ESTABLISHMENT OF 3D ORAL MUCOSA MODEL USING DIFFERENTIATED STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

NURUL HAFIZAH BINTI MOHD NOR

UNIVERSITI SAINS MALAYSIA

2020

ESTABLISHMENT OF 3D ORAL MUCOSA MODEL USING DIFFERENTIATED STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

by

NURUL HAFIZAH BINTI MOHD NOR

Thesis submitted in the fulfillment of the requirements

for the degree of

Doctor of Philosophy

May 2020

ACKNOWLEDGEMENT

Alhamdulillahi rabbil 'aalamin, peace and blessing be upon our beloved Prophet Muhammad S.A.W. and upon his family and companions. All thanks and praises to Allah, with His Blessing and Will, I managed to complete this research and thesis successfully. First and foremost, I would like to thank myself for being so strong, not quitting, and for no day-off in completing this Ph.D journey. A million thanks to my main supervisor, Dr. Zurairah Berahim for the precious guidance and advice, continually conveyed a spirit of adventure and excitement, as well as inspired me greatly to work in this research. My deepest appreciation also goes to my co-supervisor, A/P Dr. Thirumulu Ponnuraj Kannan who provided me with valuable information and willing to share the cherished knowledge, especially in writing part. Besides, the thank you goes to A/P Dr. Azlina Ahmad and A/P Dr. Khairani Idah Mokhtar@Makhtar for additional supervision. Not to forget, my family for whole support. The appreciation also goes to all laboratory colleagues and friends for insightful discussions, assistance, and guidance. Besides, I would like to thank the staff of Craniofacial Sciences Laboratory (PPSG), Central Research Laboratory (PPSP), Molecular Laboratory (PPSK) and Human Genome Centre for their assistance and providing me with good environments and facilities during my entire stay. I am also grateful to Encik Jamaruddin Mat Asan from Immunology Department (PPSP) for his valuable help in flow cytometry study. I also appreciate the financial support from the Ministry of Higher Education Malaysia for the scholarship given during my Ph.D study. Also, thanks to USM Research University Grant 1001/PPSG/812168 for the research funding as well as Toray Science Foundation, Japan for the grant award given. May Allah shower us all with His Blessing in this world and hereafter, *insha Allah.*

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celsius
µg	Microgram
µl	Microliter
µm	Micrometre
µM	Micromolar
µmol	Micromole
2D	Two-dimensional
3D	Three-dimensional
ADV	Average density value
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ASK1	Apoptosis signal-regulating kinase 1
BLAST	Basic local alignment search tool
BMP	Bone morphogenetic protein
BMMSCs	Bone marrow-derived mesenchymal stem cells
bp	Base pair
BPE	Bovine pituitary extract
BSA	Bovine serum albumin
CD	Cluster of designation
cDNA	Complementary deoxyribonucleic acid
CGC	Collagen-glycosaminoglycan-chitosan
CHO cells	Chinese hamster ovary cells

cm^2	Square centimeter
c-Met	Tyrosine-protein kinase Met (mesenchymal-epithelial transition factor)
CCN	CYR61 (cysteine-rich angiogenic protein 61 or CCN1), CTGF (connective tissue growth factor or CCN2), and NOV (nephroblastoma overexpressed or CCN3)
CNS	Central nervous system
CO_2	Carbon dioxide
COL1A1	Collagen type I
COS-1 cells	Monkey kidney fibroblast cells
CTGF	Connective tissue growth factor
Da	Dalton
DAPI	4′,6-diamidino-2-phenylindole
DDR2	Discoidin domain receptor 2
DEPC	Diethyl pyrocarbonate
DFPCs	Dental follicle progenitor cells
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPSCs	Dental pulp stem cells
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EPI	Epithelial-like cells
ERK	Extracellular signal-regulated kinase

et al.	And others
FACIT	Fibril-associated collagens with interrupted triple helices
FBS	Foetal bovine serum
FGF	Fibroblast growth factor
FIB	Fibroblast-like cells
FITC	Fluorescein isothiocyanate
FSP-1	Fibroblast-specific protein 1
g	Gram
GAG	Glycosaminoglycan
GMSCs	Gingiva-derived mesenchymal stem cells
H	Hour
H & E	Haematoxylin and eosin
HER	Human epidermal growth factor receptor
HERS	Hertwig's epithelial root sheath
HGF	Hepatocyte growth factor
HGFs	Human gingival fibroblasts
HLA	Human leukocyte antigen
HUVEC	Human umbilical vein endothelial cells
IGF1R	Insulin-like growth factor 1 receptor
IGF-II	Human recombinant insulin-like growth factor 2
IgG	Immunoglobulin G
IU	International unit
JNK	Jun N-terminal kinase
K	Keratin
KGF	Keratinocyte growth factor

L	Litre
LB	Lithium boric
MAPK	Mitogen-activated protein kinase
MEM	Minimum essential medium
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Ng	Nanogram
NGF	Nerve growth factor
NIH 3T3 cells	3-day transfer, inoculum 3×10^5 cells
Nm	Nanometre
OD	Optical density
OESCs	Oral epithelial stem cells
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PDGF	Platelet-derived growth factor
PDLSCs	Periodontal ligament stem cells
PGA	Poly (glycolic acid)
pH	Potential hydrogen
PI3K	Phosphatidylinositol 3-kinase
PLA	Poly (lactic acid)

PSCs	Periosteum-derived stem cells
r	Multiplication rate
rEGF	Human recombinant epidermal growth factor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse-transcription polymerase chain reaction
s	Second
S	Sedimentation velocity coefficient unit
SCAPs	Stem cells from the apical papilla
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGSCs	Salivary gland-derived stem cells
SHED	Stem cells from human exfoliated deciduous teeth
sqRT-PCR	Semi-quantitative reverse transcription-polymerase chain reaction
STAT	Signal transducer and activator of transcription
TE-7	Human thymic fibroblasts
TGF	Transforming growth factor
TGPCs	Tooth germ progenitor cells
USD	United States Dollar
V	Voltage
v/v	Volume/volume
α	Alpha
β	Beta

PENUBUHAN MODEL MUKOSA MULUT 3D MENGGUNAKAN SEL STEM DARIPADA GIGI SUSU MANUSIA TERKELUPAS YANG DIBEZAKAN

ABSTRAK

Mukosa oral merupakan sejenis tisu khusus yang melapisi rongga mulut. Ia terdiri daripada dua lapisan utama: epitelium skuamos berstratum dan lamina propria. Lapisan epitelium terdiri daripada sel-sel epitelium, manakala lapisan lamina propria kebanyakannya terdiri daripada fibroblas. Perkara yang dititikberatkan dalam mukosa oral *in vitro* adalah, pembentukan model ini hendaklah dijalankan mengikut seni bina ketebalan sepenuhnya menggunakan kedua-dua sel tersebut. Oleh itu, kajian ini bertujuan untuk membezakan sel stem daripada gigi susu manusia yang terkelupas (SHED) kepada sel seperti fibroblas dan epitelium yang seterusnya akan digunakan dalam pembentukan model mukosa oral 3D. Pembezaan SHED telah dijalankan dengan melibatkan faktor pertumbuhan, yang dinamakan faktor pertumbuhan tisu penghubung (CTGF) untuk pembezaan fibroblas, manakala faktor pertumbuhan keratinosit (KGF), faktor pertumbuhan epidermis (EGF), faktor pertumbuhan hepatosit (HGF), dan faktor pertumbuhan seperti insulin-2 (IGF-II) telah digunakan bagi pembezaan epitelium. Pencirian terhadap sel terinduksi dilakukan melalui pemerhatian morfologi, kadar proliferasi, analisis pengekspresan gen dan protein dengan menggunakan tindak balas berantai polimerase transkriptase balik selangkah secara semi-kuantitatif (sqRT-PCR), pewarnaan imunopendarfluor, dan sitometri aliran. Perancah kolagen-glikosaminoglikan-kitosan (CGC) telah dibina dengan

menggabungkan kolagen/kitosan/kondroitin sulfat/asid hialuronik (100/12/5/1) secara menyeluruh. Perancah berliang yang terhasil telah dicirikan melalui integriti penstrukturan, ketelapan, dan ketumpatan. Sel-sel terbeza yang telah dicirikan seterusnya dikultur bersama di atas perancah CGC untuk membentuk model mukosa oral 3D, yang kemudiannya dicirikan melalui histologi dan analisis imunopendarfluor. Keputusan menunjukkan kesan induktif faktor pertumbuhan terhadap sel seperti fibroblas dan epitelium yang terbeza daripada SHED. Sel seperti fibroblas secara morfologinya adalah sama dengan SHED, manakala sel seperti epitelium menyerupai sel epitelium asli. Analisis statistik menggunakan ANOVA sehala terhadap pengasaian proliferasi telah menunjukkan korelasi yang signifikan (p<0.05) di antara sel yang terinduksi dengan faktor-faktor pertumbuhan yang terlibat. Terdapat perbezaan yang signifikan dalam pengekspresan gen dan protein di antara SHED dengan sel-sel terbeza. Satu perancah CGC putih, liofilisasi berliang yang terhasil mampu mengekalkan integriti pengstrukturan dan ianya tidak mengalami degradasi sepanjang keseluruhan eksperimen. Perancah juga menunjukkan ketelapan dan ketumpatan yang baik. Sistem kultur bersama telah menunjukkan bahawa sel seperti fibroblas dan epitelium yang diperolehi daripada SHED berupaya untuk melekat dan membiak apabila dikultur di atas perancah CGC. Hasil pewarnaan hematoksilin dan eosin (H&E) terhadap model mukosa oral juga menunjukkan infiltrasi dan stratifikasi sel seperti fibroblas dan epitelium pada beberapa kawasan di dalam perancah CGC. Penghasilan kolagen turut dapat diperhatikan melalui pewarnaan Masson Trichrome. Pewarnaan imunopendarfluor terhadap sel seperti epitelium yang didapati di dalam perancah CGC membuktikan kehadiran sel tersebut. Oleh itu, penemuan ini telah menyediakan satu pemahaman baharu terhadap potensi SHED dalam pembentukan model mukosa oral bagi pembinaan semula tisu gigi.

ESTABLISHMENT OF 3D ORAL MUCOSA MODEL USING DIFFERENTIATED STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

ABSTRACT

Oral mucosa is a specialized type of tissue that lines the oral cavity. It consists of two main layers: stratified squamous epithelium and lamina propria. The epithelial layer is resided by the epithelial cells, while the lamina propria layer is majorly occupied by fibroblasts. As far as the *in vitro* oral mucosa is concerned, the construction of an oral mucosa model should be performed in full thickness architecture using both cells mentioned. Therefore, the present study aimed to differentiate stem cells from human exfoliated deciduous teeth (SHED) into fibroblast- and epithelial-like cells to be subsequently used in the establishment of a 3D oral mucosa model. The differentiation of SHED was carried out by the involvement of growth factors, namely connective tissue growth factor (CTGF) for fibroblastic differentiation, whereas keratinocyte growth factor (KGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF) and insulin-like growth factor-2 (IGF-II) were employed in epithelial differentiation, respectively. The characterisation of the induced cells was done by morphological observation, proliferation rate, gene and protein expression analyses using semi-quantitative reverse transcription-polymerase chain reaction (sqRT-PCR), immunofluorescence staining and flow cytometry. The collagen-glycosaminoglycan-chitosan (CGC) scaffold was constructed by combining collagen/chitosan/chondroitin sulphate/hyaluronic acid (100/12/5/1) thoroughly. The

porous scaffold produced was characterized via their structural integrity, porosity, and density. The characterized differentiated cells were then co-cultured on CGC scaffold to generate a 3D oral mucosa model, which was later characterized via histological and immunofluorescence analyses. The results demonstrated the inductive effect of growth factors in both fibroblastic and epithelial differentiation of SHED. SHED derived-fibroblast-like cells are morphologically similar to SHED, while SHED derived-epithelial-like cells resembled native epithelial cells. Statistical analysis using one-way ANOVA of the proliferation assay showed a significant correlation ($p<0.05$) between the induced cells and growth factors involved. There were significant differences in gene and protein expressions between SHED and both differentiated cells. A white, porous lyophilized CGC scaffold produced was able to maintain its structural integrity and did not degrade throughout the whole experiments. The scaffold also exhibited good porosity and density. The co-culture system showed that the fibroblast- and epithelial-like cells derived from SHED were able to attach and proliferate when being seeded on CGC scaffold. The haematoxylin and eosin (H&E) staining of the established oral mucosa model also exhibited the infiltration and stratification of the fibroblast- and epithelial-like cells in some regions within CGC scaffolds. Also, the production of collagen could be observed via the Masson Trichrome staining. The immunofluorescence staining of the epithelial-like cells grown in the CGC scaffold also supported the presence of those cells. These findings hence provide a new understanding on the potential of SHED in the establishment of oral mucosa model for dental tissue regeneration.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Oral cavity is a unique anatomical environment comprised of specialized tissues and fluids necessary for the initial food intake and speech, taste processing, as well as other sensory perceptions (McArthur, 1998). It refers to the space from the lips to the end of the hard palate (Courey and Pletcher, 2016). In the human body, the development of oral cavity occurs roughly about four weeks from the stomatodeum during the folding of the embryo in the head-tail line (Schroeder, 1991; Pelissier *et al.,* 1992; Nanci, 2017).

Anatomically, oral cavity is lined by a mucous membrane known as oral mucosa. Oral mucosa is comprised of two layers: (i) the stratified squamous epithelium (outer layer) which is attached to (ii) dense connective tissue/lamina propria (underlying layer) at the basement membrane. Both of these epithelial and dense connective tissue layers show different structural modifications in different regions of the oral cavity.

Going inside the structural modification of the epithelial layer, it is originally lined by a single layer of epithelial cells and gradually develops another two layers which takes approximately five to six weeks. Soon after, the extracellular fibres are secreted by the sparsely populated ectomesenchyme. By ten weeks, a multilayer of the epithelial cells is in a complete form (Pelissier, 1992; Winning and Townsend, 2000).

Deeper to the epithelial layer overlies the dense connective tissue, which is also known as the lamina propria layer. This layer provides support for the epithelial cell layer (Schroeder, 1991; Winning and Townsend, 2000). This connective tissue is originally from ectomesenchyme, particularly the neural crest cells that migrate from anterior rhombomeres and the midbrain to the relevant branchial arches and developing facial region (Johnston and Bronsky, 1995; Winning and Townsend, 2000).

As far as the mucosal defects are concerned, loss of integrity of the oral mucosa due to trauma a result of oral cavity tumour resections (Eckardt *et al.,* 2011), acute or chronic infections, diseases, injuries (Hafizah *et al.,* 2017), as well as cleft lip and palate is commonly reported particularly in the developing country. If this problem is left untreated, it could result in loss of water and proteins in the oral mucosa, leading to bacterial invasion in the oral mucosa (Liu *et al.,* 2010) and in due course, may cause the crucial dysfunction and aesthetic defect of the oral cavity.

Since decades ago, the oral mucosa defects have been reconstructed using guided tissue replacement, skin/autologous graft, vestibuloplasty (Izumi *et al.,* 2015), root coverage technique (Liu *et al.,* 2010), and many others. Unfortunately, the reconstructions have been challenged with the difficulty of finding an appropriate and acceptable source of the autologous grafts or transplantations. Therefore, as another alternative treatment, the development of oral mucosa *in vitro* using the tissue engineering approach has been extensively highlighted.

Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that

restore, maintain, or improve tissue function or a whole organ (Langer and Vacanti, 1993). It also denotes the development of a device in the laboratory, containing biological mediators (e.g. growth factors) and viable cells in a biological or synthetic matrix that could be implanted in patients to expedite the regeneration of specific tissues (Jaquery, 2007). Tissue engineering restores tissues that have been impaired either by trauma, injury, or disease by activating signal transduction pathways and mimicking the microenvironment. The regeneration of tissues should be engineered practically so that the regenerated tissues are as closely similar as native tissues in nature. Although researches involving this approach was carried out since early 1900s, the term "tissue engineering" was only officially coined at National Science Foundation workshop in 1988 (Akter, 2016). In 1991, the term was first recorded in an article entitled "Functional Organ Replacement: The New Technology of Tissue Engineering" in "Surgical Technology International" (Vacanti and Vacanti, 1991).

Until this date, abundant experimental and clinical studies have been done involving this approach. As far as the cost of therapy is concerned, as of the end of 2018, tissue engineering therapies have been marketed in the range of $400 in South Korea to $123,154 in Japan. The autologous cell therapies have cost around $61,500 in the United Kingdom to $169,206 in the United States. Whereas, gene therapies have been marketed around $5,501 for tonogenchoncel-L in South Korea and $1,398,321 for alipogene tiparvovec in Germany. Approximately $2,150 to $200,000 is for allogenic cell therapies in India and Canada, respectively (Shukla *et al.,* 2019). Although it is still not known how it will impact the regenerative dentistry field in the future, somehow with progressive approach in this field, it is expected to solve various health problems involving the regeneration of the destructed tissues within the next 25 years.

By focusing on the tissue engineering approach, the present study aimed on the differentiation of SHED with the involvement of growth factors to be grown on a scaffold-based 3D culture for the development of full thickness 3D oral mucosa model. The scaffold-based 3D culture is known to be anchorage-dependent featuring the cells to be embedded into the matrix. The growth of cells inside this scaffold-based 3D culture could provide an appropriate microenvironment for cell growth, optimal function, differentiation, as well as the ability to create tissue-like assemblies *in vitro.* In the present investigation, naturally-derived CGC scaffold has become a candidate of interest in the development of a 3D oral mucosa model as it has been shown to give out good fibroblasts-epithelial cells interactions and therefore, is able to produce a highly differentiated non-keratinized full thickness oral mucosa model with good multilayer stratified epithelium (Moharamzadeh *et al.*, 2007; 2012).

As far as the cell is concerned, the selection of SHED in the development of oral mucosa model is due to the fact that SHED did demonstrate the highest proliferation (Miura *et al.,* 2003) and differentiation capability (Jeon *et al.,* 2014) compared to that in other dental stem cells as well as human bone marrow-derived mesenchymal stem cells (BMMSCs). Moreover, the use of SHED provides an alternative to embryonic stem cells, where their use has been proved controversial.

The success of SHED differentiation in this study would enhance further knowledge on the SHED behaviour and their ability to be used in the future development of tissue-engineered oral mucosa model as well as in clinical applications where these cells are needed.

1.2 Justification of the study

In the oral cavity, reconstructions after tumour resection, vestibuloplasty, surgical closure of a cleft palate or treatment of gingival recessions require suitable grafting materials. Oral mucosa is limited in supply and the use of skin grafts in the oral cavity has some disadvantages. The keratinized surface of the grafted skin tends to macerate and is easily infected by fungi. Furthermore, hair growth may also occur after the transplantation into the oral cavity. Other problems include risk of donor site morbidity, inadequate tissue sources, cost, patient, surgical procedure, and time constraint. To overcome this problem, modelling of oral mucosa has been a major goal of the recent studies. Several *in vitro* tissue-engineered oral mucosa models have been developed, but no ultimate, standardized models are established neither for partial thickness nor full thickness oral mucosa models.

Apart from medical therapy, tissue-engineered oral mucosa models are being increasingly used to measure toxicity, drug delivery, as well as to investigate oral diseases. Currently, oral mucosa models are mainly comprised of normal oral keratinocytes cultured on top of a normal oral fibroblasts-containing matrix. However, the commercial supply of oral mucosa models is limited, restricting widespread use of these mucosa models. In addition, it also suffers from poor longevity and donor-to-donor variability.

Hence, this study, by using tissue engineering approach, embarked on the induction and assembly of SHED-derived-fibroblast- and epithelial-like cells in order to study their potential to be developed as an oral mucosa model. The success of differentiation

of SHED into fibroblast- and epithelial-like cells was expected to reduce the constraint of getting tissues from biopsies for oral mucosa reconstruction as well as eliminate the associated problems, thus making it a promising model in future regenerative dentistry.

1.3 Research objectives

1.3.1 General objective

This study aimed to establish a 3D oral mucosa model using fibroblast- and epithelial-like cells of SHED seeded on CGC scaffold.

1.3.2 Specific objectives

To achieve the aim of the study, several specific objectives were defined as follows:

1. To induce and characterize fibroblast-like cells from SHED by morphological and proliferation characteristic, gene and protein expressions.
2. To induce and characterize epithelial-like cells from SHED by morphological and proliferation characteristic, gene and protein expressions.
3. To construct and characterize scaffold using collagen, glycosaminoglycan, and chitosan (CGC).
4. To establish and characterize 3D oral mucosa model on CGC scaffold by co-culturing the fibroblast- and epithelial-like cells derived from SHED.

1.4 Research questions

1. Do SHED have the ability to differentiate into fibroblast-like cells?
2. Do SHED have the ability to differentiate into epithelial-like cells?
3. Can collagen, glycosaminoglycan, and chitosan be constructed into a good scaffold?
4. Do fibroblast- and epithelial-like cells derived from SHED have the ability to be co-cultured and exhibit oral mucosa architecture on CGC scaffold in the construction of a 3D oral mucosa model?

1.5 Research hypotheses

1. The fibroblast- and epithelial-like cells differentiated from SHED show significant differences in morphology, proliferation, gene and protein expressions compared to SHED.
2. It is possible to establish a 3D oral mucosa model using differentiated cells from SHED seeded on CGC scaffold.

CHAPTER 2

LITERATURE REVIEW

2.1 Anatomy of oral mucosa

Oral mucosa is a mucous membrane lining the oral cavity. Anatomically, it is located between the skin of the outer face and the mucosal lining of the gastrointestinal tract. It is about 500 mm in depth (Nanci and Ten Cate, 2003), with keratinized mucosa significantly thinner than non-keratinized mucosa (Gordon *et al.,* 1968; Markiewicz *et al.,* 2007). The structure of oral mucosa is more similar to the skin compared to any other mucosa in the body, which acts as a barrier against the external factors such as thermal, chemical, mechanical and biological damage (Izumi *et al.,* 2015), as well as protection from entry of toxic materials and microorganisms (Squier and Kremer, 2001) (Figure 2.1).

Oral mucosa is basically comprised of two main specialized tissue layers; the thick stratified epithelia overlying a thin lamina propria, in which they are divided by the undulating basement membrane (Figure 2.2). The epithelial layer forms the outer surface of oral mucosa which creates a barrier between the oral environment and deeper tissues. Therefore, when there is a disease or injury to oral mucosa, the epithelial layer is usually most affected. Underneath the epithelial layer is the lamina propria which consists of reticular and papillary layer. Structurally, reticular layer is prominent in lining mucosa whereas papillary layer is prominent in masticatory mucosa (Figure 2.2).

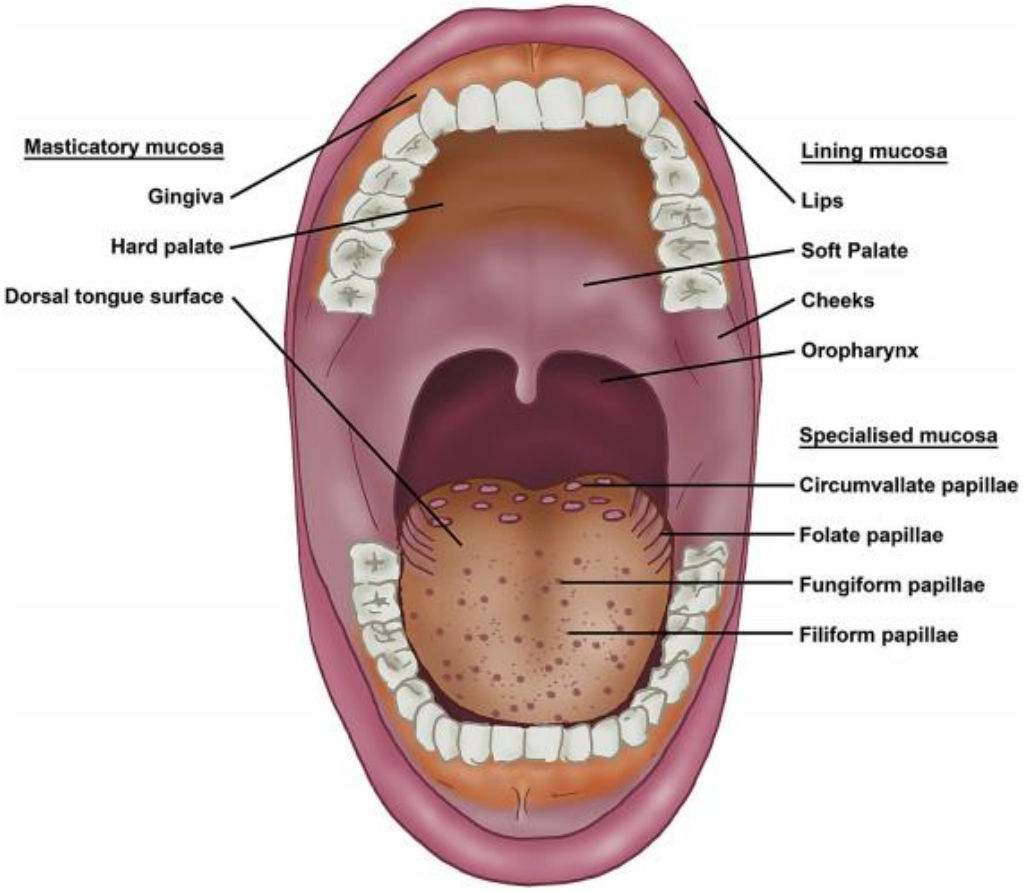

Figure 2.1 Anatomy of the human oral mucosa (Adapted from Cook *et al.*, 2017)

The structural modifications of the epithelium and connective tissue in distinct regions of the oral cavity provide three recognizable histological classifications, i.e. masticatory mucosa (keratinized mucosa), lining mucosa (non-keratinized mucosa), and specialized mucosa (both keratinized and non-keratinized mucosa). Although the keratinized mucosa is less permeable than the non-keratinized mucosa, however, it is still ten times more permeable than skin (Kinikoglu, 2010).

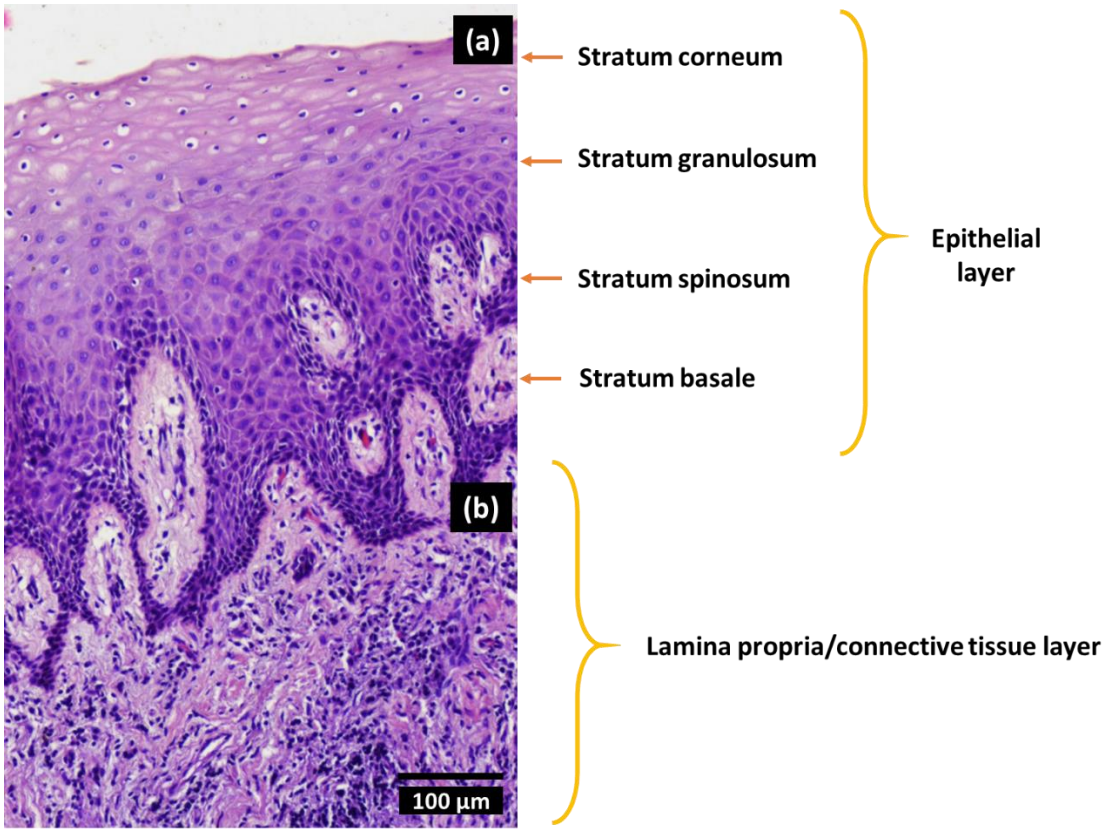

Figure 2.2 A micrograph showing the histology of oral mucosa. It consists of two main specialized tissue layers: a) the epithelial layer which is comprised of epithelial cells, and b) lamina propria layer which consists of nerves, blood, and lymphatic vessels, as well as multiple types of cells such as fibroblasts, defence cells, and other extracellular matrices (ECM). Scale bar = 100 µm. Magnification is 200X

The masticatory mucosa is the tough area which is involved with the mechanical forces during mastication, such as gingiva and hard palate. This area occupies 25% of the oral cavity (Collins and Dawes, 1987; Squier and Kremer, 2001) and is lined mostly by thick ortho-keratinized epithelium (Granado, 2012), although areas of para-keratinized epithelium might be seen. The lining mucosa, which is lined by soft and non-keratinized epithelium takes up 60% of the oral cavity (Collins and Dawes, 1987; Squier and Kremer, 2001). It covers the soft palate, alveolar processes, floor of mouth, under surface of tongue, as well as inside of cheeks and lips (Abdullah & Madeeha,

2015). This type of mucosa does not function in mastication and hence has little attrition.

The specialized mucosa is found at the dorsum of tongue and occupies 15% of the total cavity (Collins and Dawes, 1987; Squier and Kremer, 2001). This area is lined by both keratinized and non-keratinized epithelia depending on the keratinization that occurs on the papillae of the dorsum of the tongue. For example, the fungiform, circumvallate, and filiform papillae at the dorsal surface of the tongue are lined by keratinized epithelium, whereas the inter-papillary regions are lined by non-keratinized epithelium (Winning and Townsend, 2000). Although it is considered as masticatory mucosa by function, this type of mucosa is also categorized as specialized mucosa due to their cornified epithelial papillae (Granado, 2012) and high extensibility characteristic.

From this, it could be understood that although it covers oral cavity, there are variations in the types of oral mucosa which are based on their main role in oral cavity. Thus, in any case of tissue regeneration, the ideal replacement would be from tissue origin.

2.1.1 Epithelial layer

The epithelial layer of oral mucosa is comprised of the tightly packed epithelial cells originated from ectodermal embryonic germ layer (Figure 2.2a) (Kolltveit *et al.*, 2010; Queiroz *et al.,* 2010), except the tongue which arises from both ectoderm and endoderm (Winning and Townsend, 2000; Rothova *et al.,* 2012). They appear to be stratified squamous-shaped and small in size (Figure 2.3). Apart from the epithelial cells, there are several other types of cells existing in the epithelial layer including

Merkel cells, Langerhans cells, lymphocytes, as well as melanocytes (Winning and Townsend, 2000). The thickness of the epithelial layer varies depending on the location in the oral cavity. For example, the epithelial layer in the floor of the mouth is very thin, i.e. only 190 ± 40 μm, while the epithelial layer of the hard palate is about 310 ± 50 μm. Whereas, the cheek mucosa has the thickest epithelial layer compared to these two regions, which is around 580 ± 90 μm (Schroeder, 1981; Kinikoglu, 2010). The differences in the thickness of this epithelial layer could be due to the fact that the keratinized mucosa turnover is slower than that in the non-keratinized mucosa (Rowat and Squier, 1986).

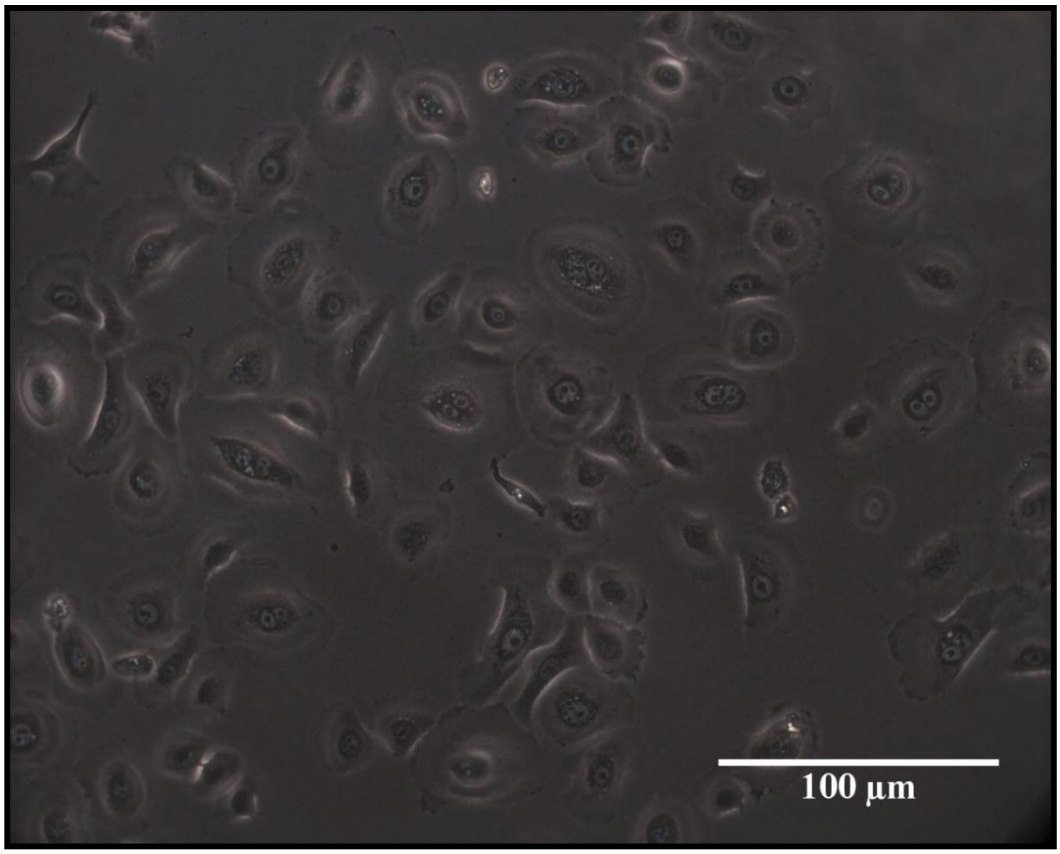

Figure 2.3 Morphology of the epithelial cells *in vitro*. The cells appear stratified squamous in shape and small in size. Scale bar = 100 μm. Magnification is 200X

The oral mucosa epithelial layer consists of four distinct layers with different degrees of differentiation, namely the stratum basale, stratum spinosum, stratum granulosum, and stratum corneum (Figure 2.2a). The stratum basale contains basal cells that are cuboidal in shape and smallest in size termed as basal cells. In this layer also resides a small population of the least differentiated, quiescent, and highly proliferative cells (Papini *et al.*, 2003; Calenic *et al.*, 2010). Cells from this layer divide, differentiate, and migrate towards the surface, into the stratum spinosum. As they move into the stratum spinosum, these cells increase in size and change in shape to appear 'prickly', hence are termed as prickle cells. The prickly appearance is due to high number of attachment that allows cells to interact with each other. These prickle cells migrate to the surface and become flatten, and the intensely staining granules at this layer give the characteristic appearance to stratum granulosum. These granules are known as keratohyalin granules. Finally, at or near the surface of the epithelium, as the granular cells migrate, they lose a lot of their structures and disintegrate into the oral cavity having the keratinized layer on the surface of oral mucosa as part of stratum corneum. However, this layer is absent in non-keratinized mucosa.

In view of keratinization, it is a maturation process that occurs in keratinocytes, a type of cells that are generated when the epithelial cells of the epithelial layer renew themselves and undergo cell turnover, which occurs approximately 25 days in the cheek and 41-75 days in the gingiva. In this process, the keratinocytes are produced by the mitotic division in the stratum basale and progress towards to the surface of the epithelium where they are shed off (Deo and Deshmush, 2018). The maturation could occur in different extents and types, which is either non-keratinization, para-keratinization (partially mature), or ortho-keratinization (fully mature) depending on

location in the oral cavity. Although these non-keratinized and keratinized epithelia are derived from the same germ layer, however, they express different sets of keratin, a protein that forms the intermediate filaments of cytoskeleton. For instance, the non-keratinized epithelia of oral mucosa express K4, K5, K13, K14, and K19, whereas the keratinized epithelia of oral mucosa express K1, K2, K5, K6, K10, K14, and K16 (Clausen *et al.,* 1986; Winning and Townsend, 2000; Moharamzadeh *et al.,* 2007; Kinikoglu, 2010).

With regards to their sources in *in vitro* studies, the epithelial cells could be collected from different areas in the body such as skin and oral mucosa biopsy (buccal mucosa, hard palate, and gingiva). Usually, the collection of these epithelial cells requires minor surgery which could be a painful and invasive process. However, they could also be obtained from other sources including adipose stem cells which could be induced to differentiate into BMMSCs, keratinocytes, umbilical cord stem cells, induced pluripotent stem cells (iPSCs), and embryonic stem cells (Liu *et al.,* 2011).

2.1.2 Lamina propria/connective tissue layer

The lamina propria or connective tissue layer is composed of numerous types of cells and fibres that are embedded in ground substances of glycoproteins and proteoglycans. This layer is majorly resided by fibroblasts, although defence cells (macrophages, lymphocytes), mast cells, plasma cells, blood vessels, nerves, as well as other ECM also exist (Figure 2.2b). The fibroblasts demonstrate the appearance of elongated cells with extended cell processes and thus, give out the spindle-like form (Figure 2.4). Their sizes range from 10-15 µm. They originate from neural crest (Enoch *et al.,* 2009)

and are known to be the least specialized type of connective tissue. They were also suggested to possess the foetal-tissue-like phenotypes (Sloan, 1991; Lee and Eun, 1999), which denote the foetal fibroblast subpopulations that have gone through the clonal expansion (Irwin *et al.*, 1994; Stephens *et al.*, 1996).

The fibroblasts function by depositing and degrading the collagen fibres, namely collagen type I, III, V, and VI in the ECM of connective tissues (Tomasek *et al.*, 2002; Driskell & Watt, 2015), in which the collagen fibres in the non-keratinized mucosa are thinner and less organized as compared to that in the keratinized one (Winning and Townsend, 2000).

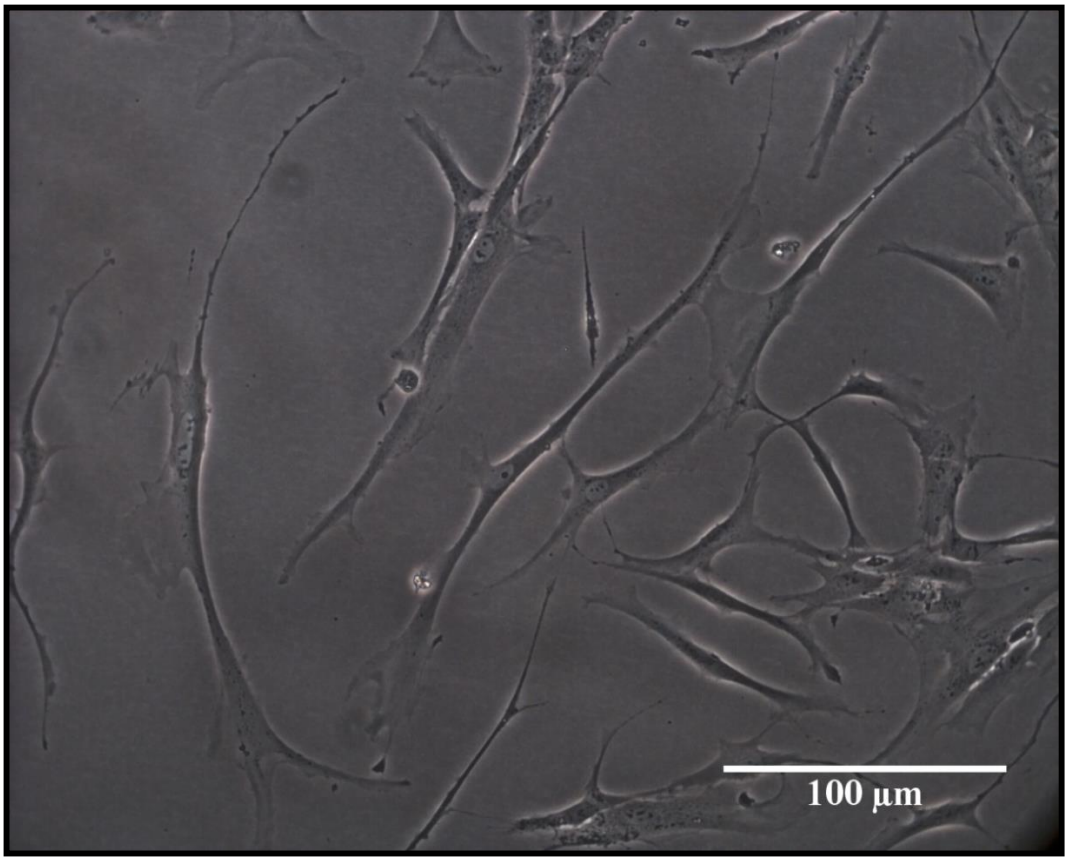

Figure 2.4 Morphology of the human gingival fibroblasts *in vitro*. The cells appear elongated and larger in size. Scale bar = 100 µm. Magnification is 200X

They also play a major role in homeostasis and wound healing of tissue (Häkkinen *et al.,* 2014). Fibroblasts facilitate the morphogenesis of the epithelium which is evidenced by a study that showed the absence of fibroblasts in the matrix terminated the proliferation of epithelial cells (Fusenig, 1994; Hafizah *et al.,* 2017) while continued the differentiation of epithelial cells (Smola *et al.,* 1998; Hafizah *et al.,* 2017). Moreover, they also influence the epithelial phenotypes and profile expressions of the cytokeratins with regard to their origin and nature. Any disturbance in activation and proliferation of fibroblasts could lead to the devastating circumstances, namely cancer and fibrosis (Kalluri & Zeisberg, 2006).

With regard to the use of these fibroblasts in cell culture study, there are no specific guidelines regarding the optimal passage range. However, a previous study reported that the fibroblasts were better to be used at early passage and less than 30 population doublings (Chen *et al.,* 2013). In fact, the use of early passage is important to mimic the *in vivo* environment more closely. Besides, the production of the ECM will decrease as the passage number of fibroblasts increases.

In addition, the late passage fibroblasts were reported to lead to the ageing of the cells, thus causing them to lose its proper functionality (Chen *et al.,* 2013; Kwist *et al.,* 2016). This phenomenon later will affect the proliferation rates, carrier-mediated transport activities, metabolic activities, cell densities, as well as transport and toxicity of exogenous and endogenous compounds (Briske-Anderson *et al.,* 1997; Ranaldi *et al.,* 2003; Hughes *et al.,* 2007). There will be decrement of RNA turnover with increment of intracellular content of RNA and protein as a result of reduced protein degradation by proteasome-mediated pathways. Also, there are changes in interactions

with the ECM or expression of secreted proteins which eventually increase the sensitivity of the cell contact (Cristofalo and Pignolo, 1993; Chen *et al.*, 2013).

2.2 Defects related to oral mucosa

Oral mucosa defects can affect people of any age, gender, and background. The majority of oral mucosa defect could primarily be due to the trauma, a result of oral cavity tumour resection (Eckardt *et al.,* 2011; Le *et al.,* 2014), cleft lip and palate, as well as chronic infection causing gingival defect such as in chronic periodontitis (Franz-Montan *et al.,* 2017). Any diseases or injuries to oral mucosa can lead to impairment of the oral functions and aesthetics. If left untreated, it will lead to the loss of integrity of the oral mucosa. Added to this problem, the limited capability of adult humans to regenerate after large tissue damage/loss will give greater impact to the structural dysfunction of the oral mucosa.

2.3 Treatment related to oral mucosa defects and development of oral mucosa model

Different approaches have been employed for the reconstruction of oral mucosa defects: from autologous/skin graft, epithelial sheet culture, to tissue-engineered three-dimensional (3D) oral mucosa model. For decades, oral mucosa defects have been reconstructed using autologous/skin grafts. This approach involved the cells collected from various parts of the oral cavity, including gingiva, hard or soft palate, and buccal surface. This approach was considered as the gold standard in craniofacial reconstruction (Cheng *et al.,* 2015), which used the cells from the same sources

(autologous), therefore it provided no risk of immune rejections. However, this model has been limited by tissue site morbidity since it leaves defects at the donor site, and it is difficult to harvest enough oral mucosa for reconstruction. In addition, the periodontal and oral maxillofacial surgeons have been frequently confronted with the problem of finding an acceptable and appropriate source of the transplantations or autologous grafts. The tendency of the skin grafts' keratinized surface to fungi infection, hair growth, and maceration following oral transplantation also need to be taken into consideration (Liu *et al.,* 2010). There are several other disadvantages including time constraint and cost problems (Izumi *et al.*, 2015). Another technique also used autologous tissue from the outside of oral cavity such as skin, however, it may not be able to lose its original characteristics and therefore, may give out its phenotype in the grafted site, e.g. the growth of hair in the oral mucosa where the skin tissue is grafted.

Due to those limitations, the researchers then started to fabricate the epithelial sheet. This model, which was established in dental researches since the 1990s uses only one type of cell layer, i.e. oral epithelial cells obtained from small oral biopsies. This approach is suitable especially in the study of the basic phenomena and biology of the oral mucosa, treatment of oesophageal ulcerations (Ohki *et al.*, 2006), as well as for cornea, trachea, skin and urinary bladder regeneration (Takagi *et al.*, 2012). The epithelial sheet culture made use of several techniques, including culturing on temperature-responsive culture dishes (Okano *et al.,* 1993), on human amniotic membranes (Nakamura *et al.,* 2003), and on collagen membranes (Imaizumi *et al.,* 2004). However, this approach showed several disadvantages like being prone to

contraction, was easily fragile, and difficult to manipulate with low engraftment rates (Feinberg *et al.,* 2005).

As an alternative treatment to overcome the limitations associated with the epithelial sheet culture, the oral mucosa model has been developed with the advancement of tissue-engineered 3D culture. This model has been developed for the purpose of *in vitro* studies of basic oral biological interactions, biocompatibility tests, understanding oral diseases and mucosal irritations, drug delivery studies, as well as for clinical applications. Oral mucosa model was developed prior to 2006, since then numerous studies have reported the development of 3D oral mucosa model with modification in cell sources, scaffolds, and media.

The architecture of tissue-engineered 3D oral mucosa model could be distinguished based on its thickness. The partial thickness model usually employs only the epithelial layer. This type of model is not suitable for advanced studies since it does not provide the anatomical structure of native oral mucosa. The structures obtained by partial thickness oral mucosa model form the basis for full thickness oral mucosa engineering. The full thickness model usually consists of epithelial and mesenchymal/connective layers. This model demonstrates a better simulation of the *in vivo* situation as it resembles normal oral mucosa as closely as possible. Several studies have reported successful assembly of full thickness human oral mucosa by culturing oral keratinocytes with fibroblasts on collagen (Moriyama *et al.,* 2001; Rouabhia and Deslauriers, 2002) or on the de-epidermized dermis (Cho *et al.,* 2000; Bhargava *et al.,* 2004). As far as the native oral mucosa composite is concerned, the construction of tissue-engineered 3D oral mucosa model in this study should be aimed to be in full

thickness architecture using the fibroblasts and epithelial cells to mimic the native oral mucosa.

2.4 Tissue engineering and its key elements

Tissue engineering is defined as an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ (Langer and Vacanti, 1993). It implies the construction of a device containing viable cells and biological mediators such as the growth factors in a synthetic or biological matrix that could be implanted in patients to facilitate the regeneration of particular tissues (Jaquery, 2007).

The basic principle of the tissue engineering triad has three pillars; the signalling molecules (growth factors), scaffolds (biomaterials), and cells (Mhanna and Hasan, 2017) (Figure 2.5). The process involves culturing cells on the biodegradable scaffolds in an optimal environment containing signalling molecules such as growth factor. Since each pillar in the triad of tissue engineering has a wide range of elements, the selection of appropriate scaffolds, signalling molecules, and methodologies is very critical since they will influence the functionality of the cells in producing the appropriate ECM.

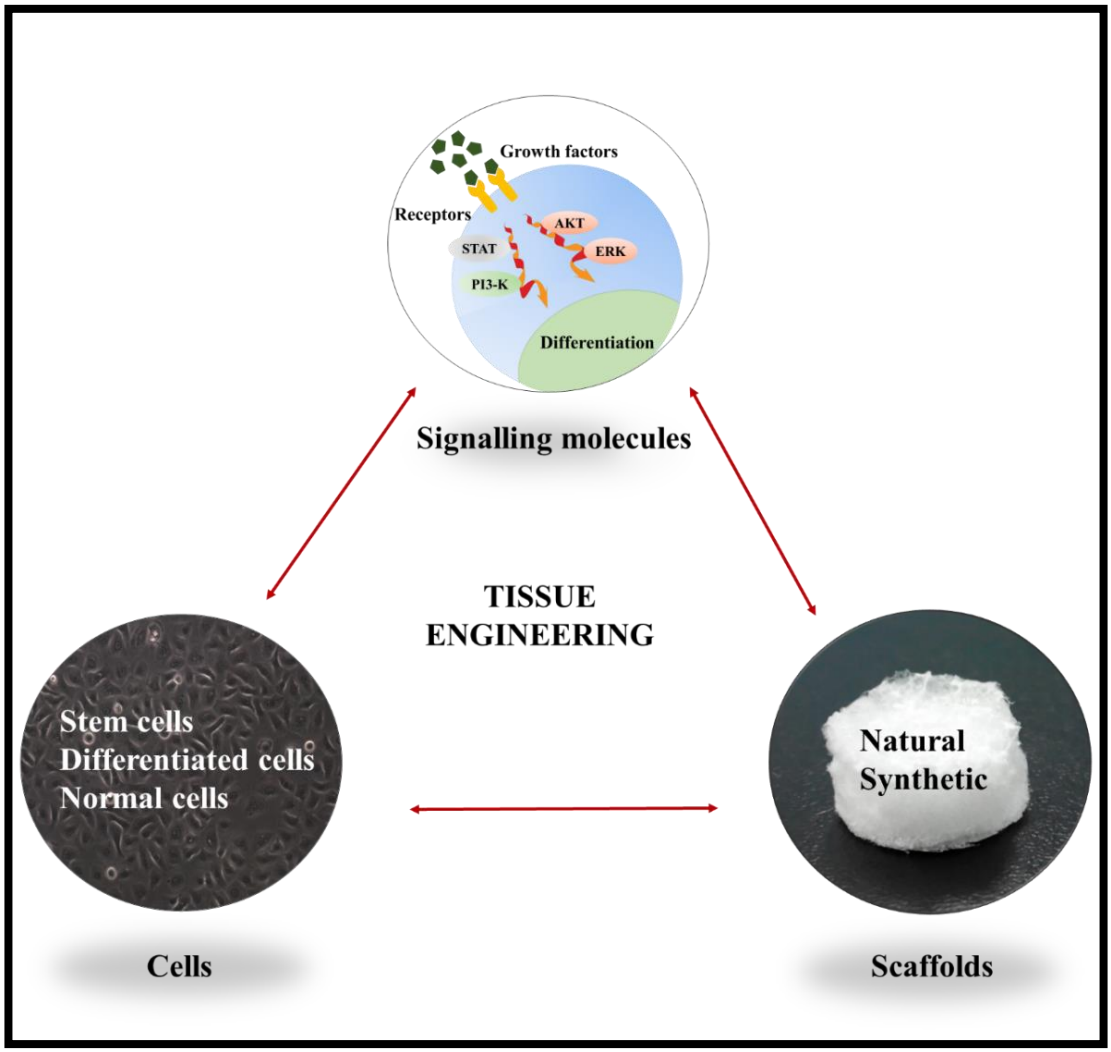

Figure 2.5 The triad of tissue engineering. The three pillars use combination of the signalling molecules (growth factors), scaffolds (biomaterials), and cells. The functionality of the cells could be influenced by the appropriate selection of scaffolds, signalling molecules, and methodologies employed

2.4.1 Signalling molecules

Signalling molecules such as growth factors are polypeptides derived from cytokine family (Goustin *et al.*, 1986) which bind to specific cell membrane receptors with high affinity (Sherbet, 2011). According to the previous studies regarding the mechanisms of biological and phenotypic effects of growth factors, there are no clearly established

grouping or classification of growth factors by far. However, based on several basic features, growth factors could be divided into several groups; (1) families, (2) species and (3) signalling pathways (Sherbet, 2011).

Growth factors generally function to up-regulate or down-regulate cell activities. These biochemical factors are responsible in the induction of cellular survival, inflammation, differentiation, growth, as well as tissue repair (Sherbet, 2011). They function as critical tools in regulating the neurite outgrowth, tissue morphogenesis, and angiogenesis. In addition, they also act as signalling molecules by means of binding to transmembrane receptors which contain cytoplasmic tyrosine kinase domains (Li and Hristove, 2010). The effect of this growth factor-receptor interaction sends morphogenic signals to the cells in order to stimulate the biological functions of cells.

Although the growth factors can be easily obtained and scaled up for major production, there are concerns regarding possible pathological side effects of the growth factors (Rose and Oreffo, 2002; Aravamudhan *et al.,* 2013). The long-term use may affect the efficacy, stability, and activity of these growth factors. This is due to the fact that the majority of the growth factors are synthesized in the prokaryotic systems such as *Escherichia coli* via recombinant DNA technology. Therefore, there is a difference in the synthesized growth factor in prokaryotic systems and the human body since the prokaryotic systems usually do not undergo the post-translational modifications, e.g. glycosylation of protein (Lee and Shin, 2007; Aravamudhan *et al.,* 2013).

Every cell type will respond differently to different growth factor, as they may have different signalling pathways to achieve certain actions. Due to this reason, the selection of appropriate growth factors is a critical factor for the induction of cell differentiation.

2.4.2 Growth factor for fibroblastic differentiation

To this date, there is dearth of studies reported on the differentiation of stem cells into fibroblast-like cells. Even to the best of available knowledge, there are only a few growth factors reported to be used in fibroblastic differentiation from various cells, such as CTGF (Lee *et al.,* 2006), EGF, and bone morphogenic protein-4 (BMP-4) (Hewitt *et al.,* 2011). The present literature focuses only on CTGF, for this growth factor was selected in this study.

2.4.2 (a) Connective tissue growth factor

CTGF is a member of the CCN cysteine-rich protein family. This 349-amino acid polypeptide (Bradham *et al.,* 1991), with a molecular weight of 38-kDa (Aikawa *et al.,* 2006) exhibits highly conserved disulphide bonding pattern (Holbourn *et al.,* 2008). It was first discovered in 1991 by Bradham and his colleagues through the screening of a HUVEC cDNA expression library using a polyclonal anti-PDGF antibody (Bradham *et al.,* 1991). Also, in the same year, the mouse CTGF (Fisp12/βIG-M2) was successfully isolated from transforming growth factor-beta (TGF-β)-stimulated mouse AKR-2B cells by Brunner *et al.* (1991), as well as from serum-stimulated NIH 3T3 cells by Ryseck *et al.* (1991) using differential cloning techniques, respectively.

Uniquely, CTGF does not behave like a cytokine or traditional growth factor, but more as a matricellular protein. This is because, this so-called CCN2 (Lipson *et al.,* 2012) does not bind to any specific receptors in order to transduce the signals. However, it does cause a change in cellular phenotype by modulating the cell-matrix interactions (Chen *et al.,* 2001; Shi-wen *et al.,* 2008).

2.4.3 Growth factors for epithelial differentiation

Within the epithelial cells, there is a collective process known as cellular migration which could influence the metastasis, development, remodelling, and wound healing (Khalil and Friedl, 2010). Such cellular cooperative movement is regulated by both biochemical signalling as well as physical interaction with neighbouring cells and underlying substrates, particularly intercellular stresses at cell-to-cell adhesion sites and traction forces at cell-substrate adhesion sites (Maruthamuthu *et al.,* 2011). Due to this reason, therefore, it is very crucial in this study to select the appropriate growth factors for the epithelial differentiation of SHED. Previous studies have reported the differentiation of SHED into epithelial cells using different means, such as cultured in serum-free KGF (Nam and Lee, 2009) and with the involvement of TGF-β1 (Azmi, 2017). As for this research, multiple combinations of growth factors were selected, namely KGF, HGF, EGF, and IGF-II.

2.4.3 (a) Keratinocyte growth factor

KGF belongs to a fibroblast growth factor (FGF) family with 30-45% homologous to other seven members of the FGF family (Finch *et al.,* 1989). This 18.9 kDa growth