INVOLVEMENT OF MEK SIGNALLING ON ENDOTHELIAL-LIKE DIFFERENTIATION OF DENTAL STEM CELLS CULTURED ON HUMAN AMNIOTIC MEMBRANE WITH VEGF TREATMENT

MUHAMMAD FUAD HILMI BIN YUSOF

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by

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LIST OF SYMBOLS

®	Registered
°C	Degree Celsius
$2^{-\Delta\Delta}C_T$	Fold change
Av	Number of average cells (total cell count divided by four)
C	Cell concentration (cells/ml)
C_T	Threshold cycle
™	Trademark
%	Percentage
α	Alpha
β	Beta
κ	Kappa

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
C	Cell concentration (cells/ml)
c	Calibrator (control sample)
IS	24h VEGF pre-induced SHED
ISAV	24h VEGF pre-induced SHED on AM with VEGF
ISAVP	24h VEGF pre-induced SHED on AM with VEGF with 1µM MEK inhibitor PD184352
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DAPI	4′,6-diamidino-2-phenylindole
APC	Allophycocyanin
AM	Amniotic membrane
Ang-1	Angiopoietin 1
bp	Base pair
BSA	Bovine serum albumin
CO_2	Carbon dioxide
cm	Centimetre
CD31	Cluster of differentiation 31
COX-2	Cyclooxygenase-2
et al.	And others
F-actin	Cytoskeletal filament actin
DF	Dilution factor
DFPC	Dental follicle progenitor cells
DSC	Dental stem cells
DNA	Deoxyribonucleic acid
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
Na_2HPO_4	Disodium hydrogen phosphate
NT5E	Ecto-5'-nucleotidase
EBM	Endothelial basal medium
EGM-2	Endothelial cell growth medium 2
EC	Endothelial cells
NOS3	Endothelial nitric oxide synthase
E-selectin	Endothelial-selectin
ECL	Enhanced chemiluminescence
ELISA	Enzyme-linked immunosorbent assay
EGF	Epidermal growth factor
EDTA	Ethylenediamine tetra acetic acid
ECM	Extracellular matrix

ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FAK	Focal adhesion kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GOI	Gene of interest
g	Gram
G	G-force or relative centrifugal force (RCF)
HSCs	Hematopoietic stem cells
HBSS	Hepes Buffered Saline Solution
HRP	Horseradish peroxidase
H	Hour
BMSC	Human bone marrow mesenchymal stem cells
HFGF-B	Human fibroblast growth factor-B
hMSCs	Human mesenchymal stem cells
HUVEC	Human umbilical vascular endothelial cells
HIF1α	Hypoxia-inducible factor-1α
ICAM-1	Intercellular Adhesion Molecule 1
IL1-β	Interleukin-1-beta
IL-6	Interleukin-6
IL-8	Interleukin-8
LacZ	LacZ encodes β-galactosidase
MMP	Matrix metalloproteinase
MALDI-TOF MS	Matrix-assisted laser desorption ionisation-time of flight mass spectrometry
MSC	Mesenchymal stem cells
mRNA	Messenger ribonucleic acid
μM	Micromolar
mm	Millimetre
ml	Millilitre
α-MEM	Minimum essential medium (MEM) alpha
min	Minutes
MAPK	Mitogen-activated protein kinase
MEKK-1	Mitogen-activated protein kinase kinase kinase 1
MEK	Mitogen-Activated Protein Kinase/ Extracellular-Signal-Regulated Kinase
MEK1	Mitogen-Activated Protein Kinase/ Extracellular-Signal-Regulated Kinase 1
NaH_2PO_4	Monosodium phosphate
BIS	N,N'-methylene-bis-acrylamide
ng	Nangogram
nM	Nanomolar
NSC	Neural stem cells

norm	normalizer
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NFAT	Nuclear factor of activated T-cells
P	Passage
PerCP	Peridinin-Chlorophyll-protein
PDLSC	Periodontal ligament stem cells
PBS	Phosphate Buffered Saline
PBST	Phosphate-buffered saline-Tween 20
PI3K	Phosphoinositide 3-kinases
p-ERK	Phosphorylated-extracellular signal-regulated kinase
PE	Phycoerythrin
PLGA	Poly(lactic-co-glycolic acid)
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride or polyvinylidene difluoride
pH	Potential of hydrogen
AKT	Protein kinase B
qRT-PCR	Real-time reverse-transcription polymerase chain reaction
RIPA	Radioimmunoprecipitation assay
Raf	Rapidly accelerated fibrosarcoma
Ras	Rat sarcoma
R3-IGF-1	Recombinant long arginine insulin-like growth factor
RCAN-1.4.	Regulator of Calcineurin 1 Isoform 4
RT-PCR	Reverse transcriptase polymerase chain reaction
RPM	Revolutions per minute
RNA	Ribonucleic acid
RT	Room temperature
s	Seconds
s	Sample of experimental
S	SHED
SA	SHED on AM
SAV	SHED on AM with VEGF
STAT3	Signal transducer and activator of transcription 3
NaCl	Sodium chloride
SDS	Sodium dodecyl (lauryl) sulphate
SC	Stem cells
SCAP	Stem cells from apical papilla
SEM	Standard error mean
SHED	Stem cells from extracted human deciduous teeth
TEMED	Tetramethyl ethylenediamine
IC_{50}	Half-maximal inhibitory concentration
3D	Three dimensional
T cells	Thymus cells

TE	Tissue engineering
TIMP3	Tissue inhibitor of metalloproteinase 3
TIMP4	Tissue inhibitor of metalloproteinase 4
TNS	Trypsin neutralizing solution
TNF-α	Tumour necrosis factor - alpha
2D	Two dimensional
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VEGF-B	Vascular endothelial growth factor-B
VEGF-C	Vascular endothelial growth factor-C
VEGF-D	Vascular endothelial growth factor-D
VE-Cadherin	Vascular endothelial-cadherin
v	Voltage
vWF	Von Willebrand factor
W	Watt
Wnt	Wingless and Int-1

PENGLIBATAN PENGISYARATAN MEK DI DALAM PEMBEZAAN STEM SEL GIGI KEPADA SEL SEPERTI ENDOTELIAL YANG DIKULTURKAN DI ATAS MEMBRAN AMNIOTIK DENGAN RAWATAN VEGF

ABSTRAK

Penyembuhan luka masih menjadi beban penjagaan kesihatan yang dikaitkan dengan peningkatan morbiditi dan mortaliti yang serius. Kejuruteraan tisu menawarkan penyelesaian yang berpotensi untuk merungkai keperluan perubatan yang tidak dipenuhi ini dengan membina konstruk hasil gabungan sel, faktor pertumbuhan, dan perancah bagi angiogenesis, iaitu suatu proses asas dalam penjanaan semula tisu. Pemahaman mekanisma molekul yang mendasari pembezaan angiogenik secara menyeluruh adalah sangat penting bagi pembangunan semula tisu dalam menyembuhkan luka. Justeru itu, kajian ini bertujuan untuk menyiasat peranan tapak jalan pengisyaratan MEK apabila teraruh dengan faktor pertumbuhan endotelium vaskular (VEGF) terhadap pembezaan sel tunjang daripada gigi susu manusia yang terkelupas (SHED) dan sel SHED yang teraruh dengan VEGF kepada sel seperti endotelium yang dikultur di atas lapisan stromal (SS) membran amnion manusia (AM). Bagi merungkai tujuan tersebut, ujikaji sitometri aliran, tindak balas berantai polimerase transkriptase berbalik (RT-PCR), tindak balas rantai polimerase transkriptase berbalik masa nyata (qRT-PCR), asai imunoserapan terangkai enzim (ELISA), dan analisis imunositokimia (ICC) telah dijalankan. Keputusan sitometri aliran menunjukkan SHED pada pasaj 10 dan 15 mengekspreskan penanda protein sel tunjang mesenkima secara positif, membuktikan SHED mengekalkan sifat ketunjangan. SHED juga tidak mengekspreskan penanda sel hematopoietik iaitu CD34, CD11b, CD19, CD45, dan HLA-DR. Hasilan Western Blot menunjukkan

penanda protein sel endotelium iaitu Ang-1 dan COX-2 diekspreskan di dalam SHED terbeza yang dikultur di atas lapisan SS AM dengan rawatan VEGF pada hari 1 dan 7. Hasil RT-PCR menunjukkan SHED terbeza mengekspreskan kedua-dua penanda sel tunjang (*Nestin*, *Nanog*, dan *CD73*) dan spesifik-endotelium (*Ang-1*, *COX-2*, dan *VE-Cadherin*) di dalam setiap kumpulan rawatan pada hari 1, 7, 10 dan 14. Pra-aruhan VEGF selama 24 jam meningkatkan pengekspresan *CD73*, *Nanog*, dan *COX-2*. Dos sub-maut sebanyak 1.0 µM perencat PD184352 telah mengurangkan kebolehidupan sel secara signifikan (ujian t sampel tidak bersandar, $p<0.05$). Analisis statistik menggunakan ANOVA sehala bagi keputusan qRT-PCR menunjukkan pra-aruhan VEGF meningkatkan pengekspresan gen *NOS3* dan *IL-8* pada hari 1 dan 10 secara signifikan ($p<0.05$). Sebaliknya, pengekspresan gen *CD31*, *vWF*, *IL1-β*, *TNF-α*, *E-selectin*, *ICAM-1*, dan *RCAN-1.4* tidak dinaikkan oleh pra-aruhan. Perencat MEK PD184352 pula menyebabkan perencatan penuh kepada pengekspresan gen *CD31* dan *NOS3* pada hari 1 dan 7, dan gen-gen tersebut telah dikesan pada hari ke-10 dan pada hari berikutnya. Sementara itu, PD184352 mengurangkan regulasi *vWF*, *IL1-β*, dan *IL-8*. Sebaliknya, PD184352 telah meningkatkan pengekspresan gen *TNF-α*, *E-selectin*, *ICAM-1*, dan *RCAN-1.4*. Hasil keputusan ELISA menunjukkan pengekspresan protein p-ERK, CD31, dan MEKK1, membuktikan bahawa pengisyaratan VEGF melalui tapak jalan MEK/ERK diperlukan bagi pembezaan angiogenik dalam konstruk yang dicadangkan. Keputusan ujikaji ICC bagi pengekspresan protein CD31, vWF, dan F-actin mengukuhkan lagi dakwaan bahawa pembezaan SHED kepada sel seperti endotelium dikawalatur oleh pengisyaratan MEK. Oleh itu, hasil kajian ini mencadangkan bahawa tapak jalan MEK mengawalatur pembezaan SHED kepada sel seperti endotelium menggunakan konstruk yang dicadangkan bagi kejuruteraan tisu untuk menyembuhkan luka.

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ABSTRACT

Wound healing continues to be a healthcare burden associated with increased morbidity and substantial mortality. Tissue engineering offers a potential solution to address this unmet medical need by building a construct combining cells, growth factor, and scaffold for angiogenesis, a fundamental process for tissue regeneration. A detailed understanding of the molecular mechanism underlying the angiogenic differentiation is vital for developing an engineered tissue for wound healing application. Therefore, this study aimed to investigate the role of the MEK signalling pathway onto the differentiation of stem cells from human exfoliated deciduous teeth (SHED) and VEGF pre-induced SHED into endothelial-like cells when induced with VEGF and cultured on the stromal side (SS) of human amniotic membrane (AM). In order to decipher the pathway involved, the current study was conducted by employing techniques such as flow cytometry, reverse transcription-polymerase chain reaction (RT-PCR), real-time reverse transcription-polymerase chain reaction (qRT-PCR), enzyme-linked immunosorbent assay (ELISA) and immunocytochemistry (ICC). Flow cytochemistry results showed that SHED at passage 10 and 15 positively expressed CD90, CD73, and CD105 mesenchymal stem cell protein markers, indicating that SHED were able to maintain their stemness property. Concurrently, SHED did not express hematopoietic cell markers, namely, CD34, CD11b, CD19, CD45, and HLA-DR. Western blot results showed that Ang-1 and COX-2 endothelial cells protein markers were expressed in differentiated SHED cultured on SS of AM

with VEGF treatment on day 1 and 7. RT-PCR findings revealed that differentiated SHED expressed both stem cells (*Nestin*, *Nanog*, and *CD73*) and endothelial-specific markers (*Ang-1*, *COX-2*, and *VE-Cadherin*) in all treatments on day 1, 7, 10, and 14. Twenty four hours VEGF pre-induction elevated the expression of *CD73*, *Nanog*, and *COX-2*. A sub-lethal dose of 1.0 μM MEK inhibitor PD184352 reduced the cell viability significantly (independent sample t-test $p<0.05$). Statistical analysis using one-way ANOVA for qRT-PCR outcomes demonstrated that VEGF pre-induction upregulated the gene expression of *NOS3* and *IL-8* significantly at day 1 and 10 ($p<0.05$). On the other hand, the expression of *CD31*, *vWF*, *IL1-β*, *TNF-α*, *E-selectin*, *ICAM-1*, *and RCAN -1.4* were not promoted by the pre-induction. MEK inhibitor PD184352 blocked the gene expression of *CD31* and *NOS3* on day 1 and 7, and the genes were detected on day 10 afterwards. Meanwhile, PD184352 downregulated *vWF*, *IL1-β*, and *IL-8*. In contrast, PD184352 promoted TNF-α, E-selectin, ICAM-1, and RCAN-1.4 gene expressions. ELISA results showed that p-ERK, CD31, and MEKK1 protein expression provided confirmatory evidence that VEGF signalling through the MEK/ERK pathway was required for angiogenic differentiation by this proposed construct. Besides, the ICC results of CD31, vWF, and F-actin protein expression enforced that SHED performed endothelial-like differentiation, and it was regulated by MEK signalling. Hence, these findings proposed that the MEK pathway regulates the differentiation of SHED into endothelial-like cells using the proposed construct for wound healing tissue engineering.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

A human's ability to heal wounds is an evolutionary advantage for survival. It is believed that humans heal faster than other forms of life, such as amphibians or unicellular organisms, to protect us from other predators and to ensure existence (Cohen, 2006). Physiologically, wound healing involves important phases; haemostasis, inflammation, proliferation, and maturation, requiring angiogenesis for nutrients and oxygen delivery to the multitude of cells (Reinke & Sorg, 2012). A deficit in angiogenesis leads to the pathological of chronic non-healing wounds. Innovations for wound healing is as old as modern human history. Retrospectively, it can be traced back to Egyptian civilisation in their record using compression for haemostasis (Broughton *et al.*, 2006). Later, after almost 3 millennia, various strategies are employed to treat acute and chronic wounds, such as third-degree burn diabetic wound ranging from non-biological materials to biological-based products.

Nevertheless, wound healing is still an unmet medical need. This gap means a massive opportunity for improvisation. According to Fortune Business Insights (2020), the global market wound care size was $ 10.43 billion in 2019 and is projected to reach USD 15.59 billion by 2027. In the US healthcare sector, more than $ 25 billion has been spent on a chronic non-healing wound.

Tissue engineering (TE) offers a solution for wound healing, especially in understanding its principles and mechanisms. TE converges three key components; stem cells (SC), growth factors and a supporting scaffold to form a 3D construct that ultimately aims in restoring the function of injured tissue (Tollemar *et al.*, 2016). Stem cells from extracted human deciduous teeth (SHED) were first discovered by Miura *et al.* (2003). This mesenchymal SC (MSC) is highly proliferative with the ability to perform neurogenic, adipogenic and odontogenic differentiation property (Miura *et al.*, 2003). Interestingly, SHED was found to express VEGF, a pro-angiogenic factor both at the mRNA and protein level (Bronckaers *et al.*, 2013). Due to the fact SHED are isolated from extracted deciduous teeth, harvesting SHED is technically non-invasive and, most importantly, with no ethical issue involved as compared to bone marrow SC and embryonic SC. Vascular endothelial growth factor (VEGF) is one of the most well studied classic pro-angiogenic growth factors for angiogenesis in humans (Ucuzian *et al.*, 2010). Hence, this makes VEGF a popular agent for angiogenic differentiation induction. A scaffold made of human amniotic membrane (AM) is an organic biomaterial rich in the extracellular matrix (ECM) clinically proven as dressing for wound healing (Bianchi *et al.*, 2018), abundantly available yet usually discarded (Ramuta & Kreft, 2018). As AM is unable to trigger an allogeneic or xenogeneic immunologic reaction, AM has attracted great interest in tissue engineering and transplantation (Malhotra & Jain, 2014). This robust performance is possible due to the combination of anti-inflammatory properties, low immunogenicity, and immunomodulatory properties (Wassmer & Berishvili, 2020).

The aim of this research was to grow the SC with the cues from growth factor and natural scaffold that mimic the natural milieu of the human body in an attempt to

create body parts such as angiogenic structures for wound healing application. Thus, assembling this triad, SHED, VEGF, and AM as a 3D construct of engineered tissue to develop a basic angiogenic structure, the endothelial cells, would be the next frontier to be pushed forward (Figure 1.1). Also, the pathway involved when SHED differentiate into endothelial-like cells by VEGF induction and cultured on the stromal side of AM was also taken into consideration. In order to evaluate the angiogenic differentiation of this proposed construct, it is necessary to clarify the effect of these two pro-angiogenic factors, VEGF and AM, in promoting SHED into endothelial-like cells at the genes and proteins expression couple with elucidating the role of MEK signalling for the differentiation regulation. The combination between VEGF and AM previously was tested by Md Hashim *et al.* (2019) and postulated the pro-angiogenic promoting effect by these factors towards angiogenic differentiation by SHED. The mechanobiological effects of these chemical and physical inductions are interesting to be deciphered as they may provide a microenvironment that can be a potential model for various applications such as angiogenesis study and evaluation of drug toxicity.

The data from the present study would enrich the information on the SHED and its differentiation capability with the designed niche. This 3D construct can be used as an angiogenic model to study angiogenesis for wound healing (Figure 1.1). Angiogenesis is also significant for the progression of tumour cells because it relies on oxygen and nutrients supplied via blood vessels, just like any normal cells (Nishida *et al.*, 2006). In order to so, cancer cells produce pro-angiogenic factors to stimulate angiogenesis to support their demands (Rajabi & Mousa, 2017). Thus, this 3D model can be used for anti-angiogenic drugs screening against cancer, not only for cellular cytotoxicity analysis but also for functional effects on the behaviour of tumour cells.

By identifying the inducer of MEK for endothelial differentiation too, this information can be manipulated to promote angiogenesis.

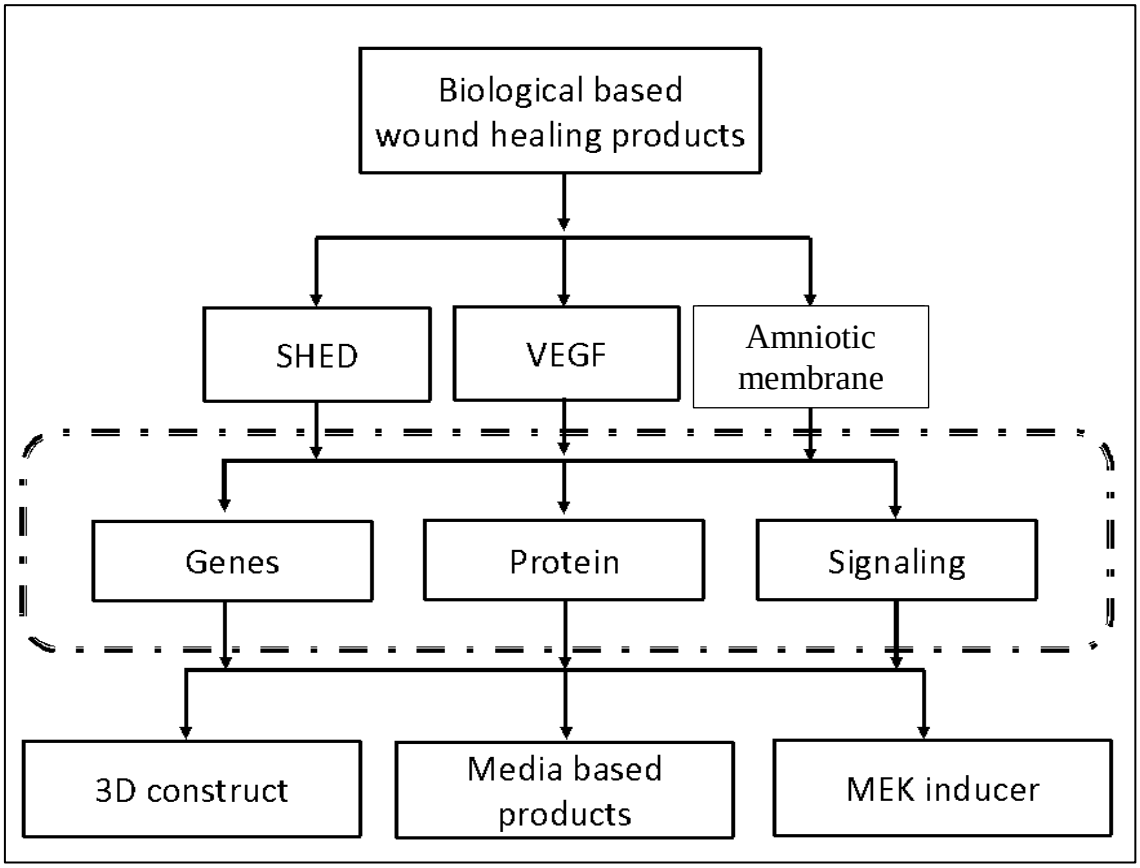

Figure 1.1: An overview of biological based wound healing products and the evaluation for the proposed construct that combined SHED, VEGF and amniotic membrane by genes and proteins expression as well as the signalling pathway.

1.2 Justification of the study

There are many studies conducted to evaluate the angiogenic differentiation potential of SHED (Sakai *et al.*, 2010; Bento *et al.*, 2013; Shi *et al.*, 2020). Md Hashim *et al.* (2019) highlighted that AM offers a microenvironment that subsequently promoted SHED differentiation into endothelial-like cells. Whilst VEGF has been established as a potent angiogenic inducer (Harmey *et al.*, 2013). Both mechanobiology and chemical cues from these pro-angiogenic factors are important to drive the SC to an appropriate fate and modulate the cell responses by tuning the signal transduction pathway (Alenghat & Ingber, 2002). Previous studies have revealed that 24 hours pre-induction and prolonged enhanced angiogenic differentiation (Stannard *et al.*, 2007; Valente *et al.*, 2014). However, to the best of our knowledge, there is no literature exploring on how the MEK signalling affects the 24 hours VEGF pre-induction on SHED angiogenic differentiation potential when treated with VEGF and seeded on the stromal side of AM. This novel information will bridge the gap in tissue engineering field as these will update the multipotent capability of SHED when cultured in this proposed 3D construct as well as the role of MEK signalling regulation within this model.

1.3 Research objectives

1.3.1 General objective

This study aimed to investigate the role of MEK signalling pathway during the differentiation of SHED into endothelial-like cells when induced with VEGF and cultured on stromal side of AM.

1.3.2 Specific objectives

1. To evaluate the stem cell properties of SHED by quantifying MSC specific protein markers at passage 10 and 15 by flow cytometry.
2. To screen the angiogenic property of cultured SHED on AM upon VEGF treatment and VEGF pre-induction by Western blot and reverse-transcription polymerase chain reaction (RT-PCR).
3. To determine the expression of angiogenic gene markers of SHED induced by VEGF and cultured on AM and treated with and without MEK inhibitor PD184352 by real time reverse-transcription polymerase chain reaction (qRT-PCR)
4. To assess the expression of protein markers related to VEGF/MEK/p-ERK pathway during the angiogenic differentiation of SHED induced by VEGF and cultured on AM and treated with and without MEK inhibitor PD184352 by ELISA and immunocytochemistry

1.4 Research hypotheses

MEK signalling regulates the angiogenic differentiation of SHED into endothelial-like cells when induced with VEGF and cultured on stromal side of AM.

1.5 Research questions

1. Do SHED cultured on AM with VEGF treatment express higher angiogenic genes and protein markers during differentiation into endothelial-like cells?
2. Does MEK signalling pathway regulate the endothelial differentiation by SHED in this proposed construct?

CHAPTER 2

LITERATURE REVIEW

2.1 Regenerative medicine and tissue engineering

Humans suffer from tissues and organs loss because of congenital defects, diseases, and trauma. Globally, many people would benefit immensely if damaged tissues can be replaced on demand (Hippen *et al.*, 2009). Heavy reliance on transplantation has caused a bottleneck effect of people waiting for their turn to get donated tissues and organs as supply cannot meet the demand (Arshad *et al.*, 2019). Moreover, the economic burden of caring for patients to society, with injured tissues and debilitating diseases, is enormous and counter-productive (Pol *et al.*, 2019). Therefore, strategies and technologies using regenerative medicine and TE to increase the supply of tissues must be developed further (Pokrywczynska *et al.*, 2014; Jain & Bansal, 2015).

Regenerative medicine appears to have been coined by Haseltine (2001) to capture his view on the future of medicine for promotional purposes. Seven years later, Mason & Dunnill (2008) defined regenerative medicine as “the process of replacing or regenerating human cells, tissues or organs to restore or establish normal function”. Regenerative medicine employs various techniques to induce organ regeneration, including cell-based therapies, immunomodulation, gene therapy, nanomedicine, and TE itself (Salgado *et al.*, 2013).

Langer & Vacanti (1993) popularised TE as a term that alludes to the combination of cells, tissue-inducing substances and placement of cells on or within

matrices used to develop functional substitutes for damaged tissue. It is one spectrum under the regenerative medicine domain (Furth & Atala, 2013), while for TE, it is a science that converges the triad; cells, growth factors and scaffolds (Figure 2.1) (Salgado *et al.*, 2004). It is an interdisciplinary field that applies engineering and life sciences principles towards the development of biological substitutes that restore, maintain or improve tissue function (Sudhakar et al., 2015). The process can involve *de-novo* growth in tissue culture (*in vitro* and *ex vivo*) or tissue regeneration *in vivo* at sites (Huang *et al.*, 2010). Eventually, due to the related objectives by regenerative medicine and TE, these two fields have been merging in recent years, originating the broad field of tissue engineering and regenerative medicine (TERM) (Salgado *et al.*, 2013). TE is also a promising strategy to restore the damages caused by COVID-19 (Aydin *et al.*, 2020).

There is an exponential growth in regenerative medicine products entering the clinical arena (Cossu *et al.*, 2018). Nevertheless, it plays a relatively minor role in patient care at present (Kaoud, 2018). One clinically proven TE product is MyDerm® to regenerate skin (Mohamed Haflah *et al.*, 2018). However, the number of current success stories may less than the public expectations. The community of tissue engineering worldwide works to address the challenges by gathering more scientific and significant evidence to translate the effort from bench to bedside. The effort continues to tune the optimal cell numbers, the effective growth factor and the best scaffold for tissue engineering application.

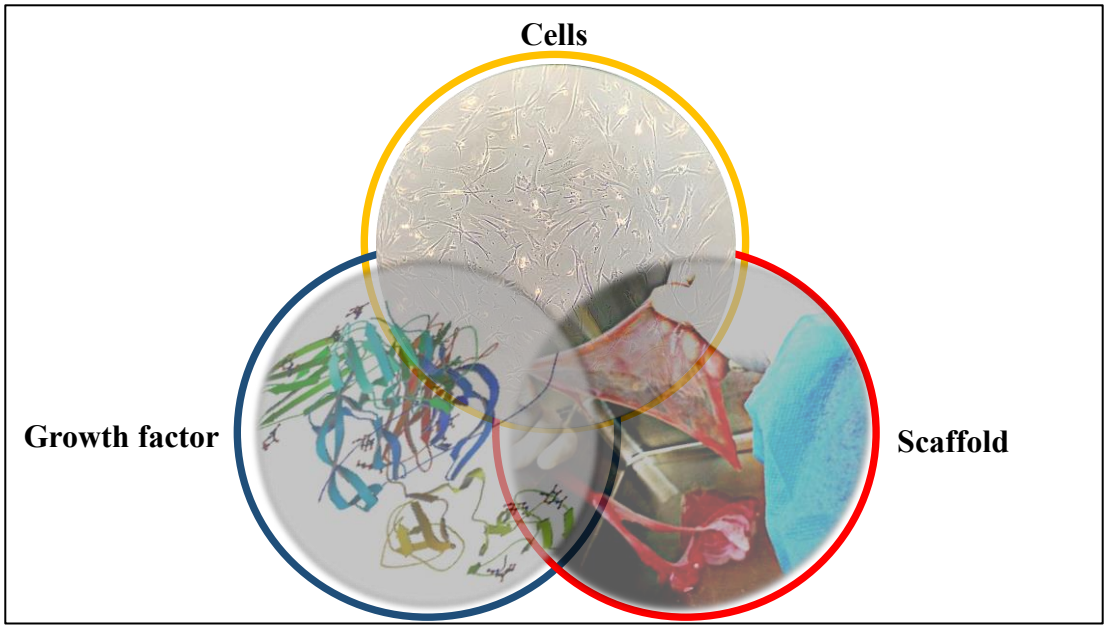

Figure 2.1 The triad of tissue engineering. Tissue engineering is a combination of three key components namely cells, biomaterial scaffold and biologically active factors

2.2 Angiogenesis

Angiogenesis plays a central role in human physiology, from reproduction and foetal development to wound healing and tissue repair/regeneration (Reddy *et al.*, 2019). Clinically relevant therapies are needed for promoting angiogenesis to supply oxygen and nutrients after transplantation (Rademakers *et al.*, 2019). By history, angiogenesis was introduced by Flint (1900) to explain the vascularisation of the adrenal gland. However, this term is arguably coined by John Hunter, a surgeon that lived circa 1728-1719 (Lenzi *et al.*, 2016).

According to Adair & Montani (2010), angiogenesis is defined as a morphogenic development for new blood vessels from the existing vasculature. It occurs throughout life in both physiological and pathological, beginning in utero and continuing postnatally. There is no metabolically active tissue inside the body located beyond a few hundred micrometres from a blood capillary, which is formed by angiogenesis. The initiation of angiogenesis begins with endothelial cell activation, matrix modulation, proliferative expansion and vascular morphogenesis (Claffey, 2002).

Angiogenesis involves a series of events, which starts with endothelial cells (ECs) responding to angiogenic factors produced either by endothelium or stromal cells (Dulak *et al.*, 2016). Initiation of angiogenesis is completed in response to hypoxia to overcome oxygen depletion and starvation. Hypoxia-inducible factor-1α (HIF1α) is one of the transcription factors that is stable and active under low oxygen tension. It is responsible for driving substantial pro-angiogenic growth factor

expression. They are a prerequisite for angiogenesis and activate EC for proliferation, survival and migration via endothelial receptors (Giaccia *et al*., 2003).

2.2.1 Endothelial cells

Endothelial cells (ECs) are monolayer cell lining the entire vascular system, from the heart to the smallest capillary, and regulating the exchanges between the surrounding tissues and bloodstream (Alberts *et al.*, 2002). ECs produce signals to organise the growth and development of connective tissue cells that form the surrounding layers of the blood vessel wall (Cleaver & Melton, 2003).

The cardiovascular system is the first organ system to develop in the embryo (Risau, 1997). The luminal surface of the circulatory system in contact with blood is a single layer of EC derived from mesoderm stem cells (Adair & Montani, 2010). Subsequently, mesodermal stem cells differentiate into "haemangioblasts". Haemangioblast was proposed almost a century ago as a term to describe the common origin of haematopoietic/endothelial progenitor cells (Murray, 1932).

This progenitor gives rise either into an angioblast, a precursor for arterial and venous EC or hemogenic EC, capable of hematopoietic cell generation (Grochot-Przęczek *et al.*, 2013). Angioblasts are a cell type with potency to differentiate into EC but have not yet acquired all EC characteristic markers (Risau, 1997). EC can also transdifferentiate into mesenchymal cells and intimal smooth muscle cells (Choi *et al.*, 1998).

The EC provide a barrier between blood and tissues and additionally act as an endocrine organ. The process of angiogenesis is entirely sustained by ECs (Munaron & Pla, 2009). ECs participate in vascular constriction and relaxation. These cells control the extravasation of fluid, hormones, macromolecules and solutes. They also guide inflammatory cells to foreign materials, defence against infections or tissue region in need of repair. Likewise, ECs are essential in governing platelet adhesion, blood fluidity, adhesion and aggregation, leukocyte activation and transmigration (Nawroth & Stern, 1986; Sadler, 1997; Cines *et al.*, 1998; Jain, 2003).

In vitro angiogenesis studies use human umbilical vascular endothelial cells (HUVEC) (Figure 2.2) as a model to represent human ECs due to their behaviour that faithfully behave like human vascular endothelium when compared to the other cell lines (Garbern *et al.*, 2013). HUVEC is used to investigate the molecular aspect and signalling cascade involving angiogenesis (Howe *et al.*, 2017; Zhang *et al.*, 2019; Zhao *et al.*, 2019). Interestingly, the application of HUVEC has been documented in a large number of published studies such as tissue engineering, diabetes and cancer (Rhim *et al.*, 1998; Onat *et al.*, 2011; Maiullari *et al.*, 2018).

The harvesting protocol for HUVEC as a source of cells requires a non-invasive method with a high number cells (Kocherova *et al.*, 2019). HUVEC are acquired from discarded umbilical cord that typically becomes “medical waste” after a child’s birth (Kadam *et al.*, 2009). Nevertheless, one major drawback of HUVEC is that these cells are terminally differentiated adult cells, site-specific phenotype property with high immunogenic response, and it is impossible to use HUVEC for auto-transplantation among adult patients (Kocherova *et al.*, 2019). Identifying a novel

cell source that would be more feasible for tissue engineering if a novel cell source for angiogenic engineering can be identified and clinically tested.

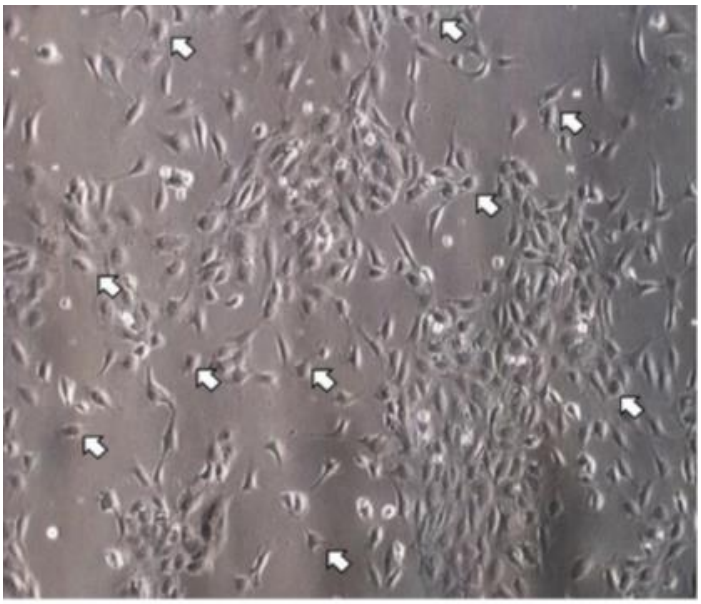

Figure 2.2: **Image of HUVEC morphology grown on the plastic surface observed using an inverted microscope.** HUVEC has a cobblestone-like shape (white arrow) (magnification at 100x). (Adapted from Md Hashim (2017)).

2.2.2 Endothelial cell markers

2.2.2 a) Angiopoietin 1 (Ang-1)

Lacking *Ang-1* resulted in defects in the vasculature (Davis *et al.*, 1996). Additionally, this gene is involved at the stage of vascular morphogenesis and maturation (Claffey, 2002). This angiogenic marker previously was suggested not only in angiogenic differentiation but also cell migration (Aziz *et al.*, 2018).

2.2.2 b) Cyclooxygenase-2 (COX-2)

COX-2 is a key enzyme in the synthesis of prostaglandins from arachidonic acid (Vane *et al.*, 1998). During angiogenesis, COX-2 initiates prostaglandins synthesis, consequently inducing the expression of pro-angiogenic factors forming new capillaries and inducing proliferation (Iñiguez *et al.*, 2003). COX-2 activity appears to be modulated by VEGF (Wu *et al.*, 2006) and can be increased mechanobiologically (Yoon *et al.*, 2015 & Khan *et al.*, 2004).

2.2.2 c) VE-Cadherin

VE-Cadherin is an endothelial cell-specific cadherin that regulates the assembly of a new blood vessel and vascular integrity maintenance (Breviario *et al.*, 1995). In an *in vitro* study by (Sakai *et al.*, 2010), VEGF induced SHED to express *VE-Cadherin*. SHED following angiogenesis and migratory induction by

supplementing angiogenic factors positively expressed *VE-Cadherin* (Aziz *et al.*, 2018).

2.2.2 d) CD31

CD31 is also described as *PECAM-1* (platelet/endothelial cell adhesion molecule-1). This gene is named after its role in maintaining and restoring the vascular cell adhesion and speed recovery of the vascular permeability barrier after thrombotic challenge function and highly expressed in endothelial cells (Lertkiatmongkol *et al.*, 2016). According to Buckley *et al.* (1996), *CD31* belongs to the immunoglobulin gene superfamily (IgSF) and associated with various function, including angiogenesis, cell differentiation, inflammation and integrin activation. The expression of this marker is highly detected on endothelium and cells of myeloid lineage (Buckley *et al.*, 1996)

2.2.1 e) Von Willebrand factor (vWF)

vWF is a multifunctional glycoprotein best known for its essential roles in primary and secondary haemostasis and as a mediator of platelet adhesion (Stockschlaeder *et al.*, 2014). ECs and megakaryocytes synthesise vWF, while congenital decrease or dysfunction of vWF causes von Willebrand disease (Randi & Laffan, 2017). This gene promotes platelets' adhesion to vascular injury sites by forming a molecular bridge between the sub-endothelial collagen matrix and platelet-surface receptor complex (Ruggeri, 2009). This highly selective angiogenic marker is claimed to be exclusively expressed by ECs and megakaryocyte (Piovella *et al.*, 1978)

2.2.2 f) Endothelial nitric oxide synthase (NOS3)

Endothelial nitric oxide synthase (NOS3; also referred to as eNOS or NOSIII) is a low output enzyme where the prototypical isoform is located in ECs (Kleinert & Forstermann, 2007). This angiogenic marker is a major determinant of vascular tone and blood pressure and several diseases such as hypertension, diabetes, and atherosclerosis (Robinson *et al.*, 1994). Beltran-Povea *et al.* (2015) revealed that ESC expressed *NOS3*. During ESC differentiation into cardiomyocytes, this gene was downregulated as observed after 14 days of the experiment (Krumenacker *et al.*, 2006).

2.3 Stem cells

Ernst Haeckel, a German biologist, coined the "stem cell" term to describe the fertilized egg that turns into an organism during the late 19th century (Reisman & Adams, 2014). Stem cells (SC) are defined as unspecialised cells with self-renewal ability through cell division (Biehl & Russell, 2009). During mitosis, a divided SC has two faith options; either to retain as a stem cell or differentiate into other kinds of cells that form the body's tissues and organs (Mummery *et al.*, 2014). SC differentiate into many types of cells in response to appropriate inductions and conditions within the body (Zakrzewski *et al.*, 2019). These properties equip SC with unique tissue repair capabilities, replacement, and regeneration (Falanga, 2012). These properties have become valuable research tools for regenerative medicine and possible stem cell therapies (Reisman & Adams, 2014).

Primarily, SC exists both in embryos and adult cells (Fortier, 2005). Embryonic SC is a pluripotent SC population that can differentiate into all types of adult cells without a limited number of times. However, this SC's creation involves the destruction of live human embryos (Landry & Zucker, 2004). Another type is the adult SC that is undifferentiated, self-renewal with multilineage property present in many adult tissues (Prochazkova *et al.*, 2015). In contrast, adult SC is a multipotent cell with limited ability to differentiate as compared to embryonic SC.

Among the type of adult SC are mesenchymal stem cells (MSC), hematopoietic stem cells (HSC) and neural stem cells (NSC) (Shi *et al.*, 2006). Adult SC can be found in dental tissue, bone marrow, foreskin, adipose tissue and umbilical cord with angiogenic differentiation potential (Gronthos *et al.*, 2000; Kang *et al.*, 2013; Lu *et al.*, 2018; Shojaeian *et al.*, 2020). For this justification, adult SC is also known as postnatal SC. This type of SC is more applicable than embryonic SC in SC therapies and regenerative medicine because SC's isolation lacks ethical concerns. Additionally, adult SC have low immunogenicity reactions and less tumorigenic potency which made adult SC a potential cell source for regenerative medicine (Potdar, 2015).

Adult SC transplants are already widely used to benefit over a million people (Gratwohl *et al.*, 2015). SC transplant has been used for many conditions, including multiple myeloma and leukaemias, have moved beyond clinical trials to become a standard medical practice to treat the patients (Gupta & Kumar, 2011; Tian *et al.*, 2015). Interestingly, SC is believed in the past; it can only differentiate specifically into adult cells of the originated cells extraction site (Rajabzadeh *et al.*, 2019). Currently, the of SC's angiogenic research is extensive and novel therapeutic strategies

are emerging utilising SC as the primary cellular component of various TE constructs (de Cara *et al.*, 2019; Wanjare *et al.*, 2019; Merckx *et al.*, 2020).

Currently, TE depends on the autologous cells from which specific cells types can be extracted, propagated and seeded onto a matrix for subsequent transplantation. However, this is for the ideal case scenario that under some circumstances, neoplasia or bad organ failure, isolation of normal cells from a patient is often problematic (Yamzon *et al.*, 2008). The ability of SC to propagate and differentiate into desired tissue types makes them an attractive alternative cell source for regenerative medicine applications (Kolios & Moodley, 2012).

2.3.1 Dental tissue-derived stem cells

Numbers of adult MSC populations have been discovered that reside in various dental tissues. These SC include dental pulp stem cells (Gronthos *et al.*, 2000), stem cells from Human Exfoliated Deciduous teeth (SHED) (Miura *et al.*, 2003), Periodontal Ligament Stem Cells (PDLSC) (Seo *et al.*, 2004), Dental Follicle Progenitor Cells (DFPC) (Morsczeck *et al.*, 2005), Stem Cells from Apical Papilla (SCAP) (Sonoyama *et al.*, 2006). Mammalian teeth originate from the embryonic source of neural crest ectomesenchyme (Huang *et al.*, 2009). Hence, this is an additional plasticity advantage for dental stem cells (DSC), displaying characteristics of both ectoderm and mesoderm. Like the other type of adult SC, these MSC are clonogenic and self-renewal postnatal SC (Chalisserry *et al.*, 2017).

In terms of the cell harvesting protocol, DSC is easily accessible by tooth extraction with a local anaesthetic or when a deciduous tooth is replaced (Sunil *et al.*, 2015). A comparative study was described by (Yusoff *et al.*, 2015) found that dental SC has differentiation higher passage numbers than amniotic membrane SC. Both SC from the dental and amniotic membrane are isolated from discarded tissue, then can be expanded for cell generation by multiple sub-cultures and differentiated to specific lineages in response to appropriate stimuli (Prisk & Huard, 2005). However, dental SC can achieve up to 25 passage number without compromising proliferative property (Jiang *et al.*, 2006). On the other hand, amniotic membrane SC ceases proliferation until passage 6 (Bilic *et al.*, 2008; Parolini *et al.*, 2008). Large-scale SC expansion with a low grade of senescence effect is substantial criteria for stem cell transplantation (Diomede *et al.*, 2017). However, continuous passages of adult SC for an extended period may affect the SC stemness properties, including proliferation and differentiation markers (Yu *et al.*, 2010). Thus, DSC has more competitiveness to be a potential SC source.

Another intriguing fact about DSC is that they can be isolated from inflamed or compromised dental tissue, yet the properties are conserved and identical those of healthy tissue (Alongi *et al.*, 2010; Sun *et al.*, 2014). In terms of multipotency, dental SC able to differentiate into five cell lineages; adipogenic, angiogenic, chondrogenic, neurogenic and odontogenic (Zhang *et al.*, 2006; Sonoyama *et al.*, 2008; Huang *et al.*, 2009; Sakai *et al.*, 2010). Clinical-grade human SC should meet essential preconditions such as normal genetic karyotype and genetically stable during long-term culturing and after cryopreserved cell banking (Bolouri, 2015).

MSC has genetic stability during culturing *in vitro* (Soukup *et al.*, 2006; Lange *et al.*, 2007). Contradict reports disclosed that an increased passage number caused MSC spontaneous genomic alternation (Borgonovo *et al.*, 2015; Stultz *et al.*, 2016). Iwanaka *et al.* (2020) revealed that DSC is not tumorigenic and maintains both the stem cell properties and therapeutic efficacy after a continuous cell expansion and tested safe for liver regeneration. Therefore, based on the previous mention of the scientific evidences, DSC is a potential source of cells for TE and regenerative medicine.

2.3.2 Stem cells from human exfoliated deciduous teeth (SHED)

Miura and colleagues (2003) isolated and identified SHED from the remnant pulp structure in the crown of incisors. As an MSC, SHED are described as a highly proliferative and clonogenic and higher number of cell population doubling when compared to bone marrow stem cells (Miura *et al.*, 2003). Hence, it offers attractive advantages over other types of MSC as these SC can be obtained from a source which non-invasive, no ethical concerns and readily accessible (Fortier, 2005). SHED exhibited good proliferation capacity at passage 40 with genetic stability and normal karyotype without tumour formation in nude mice (Yin *et al.*, 2016).

The robust differentiation plasticity of this neural crest-derived SC was also reported by various studies subject to appropriate culture conditions. The ability of SHED to undergo differentiation not only limited to osteogenic, neurogenic, odontogenic and adipogenic but also myogenic and chondrogenic cell faith (Miura *et al.*, 2003; Huang *et al.*, 2009; Sakai *et al.*, 2010; Zhang *et al.*, 2016; Yusof *et al.*, 2018). When cultured

with a basic medium alpha-MEM, SHED grow into individual fibroblastic cells adhered to the culture dish (Figure 2.3).

All these criteria, non-immunogenic, highly proliferative yet non-tumorigenic, non-invasive, genetically stable and no ethical issue, suggest that SHED could be a promising source of stem cells for TE to regenerate damaged tissue structures and possibly to treat wound injury effectively. Like any other MSC, SHED express mesenchymal markers of CD73, CD90, CD105 (Gazarian & Ramírez-García, 2017). As stipulated, SHED also positively express embryonic SC markers Nestin (Zhang *et al.*, 2016) and Nanog (Kerkis *et al.*, 2007). Furthermore, these pluripotent markers could be associated with SHED to display highly proliferative activity, clonogenic, multilineage differentiation capacities.