (Karatsaidis *et al.*, 2003; Dobaczewski *et al.*, 2011), and more studies have been performed using exogenous TGF-β1 (Elliott and Blobe, 2005; Gao *et al.*, 2009; Karaöz *et al.*, 2011; Nam *et al.*, 2014). Treatment with exogenous TGF-β1 have shown a diverse differentiation capacities such as in wound healing process in fibroblasts (Penn *et al.*, 2012; Pakyari *et al.*, 2013) and epithelial-mesenchymal transition (EMT) (Xu *et al.*, 2009a; Nam *et al.*, 2014).

TGF-β receptors involve TGF-βR1, TGF-βR2, and TGF-βR3 and the biological processes occurred when TGF-β molecule binds to the cell surface receptor TGF-βR2 (Massagué, 1998). TGF-βR1 or identified as activin receptor-like kinase (ALK) recognises the heterodimer complex of TGF-β molecule-TGF-βR2 and undergoes phosphorylation (Massagué, 2012). Out of seven known type I (Activin like kinase; ALK) receptors, ALK-4, ALK-5, and ALK-7 are structurally similar to each other (Miyazawa *et al.*, 2002). ALK-5, also known as TGF-βR1 is the specific receptor for TGF-βs (Miyazawa *et al.*, 2002; van Meeteren and Ten Dijke, 2012). Once the TGF-βR1 is phosphorylated, the downstream regulation occurred and translocated into

the nucleus, and later regulated the transcription of certain target genes (Godkin and Dore, 1998; Worthington *et al.*, 2012). However, the TGF-β signalling transduction could be inhibited in the presence of inhibitory molecules such as (SB431542) [(SB) 4-[4-(3,4-Methylenedioxyphenyl)-5-(2-pyridyl)-1H-imidazol-2-yl]-benzamide]. This chemically synthetic substance acts as a potent and specific inhibitor of ALK-5, also inhibits ALK-4 and nodal type 1 receptor ALK-7, since they are highly related to ALK-5 in their kinase domains (Inman *et al.*, 2002). Since SB431542 interacts with heterodimer complex of TGFβ type 2 and type 1 (ALK-5) receptor, the activity of TGF-β1 might also be affected to a certain extent. Hence, this study aims to study the effects of exogenous TGF-β1 and potent inhibitor SB431542 (ALK-5 inhibitor) on SHED cultured in differentiation medium enriched for promotion of differentiation process into epithelial-like cells.

The current study intended to determine the effects of exogenous TGF-β1 and its inhibitor (ALK-5) in the induction of SHED into epithelial-like cells. The SHED cultured in KGM were treated with both treatments of TGF-β1 and ALK-5 inhibitor and were analysed for cell viability using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, cell proliferation and population doubling time (PDT) using alamar blue assay, and cellular morphology on day 1, 3, 7, 14, and 21. Furthermore, gene expression of stem cell markers (*NANOG*, *nestin*, *Rex1*, and *vimentin*), epithelial markers (*E-cadherin*, Δ*Np63*, and *Keratin5*) and specific molecules (*TGFβR1*, *TGFβ1*, *Smad3*, and *Smad4*) involved in TGF-β signalling transduction together with protein expression of epithelial markers (E-cadherin and pan-cytokeratin) were also investigated.

1.2 Justification of study

Human parts such as skin, oral mucosa, and blood vessel are mainly reconstructed by epithelial cells. Formation of epithelial cells have become crucial to regenerate new cells thus replacing damaged human cells due to illness, developmental defects and accidents. SHED has been widely known as one of stem cell sources for therapeutic application. The ability of SHED to differentiate to other cell lineage such as epithelial cell, when cultured in specific medium highlights its potential for application in future tissue regeneration. Interestingly, the novel epithelial stem cell-like cells from SHED have been identified recently (Nam and Lee, 2009). This suggests the ability of stem cells from human exfoliated deciduous teeth to be differentiated to epithelial-like cells. TGF-β1 is a growth factor that is mostly produced by epithelial cells. TGF-β1 is also a growth factor that is involved during the process of epithelial-mesenchymal interaction during organogenesis. Meanwhile, ALK-5 inhibitor played a role as an inhibitor to the TGF-β type 1 receptor (ALK-5) which is involved in TGF-β signalling pathway. Although many studies have been done considering the function of TGF-β signalling pathway on cell proliferation; the role of TGF-β1 molecule especially in controlling the process of epithelial cell differentiation from stem cell is still limited. Hence, the effects of the TGF-β1, ALK-5 inhibitor, and the molecules involved in the cell signalling pathway in the differentiation process of SHED into epithelial-like cells will be identified and highlighted. This study may provide a better insight and understanding on the mechanism and cellular signalling works for stem cell and tissue regeneration process.

1.3 Objectives of the study

1.3.1 General Objective

To study the effect of TGF-β1 and its inhibitor (ALK-5) in the differentiation of SHED into epithelial-like cells when cultured in KGM.

1.3.2 Specific objectives

i. To determine the population doubling time of SHED treated with TGF-β1 and ALK-5 inhibitor when cultured in KGM.

ii. To measure the gene expression levels of specific gene markers for epithelial-like cells derived from SHED treated with TGF-β1 and ALK-5 inhibitor.

iii. To identify the protein expression of epithelial markers (E-Cadherin and pan-cytokeratin) on epithelial-like cells derived from SHED treated with TGF-β1 and ALK-5 inhibitor.

iv. To determine the gene expression levels of specific molecules associated in TGF-β cell signalling pathway (*TGFβR1*, *TGFβ1*, *Smad3*, and *Smad4*) in SHED treated TGF-β1 and ALK-5 inhibitor when cultured in KGM.

1.4 Research hypothesis

TGF-β1 and ALK-5 inhibitor treatment could affect the differentiation process of SHED into epithelial-like cells when cultured in KGM.

CHAPTER TWO

LITERATURE REVIEW

2.1 Stem cells

Stem cells are generally defined as unspecialised cells that have capabilities of both self-renewal and multi-lineage differentiation into more specialised cells with new specialised cells function (Bongso and Lee, 2005; Saxena *et al.*, 2010; Wei *et al.*, 2013). In mammals, stem cells has been broadly classified into two types; ESCs and adult stem cells (Choumerianou *et al.*, 2008; Liu and Cao, 2010; Romeo *et al.*, 2012; Venkatesan and Madhira, 2014).

ESCs originated from inner cell mass of blastocysts, which form after a few days of fertilisation between egg and sperm fusion. These cells then form three primary germ layers which consist of ectoderm, mesoderm, and endoderm (Choumerianou *et al.*, 2008; Poh *et al.*, 2014). These cells had been proven to have capability to form cells of all tissues in adult organisms (De Wert and Mummery, 2003; Can, 2008; Liu and Cao, 2010; Liu *et al.*, 2013). Therefore, ESCs are termed as pluripotent stem cells.

Although ESCs offer fully developed organisms due to its capabilities, there are some issues pertaining to ESCs that need to be concerned. The advantage of using ESCs was their unlimited differentiation into other types of cells. This unlimited potential may offer numerous medical possibilities since ESCs can generate any other part of cells and tissues, and cure any possibilities of disease that threaten human life.

Meanwhile, the main disadvantage of using ESCs was the human blastocysts were destroyed during the process of harvesting the inner mass. This purpose was unethical procedure since it involved a human life. Another disadvantage of using ESCs was that the proliferation and differentiation of the cells can lead to cancerous and unwanted growth of tissues (Baraniak and McDevitt, 2010; Herberts *et al.*, 2011; Penna *et al.*, 2015).

On the other hand, adult stem cells have been discovered in wide range of tissues of foetus and after birth. These cells have been differentiated into more specialised cells where they acted as repair system for the body by replenishing the adult tissues during normal or injured situation (De Wert and Mummery, 2003; Hsu and Fuchs, 2012). Thus, these adult stem cells had been termed as multipotent stem cells.

In 2006, Bobis and his colleagues reported that MSCs had been found to be the widest distribution among various adult stem cells in the human body and had been isolated from diverse tissues and organs (Bobis *et al.*, 2006). Since the isolated MSCs contained high mixture of stromal progenitor cells at various stages of development (Rhodes *et al.*, 2004; Maria *et al.*, 2007; Phinney, 2007; Baer and Geiger, 2012), the International Society for Cellular Therapy recommended to change the term mesenchymal stem cells to multipotent mesenchymal stromal cells (MSCs) (Dominici *et al.*, 2006).

2.2 Multipotent mesenchymal stem cells

Multipotent mesenchymal stem cells, are progenitor cells which have limited capabilities of specialisation. These MSCs have the ability to rise into tri-lineages which are osteogenic, chondrogenic, and adipogenic under a standard *in vitro* differentiation medium (Dominici *et al.*, 2006; Liu and Cao, 2010). These post-natal cells were isolated from other non-marrow tissues such as adipose tissue, placenta, amniotic fluid, tendon, synovial membrane, skeletal muscle and dental pulp (De Bari *et al.*, 2001; Shi and Gronthos, 2003; Igura *et al.*, 2004; Tsai *et al.*, 2004; Xu *et al.*, 2005; Bi *et al.*, 2007; Crisan *et al.*, 2008; Castrechini *et al.*, 2010; Levi and Longaker, 2011; de Sousa *et al.*, 2014; Machado *et al.*, 2015; Savickiene *et al.*, 2015; Zhang *et al.*, 2016). These findings were supported by Baksh *et al.* (2004), Kolf *et al.* (2007), and Porada *et al.* (2006) that in the last decade, studies on MSCs had led to the discovery of a wide range of stem cells isolated from every organ and tissue (De Bari *et al.*, 2001; Huang *et al.*, 2009b).

MSCs have been reported to be easily expanded *in vitro* although they were found in very small quantities *in vivo* (Docheva et al., 2008). Some researchers reported that in undifferentiated MSCs, there were several antibodies that react against Cluster of Differentiation (CD) 73 (membrane-bound ecto-5'-nucleotisidase), CD90 (Thy-1), CD105 (endoglin) and CD166 (ALCAM), and thus, seem to be suitable for pure isolation of MSCs population (Barry and Murphy, 2004; Bobis *et al.*, 2006; Schieker *et al.*, 2007).

Leo and Grande (2006) have mentioned that MSCs can be influenced *via* a multitude of growth factor receptors that have been identified on their surface such as Epidermal Growth Factor Receptor, basic Fibroblast Growth Factor Receptor, Insulin Growth Factor Receptor, Platelet Derived Growth Factor Receptor, Transforming Growth Factor Beta Receptor type 1 (TGFβR1) and Transforming Growth Factor Beta Receptor type 2 (TGFβR2), and these growth factor receptors are important for MSC self-renewal and differentiation (Park *et al.*, 2011b).

The proliferation and differentiation of MSCs are regulated through a variety of peptides such as *NANOG*, *Oct4*, and signalling pathways such as Transforming Growth Factor Beta (TGF-β) signalling pathway. Chambers *et al.* (2007) reported that the transcription factors *Oct4*, *NANOG* and *Sox2* are very important for the efficient maintenance of pluripotent cell identity and they found that the expression of *Oct4* and *NANOG* were detected during development such as in adult tissues; meanwhile, expression of *Sox2* was not expressed. Other than that, *Rex1* has also been suggested as one of the pluripotency markers (Son *et al.*, 2013). The expression of this gene has been reported to be limited in the inner cell mass of blastocysts, which retain the potential of pluripotency differentiation, and subsequently down regulated during the later stages of differentiation in the epiblast and primitive ectoderm (Mignotte and Vayssiere, 1998; Yu *et al.*, 2011). Scotland *et al.* (2009) also reported that the expression of *Rex1* affected the differentiation, cell cycle regulation, and cancer progression in cells. *Nestin* and *vimentin* are both intermediate filament (IF) proteins which also have been chosen as mesenchymal stem cell marker. *Nestin* expression has been associated with early stages of development of cells which have been reported in a review by Xie *et al.* (2015) where several researchers have mentioned that bone

marrow-derived MSCs expressed *nestin* before differentiation *in vitro* (Tondreau *et al.*, 2004), and this gene was enriched in embryonic stem cell-derived progenitor cells that could develop into neuroectodermal, endodermal, and mesodermal lineages (Wiese *et al.*, 2004). On the other hands, *vimentin* has been mostly utilised in EMTs which occurred during embryogenesis and metastasis (Mendez *et al.*, 2010).

2.3 Dental stem cells sources

Dental stem cells have been recognised as one of the stem cells sources that can be used for regenerative medicine. Dental stem cells were first isolated from dental pulp (DPSCs) by Gronthos and his colleagues (Gronthos *et al.*, 2000) and from exfoliated deciduous teeth (SHED) (Miura *et al.*, 2003). Other than that, dental stem cells can also be extracted from periodontal ligament stem cells (PDLSCs) (Seo *et al.*, 2004), stem cells from apical papilla (SCAP) (Sonoyama *et al.*, 2006; Sonoyama *et al.*, 2008), dental follicle progenitor cells (DFPCs) (Morsczeck *et al.*, 2008) (Figure 2.1) and the recent finding is pluripotent-like stem cells derived from dental pulp (DPPSCs) (Atari *et al.*, 2012).

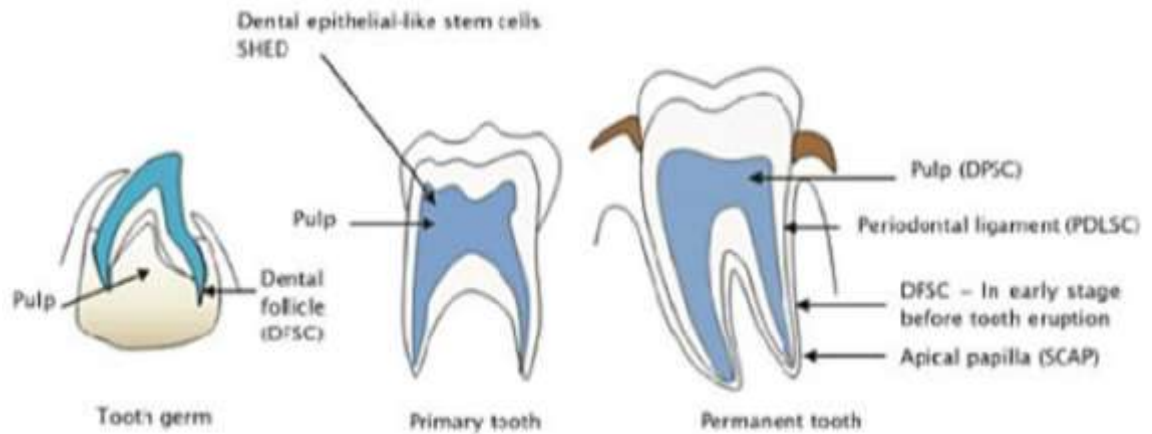


Figure 2.1 Schematic image showing the location of dental stem cells niches (Abdullah *et al.*, 2016)

2.3.1 Dental pulp stem cells

Dental pulp stem cells (DPSCs) were first discovered by Gronthos *et al.* (2000) from human adult dental pulp, which are capable to regenerate a dentin-pulp-like complex. These stem cells can easily be obtained from discarded permanent teeth and harvested with less invasive and safe manner. DPSCs have shown to have higher angiogenic, neurogenic, and regenerative possibilities as compared to stem cells from bone marrow and adipose tissue (Ishizaka *et al.*, 2012) which may serve as alternate versatile stem cell sources for cellular biological therapies.

DPSCs can be extracted from discarded permanent teeth comprising of third molars, supernumerary teeth, displaced teeth and orthodontically unnecessary teeth (Nakashima *et al.*, 2013). These stem cells have been found to display highly proliferative, self-renewal, and capacity to differentiate into other lineages (Gronthos *et al.*, 2000). Other than that, previous study on animal also has shown that DPSCs have a greater potential in repair and regeneration from various tissues, such as heart

(Gandia *et al.*, 2008), muscles (Kerkis *et al.*, 2008), teeth (Nedel *et al.*, 2009) and bone (Graziano *et al.*, 2008). Interestingly, in 2009, d'Aquino and his co-workers had successfully accomplished the first clinical trial on patient using DPSCs application for bone reconstruction (d'Aquino *et al.*, 2009). Karbanová *et al.* (2010) reported that when they cultured isolated DPSCs in a medium with low level of serum in the presence of epidermal growth factor (EGF) and platelet-derived growth factor BB simultaneously, the stem cells revealed antigenic profile of mesenchymal and neural markers with several markers of embryonic stem cells. This supported that these stem cells can differentiate into multi-lineage cells. However, DPSCs have shown to have lower proliferation rate compared to SHED (Wang *et al.*, 2012).

2.3.2 Stem cells from human exfoliated deciduous teeth

Another source of stem cell derived from dental tissues is stem cells from human exfoliated deciduous teeth or known as SHED. These stem cells have received growing attention in recent years due to its common characteristics with other MSCs pertaining to easiness of obtainment and propagation (Bluteau *et al.*, 2008). SHED was first isolated by Miura and his co-workers, and these stem cells were identified to be a population of highly proliferative, clonogenic cells capable of differentiating into a variety of cell types including neural cells, adipocytes, and odontoblasts (Miura *et al.*, 2003; Fazliah *et al.*, 2010; de Sá Silva *et al.*, 2014). Due to its advantages of a higher proliferation capability, abundant cell supply, and painless stem cell collection with minimal invasion, SHED could provide a better option as a stem cell source for potential therapeutic application.

Several studies have shown the capability of SHED to differentiate into other lineage of cells. For example, in 2009, Nam and Lee successfully induced primary SHED to differentiate into epithelial-like cells when cultured in KGM (Nam and Lee, 2009). Later, Wang and his associates differentiated SHED into dopaminergic neuron-like cells which could be the sources in treating Parkinson disease's patients (Wang *et al.*, 2010). In 2013, another achievement was achieved when SHED was successfully transplanted into full-length root canals with injectable scaffolds; these stem cells were capable to proliferate within the root canal and expressed markers of odontoblastic differentiation (dentin sialophosphoprotein, dentin matrix acidic phosphoprotein, and matrix extracellular phosphoglycoprotein) after 28 days *in vitro* (Rosa *et al.*, 2013).

There are also several signalling transductions that have been investigated related to SHED culture. TGF-β, extracellular signal-regulated kinase (ERK), protein kinase B, Wnt, and Platelet Derived Growth Factor (PDGF) signalling also have been shown to be activated in SHED cultured (Yamaza *et al.*, 2010). Besides, Bento *et al.* (2012) reported that mitogen activated-protein kinase kinase (MEK1)/ERK signalling was required for differentiation of SHED into endothelial cells. Furthermore, Notch signalling was also involved in SHED cultured in specific differentiation medium, KGM by expressing the Notch gene molecules (Taha *et al.*, 2015).

2.3.3 Periodental ligament stem cells

Multipotent PDLSCs were first described by Seo *et al.* (2004) when it was observed that these stem cells were capable to differentiate into cementoblast-like cells, which later can be used to renew the damaged tissues caused by periodontal disease. These

stem cells originated from perivascular space of the periodontium and have been found to possess mesenchymal stem cell characteristics, thus a promising tool for periodontal regeneration (Zhu and Liang, 2015). Due to PDLSCs capabilities, several researchers have mentioned that these cells also can differentiate into periodontal ligaments, alveolar bone, cementum, peripheral nerves, and blood vessels (Liu *et al.*, 2008; Huang *et al.*, 2009a; Park *et al.*, 2011a).

2.3.4 Stem cells from apical papilla

Stem cells from apical papilla (SCAP) were first discovered by Sonoyama *et al.* (2006) in human permanent immature teeth. These multipotent stem cells also have been found to express numerous neurogenic markers such as nestin and neurofilament medium, indicating that this stem cells originated from neural crest (Sonoyama *et al.*, 2008). Furthermore, SCAP has been demonstrated to own a significantly higher mineralisation potential as well proliferation rate compared to DPSCs (Bakopoulou *et al.*, 2011). Research has also been carried out to investigate signalling pathways such as Notch signalling associated with SCAP (Jamal *et al.*, 2015).

2.3.5 Dental follicle progenitor cells

Other interesting stem cells were dental follicle progenitor cells (DFPCs), which are loose connective tissues and was found by Morsczeck *et al.* (2005), Kemoun *et al.* (2007) and d'Aquino *et al.* (2011). They also reported that DFPCs acquired mesenchymal progenitor characteristics such as fibroblast-like morphology and expressed several mesenchymal markers such as Notch-1, nestin, and STRO-1.

Furthermore, also reported these stem cells also were derived from neural crest, and thus have different source from bone marrow-derived MSCs.

2.3.6 Dental pulp pluripotent-like stem cells

Recently, in 2012, Atari and his colleague successfully identified stem cell populations with embryonic-like morphology derived from human dental pulp from third molar, also known as dental pulp pluripotent-like stem cells (DPPSCs). They claimed that it was the first report available regarding DPPSCs. Based on their study, they were able to isolate these stem cells using culture media containing leukaemia inhibitor factor (LIF), EGF, and PDGF (Atari *et al.*, 2012).

2.4 Transforming Growth Factor Beta (TGF-β)

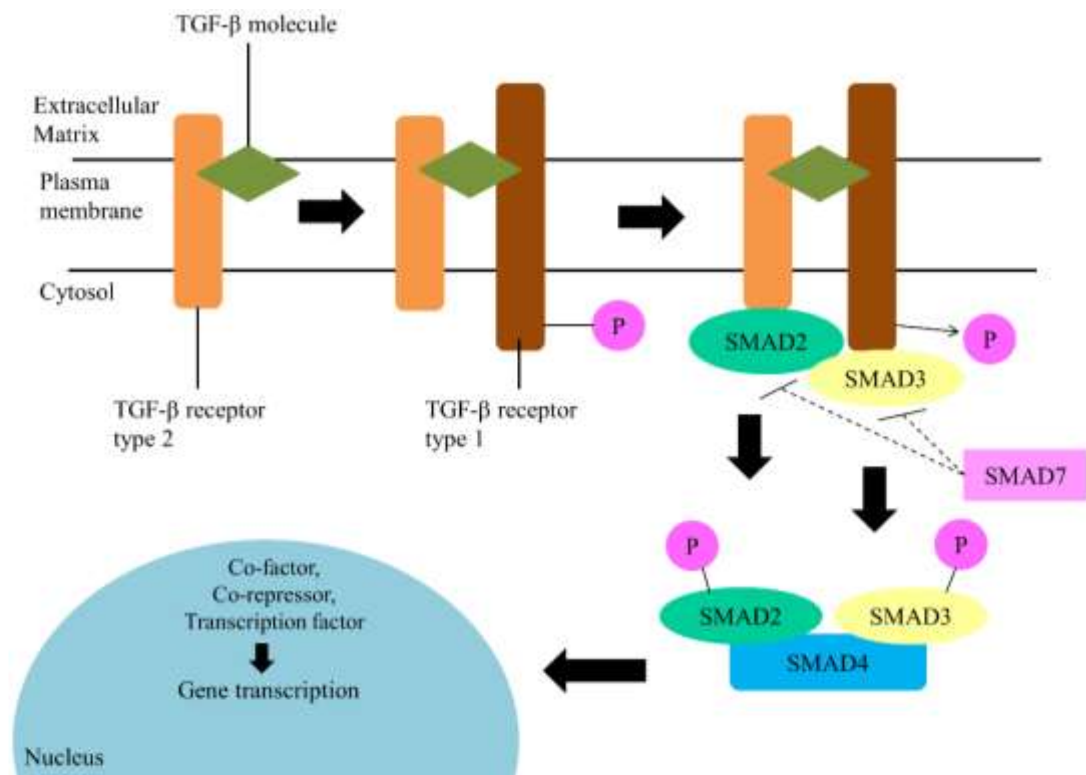
TGF-β family consists of a huge number of structurally related polypeptide growth factors, each of which can regulate an array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and cell death (Xie *et al.*, 2003; Massagué and Gomis, 2006; Massagué, 2012). The TGF-β family is highly conserved in mammals and plays a central role in regulating cell functionality for survival (Fleisch *et al.*, 2006; Taylor *et al.*, 2009). In mammals, three isoforms of TGF-β, namely, TGF-β1, TGF-β2, and TGF-β3 have been discovered (Saharinen and Keski-Oja, 2000; Massagué, 2012). These TGF-βs are multifunctional cytokines which can regulate cell proliferation and differentiation positively or negatively depending on the cell type (Hauri-Hohl *et al.*, 2008), and have been implicated in such diverse physiological events such as angiogenesis, steroidogenesis, immune function

and tissue remodelling and repair (Leask and Abraham, 2004; Bujak and Frangogiannis, 2007; Azmi *et al.*, 2013). The TGF-βs have been shown to be involved in a wide variety of cellular processes in cells and tissues such as fibroblast, epithelium, bone and extracellular matrix (ECM) (Govinden and Bhoola, 2003).

The TGF-β receptors are TGF-βR1, TGF-βR2, and TGF-βR3 and the biological processes occur when the TGF-β molecule interact with the cell surface receptors (Massagué, 2012). The TGF-β binds to the cell surface receptor TGF-βR2 but not TGF-βR1 even though both are cytoplasmic serine/threonine kinase domains. The TGF-βR1 recognises the heterodimer complex of TGF-β-TGF-βR2 and undergoes phosphorylation. Once the TGF-βR1 is phosphorylated, SMAD2 and SMAD3 proteins are activated. The process is inhibited by SMAD7. The SMAD4 proteins then attach to SMAD2 and SMAD3 proteins, undergo phosphorylation and become activated. These SMAD2/3/4 protein complexes are translocated to the nucleus and together with co-factor, co-repressor and transcription factor, and then the SMADs complex will regulate the transcription of target genes to mediate the biological process of TGF-β (Massagué *et al.*, 2005; Massagué, 2012).

In contrast to TGF-β2 and TGF-β3, TGF-β1 is being intensely studied in the TGF-β signalling pathway and this isoform has been correlated with epithelial formation in many cellular biological processes (Massagué and Xi, 2012). Gao and his colleague found that TGF-β1 was expressed in the inner of dental epithelium before the enamel matrix secretion and the expression of TGF-βRI was weekly detected in inner dental epithelium (Gao *et al.*, 2009). This finding has proved that TGF-β signalling plays a crucial role during enamel organ development. Nonetheless, there

are many more signalling pathways that can be involved in cell growth and proliferation such as mitogen-activated protein kinase, Smad, Wnt, Hedgehog and Notch signalling pathways (Derynck and Zhang, 2003; Moustakas and Heldin, 2005; Duronio and Xiong, 2013).



Modified figure reproduced from Hui and Friedman (2003)

Figure 2.2 The transforming growth factor-β (TGF-β) signalling pathway

2.5 Activin like kinase – 5 (ALK-5) inhibitor

ALK-5 inhibitor is a small molecule that has been developed to inhibit the TGF-β receptor type 1 (ALK-5). This molecule has been created for therapeutic use in cancer and other types of disease where overexpression of TGF-β1 is linked to a disease phenotype (Markell *et al.*, 2010). In this study, they found that pharmacological inhibition of TGF-β signalling also blocks tumour outgrowth in part through inhibition

of TGF-β dependent epidermal inflammation. However, they also found that the differential outgrowth of premalignant lesion with an inflammatory phenotype similar to squamous cell carcinoma following long-term treatment with SB431542 and at high risk for malignant conversion suggests that during tumour formation and premalignant progression may be different than on malignant tumours (Markell *et al.*, 2010). SB431542 has been commercialised and shown to be selective for the kinase activity of ALK-5 and to a lesser extent of activin signalling receptor ALK-4 and the nodal receptor ALK-7 (Mori *et al.*, 2004; Markell *et al.*, 2010). ALK-5 inhibitor plays a crucial role as a competitive adenosine triphosphate-binding site kinase inhibitor (Markell *et al.*, 2010; Yang *et al.*, 2016) and some researchers reported that it has been shown to inhibit the *in vitro* phosphorylation of Smad2 (Inman *et al.*, 2002) and Smad3 (Callahan *et al.*, 2002; Nyati *et al.*, 2011).

2.6 Epithelial cells

Epithelia consist of cohesive sheets of cells which line the exterior and interior surfaces of our bodies, constituting a mechanical and chemical barrier between the body and its environment (Roignot *et al.*, 2013; Le Bras and Le Borgne, 2014). These tissues play crucial roles in protection, filtration, absorption, excretion, and sensation (Kolahi and Mofrad, 2010).

Epithelia is composed of epithelial cells and there are several classifications of these cells based on the cell shape and number of layers; squamous cells which are thin and flat, cuboidal cells which are cubical to round, and columnar cells which are tall and cylindrical (Moini, 2015). Then, the number of cell layers represent by 1)

simple epithelium which is single layer cells which typically involved in absorption, filtration, and secretion, and 2) stratified epithelium consist of multi-layered of cells where can be found in body lining to withstand mechanical and chemical stress. Apart from that, pseudostratified epithelium is a simple columnar epithelial cell, giving the misleading impression as stratified epithelium when the cells were viewed in cross section since the nuclei appeared at different heights; and translational epithelium is a stratified epithelium where it can change the shape such as cuboidal or squamous stratified depending on the stretching of the cells since they have elastic properties (Moini, 2015). According to Wikramanayake *et al.* (2014), keratinocytes are specialised epithelial cells located closely to the basal layer and have been found abundantly in epithelial cells.

2.6.1 Keratinocytes

Human skin consists of three main layers which are subcutaneous tissue (stratified), underlying dermis, and cellular epidermis (Williams, 2003; McGrath *et al.*, 2004; Nelson *et al.*, 2011; Huber *et al.*, 2016). One of the components of epidermis layer includes keratinocytes which is located close to the basal layer to ensure its regeneration (Xavier Batista *et al.*, 2010). Keratinocytes have been known as specialised epithelial cells which are found abundantly in epithelial cells and these cells have been synthesised as major components of epidermal barrier through a series of differentiation process (Wikramanayake *et al.*, 2014). A typical intermediate filament (IF) protein, named keratins, are highly expressed in epithelial cells (Moll *et al.*, 2008) and these keratins are important for the mechanical stability and integrity of epithelial cells and tissues (Ramms *et al.*, 2013). In 1984, Steinert *et al.* (1984)

mentioned that there were two types of keratins; type I which was more acidic and differ from the basic type II based on their amino acid sequence. The type I keratin comprises of 28 keratin genes (K9–K10, K12–K28, and K31–K40) (including K33a and K33b) and type II consists of 26 keratin genes K1–K8 (including K6a, K6b and K6c) and K71–K86) (Moll *et al.*, 2008). In human genome, these keratin genes are designated as *KRT1*, *KRT2*, *KRT3*, etc. (Schweizer *et al.*, 2006). Other than that, several studies have also investigated the expression of epithelial markers such as E-cadherin (Van Roy and Berx, 2008), 2008), Occludin, Desmoplakin, Mucin1 (Strauss *et al.*, 2011), p63 (Yang *et al.*, 1998; Nair and Krishnan, 2013), and pan-cytokeratin (Fuertes *et al.*, 2013; Sidney *et al.*, 2015) in normal cells or cancerous cells. Hence, these epithelial markers have been used in wide range of research.

2.6.2 Keratinocyte Growth Medium

Keratinocyte Growth Medium (KGM) is a specific culture medium used to culture epithelial cells. This media has been proved by Nam and Lee (2009) where they could identify the keratinocyte cell properties cultured from a MSC of SHED through immunofluorescence technique and Reverse – Transriptase Polymerase Chain Reaction (RT-PCR). Taha *et al.* (2015) has also used this differentiation medium to culture SHED to investigate the expression of Notch signalling pathway molecules. Besides, some researchers also used KGM to culture Herwig's epithelial root sheath cells (Zeichner-David *et al.*, 2003; Farea *et al.*, 2013). Additionally, Sonoyama and his colleagues also used keratinocyte-serum-free medium to culture HERS cells (Sonoyama *et al.*, 2007). This medium is supplied without serum and bovine pituitary extract (BPE), have insulin, and growth supplements that are crucial to isolate and

growing the human keratinocyte cells. BPE acts as mitogenic supplement in serum-free growth medium (Kent and Bomser, 2003) which could induce mitosis and transformation (Xu *et al.*, 2013b).

2.7 Stem cell markers used in the analysis of SHED cultured in KGM treated with TGF-β1 and ALK-5 inhibitor

2.7.1 NANOG

NANOG is encoded by NANOG protein in human. It is a core transcription factor protein which critically involved in self-renewal of undifferentiated ESC and has emerged as one of the master regulators of stem cells pluripotency and differentiation (Boyer *et al.*, 2005; Kalmar *et al.*, 2009). Due to that, NANOG has been chosen as one of the stem cells marker and used by researchers in wide range of studies. There are several studies conducted to investigate the expression of NANOG based on its pluripotency (Abranches *et al.*, 2014), cancer cells (Park *et al.*, 2012; Shan *et al.*, 2012; Habu *et al.*, 2015), and chromatin organisation in mouse embryonic stem cells (Novo *et al.*, 2016). Other than that, NANOG collaborates with Sox2 and Oct4 to maintain the pluripotency of ESCs (Kalmar *et al.*, 2009). Overexpression of NANOG has been shown to support the self-renewal of cells, although the study was conducted in mouse ESC in the absence of LIF (Chambers *et al.*, 2003; Silva *et al.*, 2006).

2.7.2 Nestin

Nestin is a type VI IF protein which is also known as neural stem/progenitor cell marker (Guérette *et al.*, 2007). It has been found to express in undifferentiated central nervous system (CNS) cells during development, but also in normal adult CNS and CNS tumour cells (Suzuki *et al.*, 2010). The downregulation of nestin has been linked to the differentiation of cell into neurons or glial cells (Di *et al.*, 2014). Since nestin is an IF protein type, it has been shown to form homodimers and homotetramers structure, however nestin was unable to form IF protein *in vitro* without co-assembling with purified vimentin or type IV IF protein (Herrmann *et al.*, 2009; Thomsen *et al.*, 2013). Other than that, nestin has also been used as a stem cell marker since this protein emerges from neuroectoderm lineage (Noisa *et al.*, 2012). In addition, although the expression of nestin has been first reported in neuroepithelial stem cells (Lendahl *et al.*, 1990), it has been then be reported presence in other types of cells such as endothelial cells (Suzuki *et al.*, 2010), cancer cells (Krupkova *et al.*, 2010), fibroblast (Kishaba *et al.*, 2010), and other tissues such as tooth bud, testes, hair follicle sheath, skin, pancreas, and newly formed blood vessels (Zulewski *et al.*, 2001; Wiese *et al.*, 2004; Du *et al.*, 2011; Abe *et al.*, 2012).

2.7.3 Rex1

Rex1 protein has been associated with pluripotency of cells in undifferentiated ESC of both embryo and adult (Scotland *et al.*, 2009). Due to the differentiation process, the expression of Rex1 gene is downregulated (Scotland *et al.*, 2009; Glemzaite and Navakauskiene, 2016). Since *Rex1* expression has been showed to express specifically

related to pluripotency in mouse and human ES cells, this gene has been one of the well-known pluripotency marker, and has been used as a marker for multipotent adult progenitor cells and amniotic fluid cells (Masui *et al.*, 2008). Other than that, Shi *et al.* (2006) reported that NANOG is significantly associated with Rex1 since it is a transcription activator for Rex1 promoter. Thus, when NANOG is knocked out, the Rex1 expression was reduced, or Rex1 expression increased when the NANOG was stimulated. In addition, Son *et al.* (2013) reported that in human pluripotent stem cells, with depletion of Rex1, the cells lost their self-renewal capacity and their potential to differentiate, especially in their mesoderm lineage.

2.7.4 Vimentin

Vimentin is a protein that has been encoded as VIM gene in human. It is a type III IF protein which has been used widely as a marker for EMT during embryogenesis and metastasis (Mendez *et al.*, 2010). However, vimentin also is expressed in epithelial cells during the EMT under both physiological and pathological processes (Gupta and Massague, 2006), which has been supported in review by Kokkinos *et al.* (2007) and (Vechio *et al.*, 2011). Vimentin also acts as a critical player in the physiologic endothelial mechanical response towards endothelial phenotype (Conway *et al.*, 2013). Other than that, there is a study that suggests vimentin involvement in wound healing (Menko *et al.*, 2014) by regulating fibroblast proliferation, TGF-β1–Slug signalling, collagen accumulation, and EMT processing, where all these are required for keratinocyte activation (Cheng *et al.*, 2016).