EFFECT OF FIBROBLAST AND PLATELET-DERIVED GROWTH FACTORS ON CO-CULTURE OF HUMAN GINGIVAL FIBROBLASTS AND UMBILICAL VEIN ENDOTHELIAL CELLS

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UNIVERSITI SAINS MALAYSIA

2018

EFFECT OF FIBROBLAST AND PLATELET-DERIVED GROWTH FACTORS ON CO-CULTURE OF HUMAN GINGIVAL FIBROBLASTS AND UMBILICAL VEIN ENDOTHELIAL CELLS

by

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Thesis submitted in fulfilment of the requirement

for the degree of

Master of Science

November 2018

ACKNOWLEDGEMENTS

Allah (SWT), the Almighty God of all of us has sent us with a purpose and a mission so that we may reap benefit through this life, through whatever good we learn, and at the same time impart it to people with our whole might Allah has granted us with.

Any research is not an individual effort. It is a contributory effort of many hearts, hands and heads. In this work of mine, I am greatly indebted to my supervisors; Dr Zurairah Berahim, Associate Professor Dr Azlina Ahmad and Associate Professor Dr Thirumulu Ponnuraj Kannan, who have supported and praised me throughout my research, for their guidance and timely help, for encouragement and advice in every step.

I would like to express my special gratitude to Dr Nadia Aman and Dr Tayyaba Saleem, for their excellent guidance and suggestions in research writing and research studies, for always appreciating and standing side by me whenever I need help, and without whose motivation and encouragement, I would not have considered a graduate career in Periodontics research. I doubt that I will ever be able to convey my appreciation fully, but I owe them my eternal gratitude.

I am also highly obliged to Associate Professor Dr Wan Muhammad Amir for treating me always like a younger brother, for all the cheerful moments, motivation, valuable advices and constant support during my stay here in Malaysia. Thank you, Sir, for being there for me always.

I gratefully acknowledge my best friend, Muhammad Azeem, for his endless moral support, valuable advices and motivation during my studies and thesis writing. I am blessed to have him as a brother in my life. You really proved the exact meaning of the phrase; “A friend in need is a friend indeed.” Words cannot justify and express how I am thankful to you for everything.

Words fail me to express my deepest appreciation to Hassan Subhi and Victor Nna for being great friends, a constant source of support and inspiration. Their unselfish cooperation and much-needed help have helped me to complete my laboratory work.

I would also like to acknowledge all staff members at Craniofacial Science Laboratory whose assistance made me able to work smoothly, their timely help and guidance in every step to complete my research.

My sincere thanks go to Mr Abdul Ghani for his caring nature and good advices whenever I need help with administrative matters. Sir, I am grateful in every possible way.

I would also like to thank the members of my ‘cabin-crew’ in IPPT; Hafizah, Hamsha, Wafa, Fuad, Faiz, Shafiq, Arif, Adawiyah, Naziah, Ayman, Athiya, Ching, Hazrini, Vivian, Siti, Abdallah, Belal and Rigzin for being good friends and constant support of inspiration.

I am very thankful to a group of fellow students and friends in Block A, International House, specially, Dr Asrar, Dr Jawaad, Dr Tahir, Dr Syed Mudassar, Dr Farooq, Dr Zahoor, Dr Usman Rashid, Ali, Khaled, Dr Riaz, Dr Rehan (late), Dr Mahmood, Dr

Shahid, Dr Irfan Ullah, Dr Badr, Dr Asif Ul Islam, Dr Anas, Dr Sohaib, Dr Saleh, and Dr Mustafa who was always willing to help and give me their valuable suggestions.

Special thanks go to my family. Words cannot express how grateful I am to my mother, father, and siblings for all the sacrifices that they've made on my behalf and make it possible for me to achieve a level of higher education. I also cannot forget the love of my dear grandfather; Abba Jee, my late grandmother; Ammi Jee, my late Nana Abbu and my late Nani Ammi Jan. Your prayers for me was what sustained me thus far. Thank you for supporting me for everything, and especially I cannot thank you enough for encouraging me throughout this experience. Last but not the least, I would like to dedicate this thesis to all my lovely nephews and niece; Ahmed, Hamza, Maryam, Ismayial and Mustafa.

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ACE	Angiotensin-converting enzyme
AC-LDL	acetylated low-density lipoprotein
ALP	Alkaline phosphatase
Ang	angiopoietins
ANOVA	Analysis of variance
α-MEM	Alpha modified eagle's medium
Bp	Base pairs
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSC	Biosafety cabinet
BMP	Bone morphogenetic proteins
CO_2	Carbon dioxide
COL1A1	Collagen, type I, alpha 1
CD-31	Cluster of differentiation-31
cm^2	Square centimetre
C_T	Threshold cycle
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
D-PHI	Degradable/ polar/ hydrophobic/ ionic polyurethane
DPEC	Diethyl pyrocarbonate
°C	Degree Celsius
ECM	Extracellular matrix
EGF	Epidermal growth factor
ECs	Endothelial cells

epiCs	Epithelial cell system
EPC	Endothelial progenitor cells
EGM™-2	Endothelial cell growth medium-2
EBM™-2	Endothelial cell basal medium-2
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
et al	And other workers
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FGFR	Fibroblast growth factor receptor
FM	Fibroblast medium
FN	Fibronectin
FGS	Fibroblast growth supplement
FSP-1	Fibroblast specific protein-1
GFs	Growth factors
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HARP	Heparin affin regulatory peptide
HDMECs	Human dermal microvascular endothelial cells
HPDLFs	Human periodontal ligament fibroblasts
hFBs	Human primary fibroblasts
HEPES-BSS	Hydroxyethyl-piperazineethane-sulfonic acid- buffered saline solution
HGFs	Human gingival fibroblasts
HUVECs	Human umbilical vein endothelial cells
HBMFs	Human bone marrow-derived fibroblasts
HBMEC	Human bone marrow endothelial cell line

HO	HGF only
HHUO	HGF co-cultured with HUVEC only
HHUGF	HGF co-cultured with HUVEC and GFs (FGF-2 and PDGF-BB)
HUO	HUVEC only
HUHO	HUVEC co-cultured with HGF only
HUHGF	HUVEC co-cultured with HGF and GFs (FGF-2 and PDGF-BB)
iPS	Induced pluripotent stem cells
IGF	Insulin-like growth factor
IL	Interleukin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide
MMP	Matrix metalloproteinase
Ml	Millilitre
Mg	Milligram
mM	Millimolar
µm	Micrometre
µg	Microgram
µl	Microliter
Nmol	Nanomole
Ng	Nanogram
NTC	No-template control
OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDGF-BB	Platelet-derived growth factor-BB

PDGFR-α	Platelet-derived growth factor receptor-α
PDGFR-β	Platelet-derived growth factor receptor-β
PDL	Periodontal ligament
Pen-Strep	Penicillin-streptomycin
RT-PCR	Reverse transcriptase-Polymerase chain reaction
RPM	Revolutions per minute
RNA	Ribonucleic acid
R^2	Correlation coefficient
RT	Room temperature
Std. Error	Standard error
TE	Tissue engineering
TGF-β	Transforming growth factor-β
TNS	Trypsin neutralizing solution
2D	Two-dimensional
3D	Three-dimensional
V	Voltage
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
VE-CAD	Vascular endothelial-cadherin
VIM	Vimentin
v-WF	Von-Willebrand factor
S.E.M	Standard error of the mean
UK	United Kingdom
USA	United States of America
USM	Universiti Sains Malaysia

KESAN FAKTOR PERTUMBUHAN FIBROBLAST DAN TERBITAN PLATELET KE ATAS KO-KULTUR SEL FIBROBLAS GINGIVAL DAN ENDOTELIAL VENA UMBILIKAL MANUSIA

ABSTRAK

Banyak jenis sel tunggal dalam kultur *in-vitro* telah digunakan dalam kejuruteraan tisu, tetapi kajian mengenai interaksi parakrin langsung di antara populasi sel heterotip adalah kurang. Pendekatan ko-kultur mewujudkan atmosfera yang sangat baik untuk mengkaji interaksi ini. Objektif kajian eksperimen *in-vitro* ini adalah untuk menentukan kesan faktor pertumbuhan fibroblast (FGF-2) dan terbitan platelet (PDGF-BB) dalam ko-kultur sel fibroblas gingival manusia (HGF) dan sel endothelial vena umbilikal manusia (HUVEC). Untuk tujuan ini, medium yang sesuai untuk pertumbuhan sel dalam teknik ekalapis dan ko-kultur perlu dioptimumkan terlebih dahulu. Selepas itu, kepekatan optimum faktor-faktor pertumbuhan ini ditentukan dalam ekalapis dan digunapakai untuk pertumbuhan di dalam ko-kultur kedua-dua sel. Keberkesanannya dinilai dengan menggunakan ujian MTT. Seterusnya, analisis ekspresi gen untuk penanda-bio HGF dan HUVEC ditaksir menggunakan ujian RT-PCR untuk mengkaji kesan stimulasi faktor-faktor pertumbuhan dalam ko-kultur HGF dan HUVEC. Seterusnya, penilaian statistik ke atas hasil kajian dilakukan menggunakan ujian ANOVA satu arah dan Kruskal-Wallis dengan p <0.05 dianggap signifikan secara statistik. Keputusan ujian MTT menunjukkan bahawa kesan FGF-2 kepada HGF bergantung kepada dos dan optimum pada kepekatan 5 ng/ ml (p = 0.001), manakala PDGF-BB keatas HUVEC adalah optimum pada kepekatan 20 ng/ml (p = 0.004). Kesan stimulasi FGF-2 dan PDGF-BB terhadap HGF dan HUVEC disokong oleh keputusan analisa RT-PCR yang menunjukkan bahawa,berbanding

kumpulan kawalan, terdapat peningkatan gen penanda-bio yang signifikan (p <0.05) dalam kumpulan rawatan kedua-dua sel selepas tiga hari diko-kultur. Oleh itu, disimpulkan bahawa kemungkinan terdapat kesan sinergistik kedua-dua faktor pertumbuhan pada ko-kultur HGF dan HUVEC yang mempunyai potensi mencetuskan aktiviti proangiogenik.

Kata kunci: Ko-kultur, FGF-2, PDGF-BB, Fibroblas gingival manusia, sel-sel endothelial vena umbilik manusia, Kejuruteraan tisu, PCR masa nyata

EFFECT OF FIBROBLAST AND PLATELET-DERIVED GROWTH FACTORS ON CO-CULTURE OF HUMAN GINGIVAL FIBROBLASTS AND UMBILICAL VEIN ENDOTHELIAL CELLS

ABSTRACT

Numerous types of single cells in *in-vitro* cultures have been studied in tissue engineering, but the study on direct paracrine interactions between heterotypic cells population is lacking. Co-culture approach establishes an excellent atmosphere to study these interactions. The objective of this *in-vitro* experimental study was to determine the effects of fibroblast and platelet-derived growth factor ((FGF-2 and PDGF-BB) in a co-culture of human gingival fibroblasts (HGFs) and human umbilical vein endothelial cells (HUVECs). To this end, the medium for the establishment of monolayer and co-culture of these cells were first optimised. Thereafter, the optimal concentrations of these growth factors were determined in a monolayer and then in a co-culture medium by assessing the cell viability using MTT assay. Next, gene expression analysis for fibroblast and angiogenic biomarkers was assessed using real-time RT-PCR to study the stimulatory effect of these growth factors by using 6 well-plate with transwell inserts. Afterwards, statistical analysis of the results was performed using one-way ANOVA and Kruskal-Wallis test with $p < 0.05$ considered statistically significant. Results of cell viability assay showed that the effect of FGF-2 on HGF was dose-dependent and was optimum at a concentration of 5 ng/ml (p =0.001), while that of PDGF-BB on HUVEC was optimum at a concentration of 20 ng/ml (p =0.004). The stimulatory effect of FGF-2 and PDGF-BB on HGF and HUVEC was supported by the real-time RT-PCR results which showed that there is a significant upregulation ($p < 0.05$) of gene biomarkers in the treatment group of both

cells after three days of co-culture experiment, compared to control group. Therefore, it is concluded that there is the possibility of a synergistic effect of these two growth factors on a co-culture of HGF and HUVEC which were suggestive of a proangiogenic activity.

Keywords: Co-culture, FGF-2, PDGF-BB, Human gingival fibroblasts, Human umbilical vein endothelial cells, Tissue engineering, Real-time RT-PCR

CHAPTER 1

INTRODUCTION

1.1 Background of the study

The periodontium consists of specialised tissues that surround and support the tooth. These include root cementum, periodontal ligament, alveolar bone and gingiva. The gingiva consists of two specific tissue types namely an outer gingival epithelium and underlying fibrous connective tissue (Cho and Garant, 2000). Oral soft tissue deformities of which gingival recession is more prevalent affects more than 20% of adults in First World countries (Kassab and Cohen, 2003; Susin *et al.*, 2004; Sarfati *et al.*, 2010). Gingival recession is defined as an apical shift of the gingival margin, causing exposure of the root surface of a tooth (Jati *et al.*, 2016). Traditional approaches being tailored to treat the lost tissues usually include the use of tissue grafts. However, they are often limited by certain drawbacks such as lack of adequate blood supply, insufficient amount of available donor tissue to cover the recession area and high-cost (Chambrone *et al.*, 2010; Tonetti and Jepsen, 2014). To repair or regenerate the damaged/lost gingival connective tissues, the concept of gingival tissue engineering has emerged as a promising treatment and has generated significant interest in the factors and cells that regulate their formation and maintenance.

Gingival tissue consists of collagen and blood vessels. Fibroblast and endothelial cell are the common cells in this tissue. Endothelial cells (ECs) are the most widely distributed cell type in the human body and forms the inner cellular lining of the entire

vascular system (Cines *et al.*, 1998). Fibroblasts, on the other hand, play an essential role in the angiogenic process through their production of extracellular matrix (ECM) molecules (Newman *et al.*, 2011). In addition, fibroblast releases essential angiogenic growth factors (GFs) such as transforming growth factor-β (TGF-β) (Paunescu *et al.*, 2011), vascular endothelial growth factor (VEGF) (Kellouche *et al.*, 2007) and platelet-derived growth factor-BB (PDGF-BB) (Antoniades *et al.*, 1991). The growth of these cells *in-vitro* requires the addition of exogenous molecules such as GFs that are known to stimulate the proliferation and differentiation of these cells.

Growth factors are a group of naturally occurring polypeptides that are capable of initiating and transmitting distinctive cellular responses in a biological milieu (Babensee *et al.*, 2000; Lee *et al.*, 2011). The unique response triggered by GFs signalling can result in a diverse range of cell actions, including cell survival, and control over migration, differentiation or proliferation of a specific cells subset (Tayalia and Mooney, 2009; Brochhausen *et al.*, 2010). Successful tissue growth often relies on the delivery of GFs to cells within regenerating tissues (Tabata, 2003) and, hence, they play a pivotal job in tissue engineering strategies (Nimni, 1997; Kaigler *et al.*, 2006). Numerous GFs are known for their ability to actively regulate various functions of cells in regeneration and *in-vitro* culture. Of these, the fibroblast growth factor-2 (FGF-2), insulin-like growth factor (IGF), epidermal growth factor (EGF), PDGF, VEGF, and TGF-β appear to have an important role in oral tissue repair and reconstruction (Chen and Jin, 2010). Among these, PDGF-BB and FGF-2 are known to play vital roles in endothelial and fibroblast activity (Dereka *et al.*, 2006; Li *et al.*, 2017). They also support cell proliferation and migration, thus enhancing the formation of cell-cell

connections in a dose-dependent manner (Battegay *et al.*, 1994; Sukmana and Vermette, 2010).

To explore the cellular based strategy on cell reactions towards certain stimuli, many *in-vitro* culture experiments using one type of cell have been conducted. However, to study the interaction between more than one cell (direct or in-direct interactions), the concept of heterotypic culture (also known as co-culture system) has been established (Alfaro-Moreno *et al.*, 2008; Paschos *et al.*, 2015; Kimura *et al.*, 2017). In the co-culture system, apart from the paracrine factors released by the cells, exogenous molecules such as GFs can be added (Rodrigues *et al.*, 2010b). Using a co-culture approach, the focus of the current study is to investigate the interaction of endothelial cells with gingival fibroblast. In this study, we evaluated the effect of FGF-2 and PDGF-BB on the co-culture of human gingival fibroblasts (HGFs) and human umbilical vein endothelial cells (HUVECs).

1.2 Justification of the study

In tissue engineering, two options have been widely used by researchers when vascularising tissue-engineered constructs. Either the tissue-engineered construct implant *in-vivo* whereby host microenvironment majorly guide vascularization or *in-vitro* organisation/culture of cells under controlled conditions focussed in order to develop functioning vascular network before implantation. The latter strategy offers more control as researchers can modify and optimise parameters under specific conditions prior to implantation. In most tissue-engineered constructs, vascularisation is achieved by using ECs. Moreover, apart from ECs, different cells population have

been used within the same culture environment depending upon the tissue of interest. Co-culture systems have long been used to study the communication between different cell populations and are fundamental to cell-cell interaction studies of any kind. Previously, *in-vitro* pre-vascularization has been achieved in a co-culture approach using different cells population, for example, a study has been done using dermal fibroblasts and HUVEC in a co-culture system for microvascular maturation (Sukmana and Vermette, 2010). However, there is a limited knowledge on the interaction of the cells in a co-culture system especially between HGF and HUVEC, which is very important to understand angiogenesis, specifically in gingival tissue. Apart from using heterotypic cell population in a co-culture, exogenous molecules such as GFs are used to achieve stable and mature vasculature within a construct (Buranawat *et al.*, 2013). FGF-2 and PDGF-BB are known to play important roles in fibroblast and EC activity, however, there is a dearth of information in the literature that assesses the effect of these two angiogenic GFs on an *in-vitro* co-culture of HGF and HUVEC. Using the tissue engineering technology, this preliminary study will provide further understanding and aid in developing functional tissue graft for gingival regeneration.

1.3 Objectives of the study

1.3.1 General Objective

To study the effect of exogenous GFs; FGF-2 and PDGF-BB on the co-culture of HGFs and HUVECs.

1.3.2 Specific Objectives

1. To optimise the culture medium for the establishment of monolayer and co-culture of HGF and HUVEC.

2. To determine the optimal concentration of FGF-2 and PDGF-BB for HGF and HUVEC culture respectively, by assessing the cell viability.

3. To determine the gene expression levels of fibroblast biomarkers i.e. Collagen, type 1, alpha 1 *(COL1A1)*, Fibronectin *(FN)*, and Vimentin *(VIM*) and angiogenic biomarkers i.e. Cluster of differentiation-31 *(CD-31)*, Von-Willebrand factor *(v-WF)*, and Vascular endothelial-cadherin *(VE-CAD)* on a co-cultured HGF and HUVEC with FGF-2 and PDGF-BB.

1.4 Research hypothesis

1. Addition of FGF-2 and PDGF-BB have a significant effect on the viability of HGFs and HUVECs in a monolayer cell culture, respectively.

2. Combination of growth factors (FGF-2 and PDGF-BB) significantly expressed the gene expression levels of fibroblast biomarkers (*COL1A1, FN, and VIM*) and angiogenic biomarkers (*CD-31, v-WF, and VE-CAD*) in a non-contacting co-culture system of HGF and HUVEC, respectively.

CHAPTER 2

LITERATURE REVIEW

2.1 The human gingiva

The periodontium is a complex structure, consisting of hard and soft connective tissues. The primary functions of the periodontium are to provide structural support at the interface between teeth and jaw and to serve as a protective barrier against the microbes of the oral cavity (Katancik *et al.*, 2016). The hard-connective tissues comprise of the cementum and the alveolar bone whereas the soft connective tissues include the gingiva and the periodontal ligament (PDL) (Fig. 2.1-A) (Schroeder, 1986). The part of gingiva that facing the oral cavity is covered by the gingival epithelium which is capable of continuous renewal (Mackenzie and Tonetti, 1995; McKeown *et al.*, 2003).

Microscopically gingiva is composed of a stratified squamous epithelium and a dense network of collagenous lamina propria (connective tissue) that includes the supra-alveolar fibre apparatus, blood, lymphatic vessels and nerves (Fig. 2.1-B) (Melcher and Bowen, 1969; Taba *et al.*, 2005). The epithelium of the gingiva depicts some morphological and regional variations that show tissue adaptation to the tooth and underlying alveolar bone (Schroeder, 2012). These include outer (oral) epithelium also called gingival epithelium, sulcular epithelium and junctional epithelium. The gingival epithelium faces the oral cavity and extends from gingival margin to the mucogingival junction. Thereby, it covers the outer surface of the free gingiva and the attached gingiva. The non-keratinized sulcular epithelium lines the gingival sulcus and acts as a protective

layer to prevent the entry of injurious bacterial products. The gingival sulcus is a shallow groove/space between the sulcular epithelium and tooth surface and encompasses the newly erupted tip of the crown. It is bound apically by the coronal aspect of the junctional epithelium, laterally by the sulcular epithelium, and medially by tooth surface, and superiorly exits into the oral cavity. The junctional epithelium is firmly attached to the enamel (or cementum in gingival recession) and composed of a collar-like band of the stratified squamous non-keratinizing epithelium. It acts as an epithelial barrier against plaque-bacteria and protects the underlying periodontal ligament from invasion by noxious substances. Thus, plays an extremely significant role in periodontal health and disease (Nanci, 2013). Together, the sulcular epithelium and junctional epithelium form the dentogingival junctional tissue. The epithelial layer of the gingiva is inflexible, tough, resistant to abrasion and tightly bound to the underlying lamina propria through hemidesmosomes and a basement membrane, which consists of type IV collagen, laminin, and fibronectin (Moharamzadeh *et al.*, 2007). The junctional epithelium is supported by the supracrestal connective tissue fibres of the gingiva. Clinically, healthy vestibular gingiva consists, on average, of 4% junctional epithelium, 27% oral gingival epithelium and 69% connective tissue that includes a cellular infiltrate occupying about 3-6% of the gingival volume (Schroeder *et al.*, 1973).

The human gingiva is also known to be rich in cellular niche and composed of a variety of cells including epithelial cells (keratinocytes) which are the main resident of gingival epithelium and is responsible for protecting the underlying connective tissues (Schroeder, 1986). Besides, fibroblasts are the main cell type residing in the lamina propria, along with ECs, pericytes, nerve cells, and a small number of macrophages, mast cells, monocytes and lymphocytes (Schroeder, 1986; Moharamzadeh *et al.*, 2007).

Recent studies showed that the lamina propria also contains a novel mesenchymal stem cells population that can serve as a replacement source for the fibroblasts (Marynka-Kalmani *et al.*, 2010; Fawzy El-Sayed and Dörfer, 2016; Venkatesh *et al.*, 2017). From the underlying connective tissue of the lamina propria to the surface of the gingiva, the keratinized epithelium consists of four distinct layers i.e. the basal layer, the prickle cell layer, the granular layer and the keratinized layer. Each layer depicts specific arrangement of cells and plays significant role in epithelial maturation. Figure 2.2 shows the schematic representation of cells in the different layers of gingival epithelium and lamina propria (connective tissue).

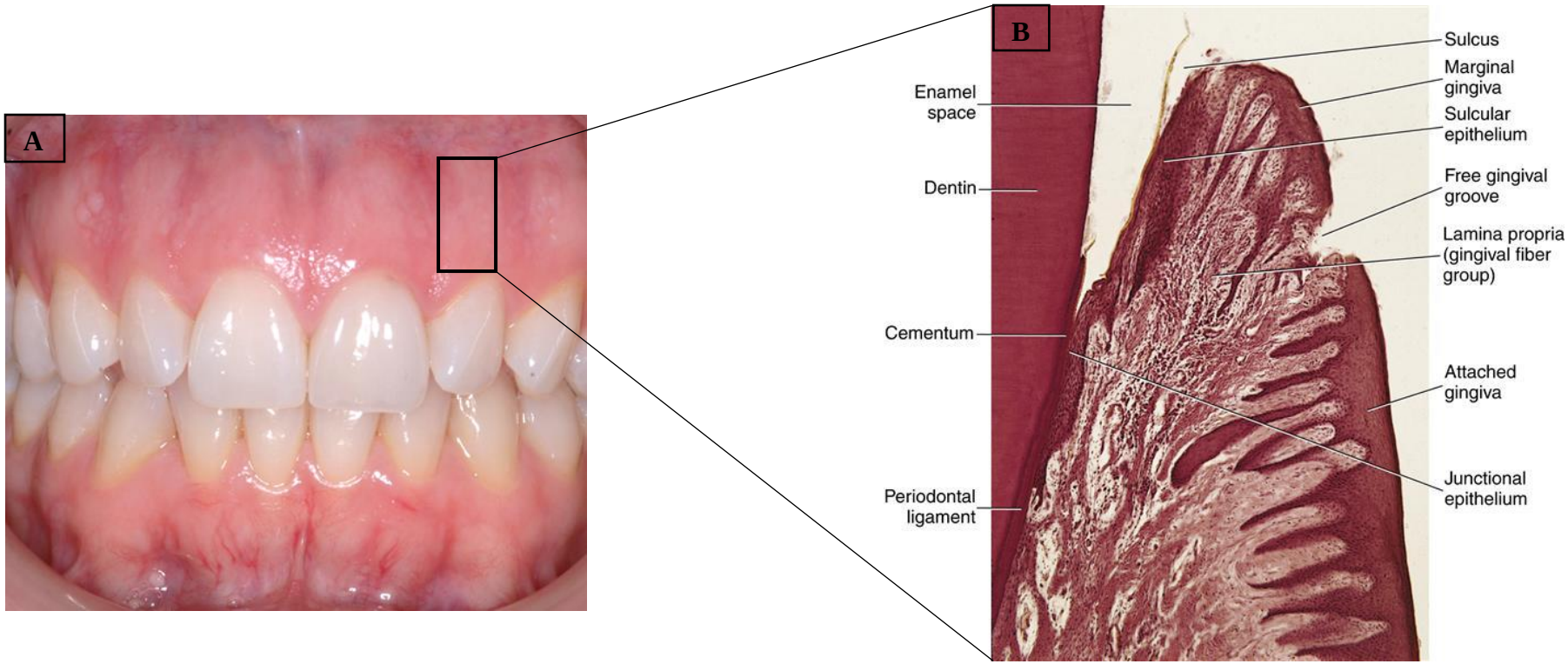

Figure 2.1: (A) A photograph of a clinically healthy human gingiva; (B) A photomicrograph of the cross-section of gingival tissue (Fehrenbach and Popowics, 2015). The gingiva is covered by oral epithelium, deep to the epithelium is the underlying lamina propria, which is continuous with the periodontal ligament that anchor the tooth to the alveolar bone.

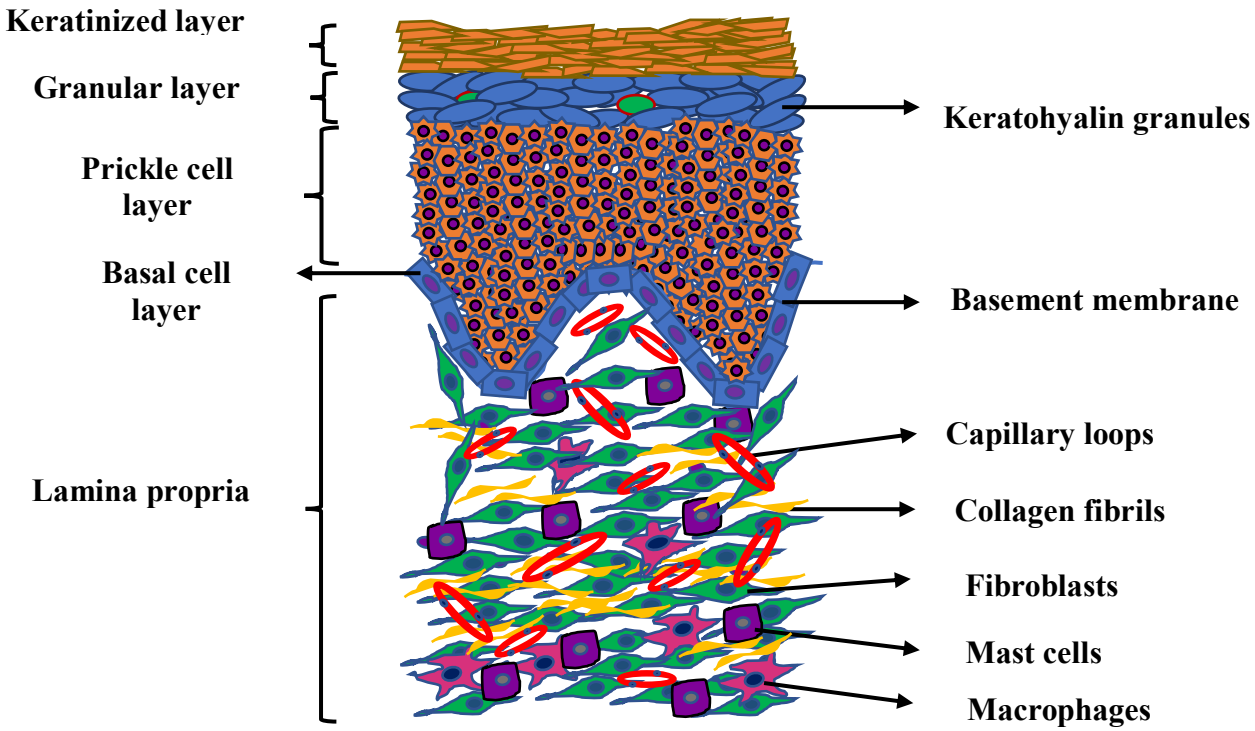

Figure 2.2: Schematic representation of cells in gingival epithelium and lamina propria. The gingival epithelium is a stratified squamous epithelium consisting of cells tightly attached to each other and arranged in several distinct layers. The keratinized layer comprises essentially of keratin proteins along with few flat squamous cells in which all organelles have been lost. The granular layer consists of larger flattened cells containing small granules called keratohyalin granules. Next to this layer is prickle cell layer which consists of larger ovoid cells with membrane-coating granules. Adjacent to the lamina propria is a basal layer which consists of cuboidal or columnar layer of cells (mostly consists of Melanocytes, Merkel cell and Langerhans cell). Most of the cell divisions occur in this layer. The epithelium is tightly bound to the underlaying dense connective tissue (lamina propria). The lamina propria consists of several different cells (fibroblasts, macrophages, endothelial cells, mast cells), wide capillary loops, neural elements and anchoring fibrils (e.g. collagen fibrils; mostly type I and III collagen). Adapted from (Nanci, 2013).

2.2 Gingival recession

Chronic inflammation of the periodontium may cause the gingiva to recede and expose the root surface (Fig. 2.3). Gingival recession is highly predominant (Sarfati *et al.*, 2010) and is defined as "an irreversible displacement of the gingival margin apical to the cementoenamel junction causing exposure of the root surface of a tooth" (Chambrone *et al.*, 2010; Graziani *et al.*, 2014; Tonetti and Jepsen, 2014). The exposed root surface may be associated with hypersensitivity, non-carious cervical lesions and root caries etc. (Chambrone *et al.*, 2010). This gingival condition if left untreated may also lead to tooth loss, and it has a negative impact on the quality of life with regards to impaired aesthetics due to the appearance of elongated teeth and pain due to hypersensitivity. The multiple causative factors in gingival recession include chronic trauma, tooth malalignment, alveolar bone dehiscence, frenum pull, ageing, and smoking, etc. (Graziani *et al.*, 2014; Jati *et al.*, 2016).

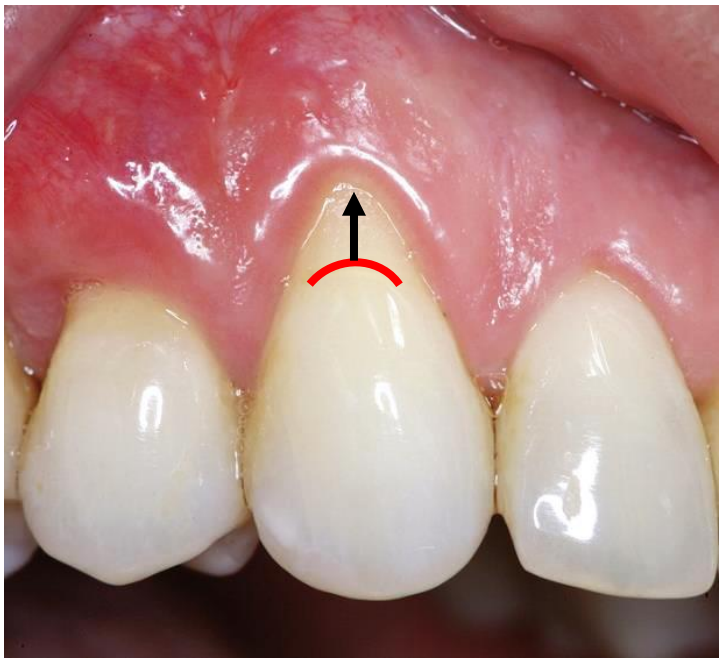

Figure 2.3: A tooth with gingival recession. The red curve on the canine depicts the actual position of a healthy gingiva margin. The black arrow shows the apical shift of the gingival margin causing exposure of the root surface.

2.2.1 Current treatment for gingival recession

Treatment for gingival recession involves various methods including laterally positioned flap, coronally advanced flap, guided tissue regeneration with membranes, soft connective tissue grafts, free gingival grafts, acellular dermal matrix, enamel matrix derivative, platelet-rich plasma, or combination techniques (Pierpaolo and Giovanpaolo, 2012; Hofmanner *et al.*, 2012; Aroca *et al.*, 2013; Graziani *et al.*, 2014). Among these, the connective tissue grafts are widely used and considered as a "gold-standard" due to its high predictability (Ricci *et al.*, 1996; Roccuzzo *et al.*, 2002). Soft connective tissue grafts are usually harvested from the palate and transplanted at the recession area to replace the receding tissue (Thoma *et al.*, 2014; Thoma *et al.*, 2016). Besides the root coverage is achieved, these grafts are not fully sufficient to regain the physiological functions and coupled with certain limitations. These limitations include; lack of adequate vascularization, limited amount of available donor tissue and demand of a second surgical site, resulting in additional trauma to the patient and associated risks such as pain, infection, donor-site morbidity and risks of rejection by the patient's immune system (Hughes *et al.*, 2010; Chen and Jin, 2010; Chambrone *et al.*, 2010; Amini *et al.*, 2012). The study of Rastogi and co-workers (2009) demonstrated that tissue grafts from the oral mucosa can potentially cause secondary defects which cannot be closed; these opened defects are highly susceptible to bacterial infections in the moist oral cavity (Rastogi *et al.*, 2009). Collagen (Mucograft) and acellular (AlloDerm) matrices have been used by clinicians as an alternative to the tissue grafts but the clinical outcome (e.g. complete root coverage) was not significantly promising when compared with tissue grafts itself (Cardaropoli *et al.*, 2012). Pertaining to the disadvantages of current treatments, tissue-engineered

constructs are currently being explored in the field of biomedical engineering, however, desirable biocompatibility and bio-functionality still need to be explored.

2.3 Gingival tissue engineering

Tissue engineering (TE), first described in the late 1980s, is a field that is contributing to the regenerative medicine. This area covers the principles of autologous, allogenic and syngeneic cell transplantation, biomaterials sciences, and engineering to develop a substitute of biological origin that can help in the restoration, maintenance and improvement of normal tissue functions (Berthiaume *et al.*, 2011). TE aims to regenerate functional tissues and organs with the help of certain key tools including cells, GFs or signalling molecules, and biomedical scaffolds (Nerem, 1991; Galler and D'Souza, 2011). Advancement in the field of TE has transformed the concept of two-dimensional (2D) to three-dimensional (3D) tissue reconstruction that has found its reliable applications in both *in-vitro* and *in-vivo* studies.

When comes to gingival TE, the goal is to treat the gingival tissue defect by using tissue-engineered constructs manufactured *ex-vivo*. Later, these tissue-engineered constructs can be implanted back to the lost/diseased site to restore the anatomy, physiology, mechanical properties and aesthetic nature of the gingiva that existed before the damage (Taba *et al.*, 2005; Saxena, 2008). Vascular TE encompasses the use of appropriate cells, cellular interactions using biologically active molecules and microvasculature to deliver oxygen and nutrient supply (Moharamzadeh *et al.*, 2007; Chen *et al.*, 2010). The regeneration of gingiva involves two layers of tissues which is the epithelial layer and the connective tissue (gingiva lamina propria) layer.

The epithelial layer consists essentially of keratin proteins surrounded by lipids which along with other proteins (involucrin, loricrin and trichohyalin) formed the keratinocytes. Apart from keratinocytes, non-keratinocytes also present which includes Langerhans cells, Merkel's cells, melanocytes and inflammatory cells including lymphocytes. Cells from the epithelial layer are continuously shed and replaced by the underlying layers which shows that this layer is capable of self-renewal (Mackenzie and Tonetti, 1995; McKeown *et al.*, 2003) and progressive maturation (Berkovitz *et al.*, 2016). On the other hand, the gingival lamina propria is highly vascular and contains wide capillary loops, several different cells population including fibroblasts (principal cell of the lamina propria), ECs, histiocytes, mast cells, macrophages as well as an ECM comprised of collagenous and non-collagenous proteins (Bartold and Narayanan, 2006; Moharamzadeh *et al.*, 2007). A recent study has revealed that vascularity of gingival lamina propria can be achieved by co-culturing fibroblasts and ECs (Cheung *et al.*, 2015). Section 2.5.1 discussed the co-culture of these cells in detail.

2.4 Cells for gingival tissue engineering

Recent advancements in tissue engineering technology have enabled the development of cell-based therapeutics that aimed at achieving the regeneration of oral soft tissues with greater efficacy and predictability (Lin *et al.*, 2009; Chen *et al.*, 2012). In this context, a variety of cell types, including fibroblasts (Scanlon *et al.*, 2011), osteoblasts progenitor (Yu *et al.*, 2017), bone marrow mesenchymal stem cells (Yang *et al.*, 2010), and dental follicle cells (Guo *et al.*, 2012) have been shown to promote regeneration of gingival tissues to various degrees in *in-vitro* and *in-vivo* models.

To develop functional vascular grafts, many studies have been done using endothelial and smooth muscle cells (Bhattacharyya, 2012; Wang *et al.*, 2014; Kolster *et al.*, 2017). ECs are the building block of the vascular system and expected to form functional capillary networks in the tissue construct (Song *et al.*, 2015). On the other hand, fibroblasts play an essential role in the angiogenic process through their production of ECM molecules (Newman *et al.*, 2011). The culture of these cells was conducted in monolayer and co-culture approach or both to study the process of vascularisation in tissue-engineered constructs. These cells are normally obtained from the biopsies of the oral tissues during surgeries. In this present study, which intended to broaden our knowledge of gingival tissue engineering, we used HGFs and HUVECs and are discussed in subsequent sections.

2.4.1 Fibroblasts

Fibroblasts are mesenchymal cells, commonly found in connective tissue that is usually characterised by their morphology and the secretion of the components of the ECM for tissue maintenance and repair (Hinz, 2007; Wipff and Hinz, 2009). Apart from their role as synthesisers and modifiers of the ECM, fibroblasts have a strong potential to induce an angiogenic response in the culture (Eckermann *et al.*, 2011). Numerous angiogenic GFs (VEGF, TGF-β and FGF-2), as well as matrix proteins (collagen I, fibronectin, and proteoglycans), are known to be secreted by these cells that have been shown to modulate EC sprouting and the expansion of capillary-like networks *in-vitro* (Berthod *et al.*, 2006; Kunz-Schughart *et al.*, 2006; Newman *et al.*, 2011). Gene expression analysis study revealed that fibroblasts are quite different cells, depending on their tissue of origin (Grant *et al.*, 1989) and each cell represent its own genetic makeup. For example, expression of fibronectin, vimentin, fibroblast

specific protein (FSP-1), hyaluronic acid, COL1A1 are characteristics gene biomarkers studied for human gingival fibroblasts (Mohd Nor *et al.*, 2017).

2.4.1 (a) Human gingival fibroblasts (HGF)

Typically, there are three potential sources where fibroblasts can be harvested in the oral cavity for the regeneration of gingival connective tissue. These include the gingiva itself (Jin *et al.*, 2012), the periodontal ligament (Giannopoulou and Cimasoni, 1996), and the dental pulp (Buurma *et al.*, 1999). From these, gingiva is the easiest source for fibroblast due to its superficial location and greater distribution. HGFs are the major cell type of the gingival lamina propria. They are known to contribute towards the pathogenesis of periodontal disease in the inflammatory periodontium by an exuberant secretion of inflammatory mediators, matrix metalloproteinases, and cytokines (Daghigh *et al.*, 2002; Moharamzadeh *et al.*, 2007). HGFs are the common cell type used for assessing the biocompatibility of implant prosthesis in the orofacial region (Jin *et al.*, 2011; Ma *et al.*, 2011), for populating *in-vitro* models of gingival connective tissues (Blackwood *et al.*, 2008), soft tissue constructs (Chung *et al.*, 2009), and can be a source of induced pluripotent stem cells (iPS) for periodontal tissue engineering (Egusa *et al.*, 2010; Fournier *et al.*, 2010; Wang *et al.*, 2011; Fournier *et al.*, 2013; Ferré *et al.*, 2014).

When compared human periodontal ligament fibroblasts (HPDLFs) with HGF, several investigators have shown that the morphology and growth rates of both types of fibroblasts are similar (Somerman *et al.*, 1988; Ohshima *et al.*, 1988; Somerman *et al.*, 1990; Chou *et al.*, 2002). However, their functional characteristics differ a little. An *in-vitro* study has been done by Giannopoulou & Cimasoni (1996) to study the

functional characteristics of both cells. It has been shown that collagen types I and IV promoted the attachment of HGF, while gelatin, laminin, and vitronectin promoted that of HPDLF. Moreover, most ECM components increased the proliferation rate of HGF and the biosynthetic activity of HPDLF. When compared biochemical markers, it has found that they are similarly distributed between the two cell types, except for alkaline phosphatase, which was greater in a cellular extract of HPDLF. Table 2.1 shows the important parameters being used for the characterisation of HGFs.

In this study, Fibronectin *(FN)*, Collagen, type 1, alpha 1 *(COL1A1)*, and Vimentin *(VIM)* were used as a fibroblast biomarker. Fibronectin is a type of non-collagen glycoprotein with an important bioactivity that appeared as a fibrillar structure in the lamina propria of the healthy gingiva (Manimegalai *et al.*, 2016). Collagen, type 1, alpha 1 is a characteristic collagen type of the hard tissues that has been demonstrated by thick collagen fibres in the alveolar bone and in the gingival connective tissue (Romanos and Bernimoulin, 1990). Vimentin is the intermediate filament protein of mesenchymal cells, abundantly found in subgingival connective tissue (Mussig *et al.*, 2005). Usually, expression of these proteins is linked to support and facilitate cellular attachment and communication by activating signalling pathways and serve as a functional unit to maintain the periodontal attachment (Albelda and Buck, 1990; McCulloch *et al.*, 2000).

Table 2.1: Parameters for the characterisation of human gingival fibroblasts (Mohd Nor *et al.*, 2017)

Growth characteristics	Metabolism	Genetic makeup
• Spindle-shaped morphology having elongated cytoplasmic projections and nucleus • Lower growth rate but proliferation rate is higher	• Show reduced p38 but not extracellular signal-regulated kinase phosphorylation • Greater expression of *COL1A1* • Increase expression of matrix metalloproteinases (MMP) -1,-3 and -10 • Increase TGF-β and VEGF-α expression • Lower ALP expression	• Greater expression of cell-cycle regulatory proteins and metabolism-related proteins • Osteoblastic differentiation through the expression of osteonectin, osteopontin and bone sialoprotein • Expression of vimentin and fibroblast-specific protein (FSP-1)

2.4.1 (b) Culture of HGFs

HGFs culture in different matrices (such as collagen, fibrin or 3D scaffold) has shown promising results in soft tissue regeneration (Jhaveri *et al.*, 2009; Rodrigues *et al.*, 2010a; Maia *et al.*, 2011) and exhibit greater functional and biochemical activity *in-vitro* such as increased cell adhesion, cell number and total protein count (Pelegrini *et al.*, 2013). Mariotti and Cochran (1990) compared the growth characteristics and macromolecular synthesis of HGF and HPDLF. They reported that in *in-vitro* cell culture, HGF showed higher proliferative rate, total protein content and grew more rapidly than HPDLF. However, the distribution of glycosaminoglycan, hyaluronic acid, and heparin was more dominant in the cellular segment of PDL tissue, which is indicative of fibroblasts heterogeneity.

In another study by Yoshino *et al.* (2003), the relationship between mechanical stress and biochemical phenomena on angiogenic stimulator and inhibitor has been studied with HGFs and HPDLFs. It has been shown that when cultured on a flexible substrate (flexible-bottom elastomer coated with type I collagen), there is an increased production of VEGF by both cells ($P < 0.01$). Adherence and proliferation of HGFs on polyglactin matrices (Bio-Gide and Ethisorb tamponade) has been studied to understand the effect of specific biomaterial on gene expression analysis (Hillmann *et al.*, 2002). It has been shown that after 4-weeks of *in-vitro* culture, cells were able to express type I collagen, bone morphogenetic proteins (BMP) -2, -4, -7, the BMP type I and the type II receptor. Moreover, they also revealed that static seeding favours (as the significantly higher number of cells observed) the adherence and proliferation of

primary gingival cells on these biodegradable matrices which could serve as a valuable tool for periodontal tissue engineering (Hillmann *et al.*, 2002).

2.4.2 Endothelial cells

ECs are known to be the major cellular resident of the entire vascular system (arteries, veins, and capillaries). They form a continuous lining at the interface between blood and tissue and are present in all blood vessels. Due to its unique strategic position at the interface between the blood and the tissue, it plays a vital role in providing the proper haemostatic balance. ECs from various sources (retinal, foreskin, umbilical vein, aortic and human coronary artery etc.) have been used for promoting angiogenesis and vasculogenesis *in-vitro* (Bouis *et al.*, 2001; Vailhe *et al.*, 2001; Zheng *et al.*, 2012; Morin and Tranquillo, 2013; Heiss *et al.*, 2015) and *in-vivo* (Fràter-Schröder *et al.*, 1987; Cao *et al.*, 1998; Ribatti and Vacca, 1999; Donovan *et al.*, 2001; Staton *et al.*, 2009).

Among the mature EC types, HUVECs and human dermal microvascular ECs (HDMEC) are the most widely used cells in the tissue culture experiments (Unger *et al.*, 2007; Bidarra *et al.*, 2011). Besides its crucial role in providing the lining of the vessel walls, ECs also exhibit certain essential functions. They are known to be involved in the blood coagulation cascade (thrombosis and thrombolysis), platelet-blood vessel interaction, and act as a potential source of growth promoters (PDGF, endothelin-1, thrombin, FGF-2, and interleukin-1 (IL-1) and inhibitors (heparin sulphates, nitric oxide, TGF-β) (Rudijanto, 2007; Rajendran *et al.*, 2013). The migratory and proliferative capacity of ECs is regulated by these factors that play a

vital role in the regulation of vascular growth. Thus, the endothelial layer can regulate and help in vascular tone and growth (Verhamme and Hoylaerts, 2006; Rajendran *et al.*, 2013).

The ability to identify and distinguish ECs in culture is based on the structural and functional properties of these cells *in-vitro* and *in-vivo*. ECs display a distinctive pattern of growth in culture and possess many typical ultrastructural features such as typical cobblestone appearance and formation of capillary tube-like structures angiogenesis assays (Table 2.2). The typical markers for identification include expression of v-WF, CD-31, angiotensin-converting enzyme (ACE), prostacyclin production, and uptake of acetylated low-density lipoprotein (AC-LDL). Table 2.2 shows the important parameters being used for the characterisation of ECs.

Cluster of differentiation- 31 *(CD-31),* Von-Willebrand factor *(v-WF),* and Vascular endothelial cadherin *(VE-CAD)* were used as an angiogenic biomarker for ECs in this study. Cluster of differentiation- 31 is a glycoprotein known to be used as an EC specific marker and is localised to cell-cell borders of confluent monolayers and, in addition, to lumen-facing areas of blood vessels or tube-like endothelial structures formed *in-vitro* (Ilan *et al.*, 2000). Von-Willebrand factor is a multimeric plasma glycoprotein synthesise specifically by ECs that mediates platelet adhesion to both the subendothelial matrix and endothelial surfaces and acts as a carrier for coagulation factor VIII in the circulation (Sporn *et al.*, 1986; Huang *et al.*, 2009). Vascular endothelial cadherin is a strictly endothelial specific adhesion molecule located at junctions between ECs. They are known as a major determinant of EC contact integrity and regulation of its activity or its presence at cell contacts is an essential step that

controls the permeability of the blood vessel wall for cells and substances (Vestweber, 2008). Usually, the expression of these EC specific markers is majorly associated with vascular biology and angiogenesis (Vestweber, 2008; Goncharov *et al.*, 2017).