GENE EXPRESSION ANALYSIS OF STEM CELL FROM EXFOLIATED DECIDUOUS TEETH IN MIGRATION AND LOCAL ANGIOGENESIS OF TISSUE REPAIR

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percentage
(+A+M)	SHED cultured in angiogenic medium with insert
(+A-M)	SHED cultured in angiogenic medium
(-A-M)	SHED cultured in CCM
°C	Degree celsius
µg	Microgram
µl	Microlitre
2D	Two dimensional
3D	Three dimensional
ADO	Adenosine
ADSC	Adipose derive stem cell
ADV	Average density value
Ang	Angiopoietin
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BLAST	Basic local alignment tools
bp	Base pairs
CAM	Chorioallantoic membrane
CBSC	Neural-crest-derived adult carotid body stem cells
CCM	Complete culture medium
cm	Centimetre
cm^2	Centimetre squared
CNC	Cranial neural crest
DEPC	Diethyl pyrocarbonate

DFPC	Dental follicle precursor cells
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cells
EC	Endothelial cell
ECM	Extracellular matrix
ECPF	Endothelial colony-forming cells
EGM	Endothelial growth medium
EGM	Endothelial cell growth medium
EPC	Endothelial progenitor cells
EPO	Erythropoietin
ESC	Embryonic stem cells
ET	Endothelin
et al.	and others
exo	Exosome
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF	Fibroblast growth factor
FSC	Follicle precursor cells
g	Gram
g	Gravity
GA	Gentamicin sulfate amphoterin
HBSS	HEPES buffered saline solution
HDPF	Human dental pulp fibroblasts
HFSC	Hair follicle stem cells

HGF	Hepatocyte growth factor
HMEC	Human microvascular endothelial cells
HSC	Hematopoietic stem cell
HUVEC	Human umbilical vein endothelial cell
HUVEC	Human umbilical vein endothelial cells
ICA	Internal carotid artery
Il	Interleukin
iPSCs	Induced pluripotent stem cells
KCl	Potassium chloride
KH_2PO_4	Potassium phosphate
L	Litre
LB	Lithium borate
M	Mole
MANOVA	Multivariate analysis of variance
mg	Milligram
min	Minute
ml	Millilitre
MRI	Magnetic resonance imaging
MSC	Mesenchymal stem cell
$Na_2HPO_4.2H_2O$	Sodium phosphate dibasic
ng	Nanogram
nm	Nanometer
PA	Peptide-amphiphile
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor

PDL	Periodontal ligament
PDLSC	Periodontal ligament stem cell
PFA	Paraformaldehyde
pH	Potential hydrogen
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
RT-CIM	Real-time cell invasion and migration
RT-PCR	Reverse-transcriptase polymerase chain reaction
s	Second
SC	Satellite cells
SCAP	Stem cells from apical papilla
SHED	Stem cells from exfoliated deciduous teeth
TGF	Transforming growth factor
TNF	Tumour necrosis factor
VE	Vascular endothelial
VEGF	Vascular endothelial growth factor
α	Alpha
α-MEM	Alpha minimum essential medium
β	Beta

ANALISIS EKSPRESI GEN SEL TUNJANG DARIPADA GIGI SUSU TERKELUPAS DALAM PROSES MIGRASI DAN ANGIOGENIK SETEMPAT UNTUK PEMBAIKIAN TISU

ABSTRAK

Sel tunjang memainkan peranan penting semasa proses angiogenik (pembentukan tisu salur darah) semasa proses pembaikan tisu. Sel tunjang daripada gigi susu yang terkelupas (SHED) memiliki ciri-ciri sel tunjang mesenkima (MSC) yang menunjukkan kebolehan untuk membentuk pelbagai susur galur sekaligus menunjukkan potensinya yang besar untuk bermanfaat kepada terapi berasaskan sel dan pemulihan tisu. Walau bagaimanapun, keupayaan migrasi dan ekspresi gen oleh SHED semasa proses angiogenik belum dapat difahami sepenuhnya. Kajian ini menyasarkan untuk menentukan perbezaan pola ekspresi gen SHED yang bermigrasi dan melalui perubahan angiogenik. Kesan perbezaan ketumpatan awal pembenihan dan media aruhan ke atas migrasi SHED turut dikaji. Secara ringkasnya, SHED dikembang biakkan secara *in vitro* dan kemudian dikultur menggunakan 10 ng/ml faktor pertumbuhan endotelium vaskular (VEGF) dalam 2 ml medium pertumbuhan endotelium 2 (EGM2). Kajian gores dijalankan di dalam ruang transwell untuk menentukan kadar ketumpatan pembenihan permulaan SHED yang optimum dengan membandingkan 5,000 sel/cm^2 dan 10,000 sel/cm^2 sebelum aruhan angiogenik. Perbandingan lanjut telah dibuat untuk melihat kesan media aruhan angiogenik ke atas kadar migrasi SHED. Tiga kumpulan SHED telah dikembangkan untuk mengkaji ekspresi gen; kumpulan aruhan angiogenik (+A-M), kumpulan aruhan angiogenik dengan migrasi (+A+M), dan kumpulan pemalar (-A-M). RNA diekstrak pada titik masa yang berbeza (hari 1, 3, 7, 10 dan 14). RT-PCR Satu Langkah telah dijalankan

untuk menaksir tahap ekspresi gen untuk siri penanda gen angiogenik, migrasi dan MSC. Secara umum, data menunjukkan keupapayaan SHED untuk membentuk sel daripada susur galur angiogenik. Ketumpatan pembenihan yang tinggi dapat meningkatkan kadar migrasi SHED manakala proses aruhan angiogenik didapati telah mengurangkan kadar migrasi SHED. SHED didapati positif untuk penanda gen angiogenik; *Ang-1, IL-8* dan *VE-Cadherin* serta gen migrasi; *CCR1, CXCR4* dan *CCL28*. Malah, SHED juga mengekalkan ekspresi semua penanda gen MSC iaitu *CD73, CD90* and *CD105*, walaupun selepas aruhan. Pola ekspresi gen angiogenik dan gen migrasi oleh SHED menunjukkan cabaran dalam memahami kedua-dua proses tersebut yang terlibat dalam proses pembaikan tisu. Namun, kefahaman yang lebih baik tentang interaksi SHED-chemokine adalah amat diperlukan untuk membolehkan penggunaan SHED dengan lebih berkesan dalam terapi berasaskan sel.

GENE EXPRESSION ANALYSIS OF STEM CELL FROM EXFOLIATED DECIDUOUS TEETH IN MIGRATION AND LOCAL ANGIOGENESIS OF TISSUE REPAIR

ABSTRACT

Stem cells play essential role during the angiogenesis process of wound healing. Stem cells from exfoliated deciduous teeth (SHED) possess mesenchymal stem cell (MSC) characteristics, showing the ability to differentiate into various lineages, indicating their massive potential to benefit cell-based therapy and tissue repair. Nevertheless, the behaviour of SHED engaging in angiogenesis in terms of its migratory capacity and gene expression regulation remains questionable. The present study aims to analyse the gene expression pattern of SHED undergoing migration and angiogenic differentiation. Together, the effect of different initial seeding density and induction medium on the migration of SHED were assessed. SHED were expanded *in vitro* and induced for angiogenesis by supplementation of 10 ng/ml of vascular endothelial growth factor (VEGF) in 2 ml of endothelial growth medium 2 (EGM2). Scratch test assay was conducted in the transwell chamber to determine the optimum initial seeding density for SHED by comparing 5,000 cells/cm^2 and 10,000 cells/cm^2 prior to angiogenic induction. A further comparison was made to assess the effect of angiogenic induction media on SHED migration rate. Three groups of SHED were assessed the gene expression analysis: the angiogenic induction group (+A-M), angiogenic induction with migration group (+A+M), and the control group (-A-M). RNA was extracted at different time points (day 1, 3, 7, 10 and 14). One-step RT-PCR was then performed to assess the gene expression level of a series of angiogenic, migration and MSC gene markers. Overall, the data demonstrated a high capability of

SHED committing to angiogenic lineage. Higher seeding density (10,000 cells/cm^2) increased SHED migration, whereas angiogenic induction suppressed SHED migratory capacity. SHED positively expressed *Ang-1, Il-8,* and *VE-Cadherin*, the angiogenic markers, as well as the migratory gene markers (*CCR1, CXCR4* and *CCL28*). SHED also maintains the stemness level by positive expression of *CD73, CD90* and *CD105* during the induction protocol. The gene expression pattern of both angiogenic and migratory gene markers observed within this study indicate the complexity of understanding these two events during tissue repair. Indeed, a better understanding of these SHED-chemokine interactions is needed to enable the effective use of SHED in cell-based therapies.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Wound healing begins when bleeding occurs. Angiogenic blood and coagulant proteins enter the wound site, followed by growth factors that were produced by inflammatory cells, metabolic by-products and coagulation proteins (Hunt *et al.*, 2008). Macrophages were recruited to the wound bed and stimulate the proliferation of fibroblasts, keratinocytes, endothelial cells, epithelial cells and connective tissues to induce extracellular matrix formation, neovascularisation and reepithelialisation (Delavary *et al.*, 2011). Next, in response to high metabolic needs at the wound area, fibroblast will help to form the extracellular matrix. At the same time, endothelial cells will line the capillary tubes to repair the blood flow (Schugart *et al.*, 2008). The final stage of wound healing involves the maturation of the granulation tissue which forms mature connective tissues or an avascular scar (Enoch and Leaper, 2008; Strodtbeck, 2001). Positive outcomes in tissue repair can be achieved through an effective approach to wound healing.

Efficient wound management is fundamental in all living organisms. The discovery of the importance of stem cells as therapeutic agents in wound treatment has gained much interest from the public in recent years. The used of stem cells in cell-based therapies helps in achieving the primary aim of wound treatment; a rapid closure of the lesion along with a functional and aesthetically satisfactory scar (de Mendonça, 2012). Moreover, the transplanted stem cells can assist the healing of an acute and chronic wound by differentiating into multiple types of cells and providing cytokines and growth factors needed by the healing area, making the process simple and less time-consuming (Kim and Suh, 2010; You and Han, 2014).

Stem cells are unspecialised biological cells that can differentiate into committed cells by undergoing self-regeneration. In dentistry, stem cells offer great potential in providing better treatment modalities for the patients. Stem cell-based therapies could help in new advances in treating damaged teeth, inducing bone regeneration and treating neural injury (Narang and Sehgal, 2012). In general, there are three significant types of stem cells in regenerative medicine; the embryonic stem cells, adult stem cells and induced pluripotent stem cells.

One of the most advanced types of medical-scientific approach in clinical applications includes adult stem cells research. Among the various sources of adult stem cells, an immature stem cell population from human exfoliated deciduous (primary) teeth (SHED) were successfully isolated and characterised (Martinez Saez *et al.*, 2016). SHED are progenitor cells isolated from the pulp remnant of exfoliated (natural resorption of primary tooth's root) deciduous teeth. SHED are highly proliferative cells, possessing a clonogenic capacity to differentiate into a variety of cell types, such as neural cells, adipocytes, and odontoblasts (Goomer *et al.*, 2014; Park *et al.*, 2016). SHED remain feasible, convenient and affordable to collect, which hold promise for a range of potential therapeutic applications (Bansal and Jain, 2015).

Transplanted SHED were found to provide therapeutic potential in liver regeneration since it improves the hepatic dysfunction in primary and secondary recipients and directly transform into hepatocytes without cell fusion in carbon tetrachloride-treated mice (Yamaza *et al.*, 2015). SHED may potentially be used in the treatment of immune disorders such as SLE and ischemic kidney injury by reversing the damaging effects of the disease (Hattori *et al.*, 2015; Yamaza *et al.*, 2010). Despite the increasing researches conducted on SHED, the information is still lacking to enable SHED usage in regenerative therapy.

Angiogenesis is the physiological and pathological formation of blood vessels from the existing vasculature (Adair and Montani, 2010; Michaelis, 2014). In the early stage of tissue repair, angiogenesis helps to provide nutrients and oxygen to the reparative cells through the restoration of blood flow to the damaged tissue, marking the beginning of a healing response (Johnson and Wilgus, 2014; Willenborg *et al.*, 2012). Increasing the efficiency of directional stem cell homing might directly increase the process of angiogenesis and subsequently accelerate wound healing time. Since uncontrolled angiogenesis has long been associated with invasive tumour growth, metastasis, and cancer progression, the process of angiogenesis in tissue repair must be precisely controlled and monitored to avoid such events.

Another vital aspect in tissue reconstruction is the homing process, which refers to the migratory capacity of progenitor cells to travel from the neighbouring area to the damage site and further support the process of new tissue formation. The delivery or migration of stem cells to the damaged area is influenced by multiple factors including age and passage number of the cells, culture conditions, and the delivery method (Khaldoyanidi, 2008; Sohni and Verfaillie, 2013). Studies on angiogenesis have gained much interest in recent years due to their essential role in regulating the critical event in tissue regeneration. Endothelial cell migration is essential to angiogenesis as chemotactic, hepatotactic and mechanotactic stimuli directionally regulate this process and further involves in the degradation of the extracellular matrix to enable the progression of the migrating cell (Lamalice *et al.*, 2007).

Previously, cellular migration during angiogenesis has been performed using various type of cells, including the human umbilical vein endothelial cells (HUVEC), endothelial cells (EC) and human dental pulp stem cells (hDPSC) (Ausprunk and Folkman, 1977; Bronckaers *et al.*, 2013; Daub and Merks, 2013; Dissanayaka *et al.*,

2014; Kang *et al.*, 2016). HUVEC proved to have a high potential to be used in therapy, but the application may be constrained by both medical and ethical issues (Seidl *et al.*, 2012). As an alternative, SHED can be used without arising any ethical concern. However, there are a limited number of studies conducted to investigate the migration of SHED during angiogenesis. The present study, aims to investigate the migratory capacity of SHED within an *in vitro* environment, as well as analysing the regulation of gene that involved in cell migration and angiogenesis.

1.2 Justification

The impeccable cellular properties of SHED in term of its migratory capacity and gene expression level during the proses of angiogenesis have not been fully understood. Despite the numerous studies conducted on other types of dental stem cell, the cellular migration of SHED during angiogenesis has not been documented in details. This study aims to obtain an insight into SHED's behaviour during the formation of blood vessels and migration in wound healing. The simple monolayer model will mimic the condition during the process of wound healing. This process involves the migration of endothelial cells to the infected area but at the same time undergoing angiogenesis to produce new blood vessels. Screening of the gene markers expressed during these processes will help us to understand more about the complicated gene regulatory of SHED during the repair process. The gene markers selected for this study were rarely screened on SHED and some of them has never been detected on SHED before. The completion of this study will help researchers to understand the regulatory process involved and further exploit the process with the identification and formulation of new therapeutic options to enhance tissue repair.

1.3 Objective

1.3.1 General objective

To investigate the chemotaxis of stem cells from human exfoliated deciduous teeth (SHED) in 2D culture system with angiogenic differentiation medium.

1.3.2 Specific objectives

1. To determine the optimal seeding density of SHED in association with stem cell migratory capabilities during angiogenic differentiation.
2. To assess the gene expression level of angiogenic, migratory and stem cell gene markers of SHED cultured in angiogenic differentiation medium.

1.4 Research questions

1. Does the migratory rate of SHED proportional to high/low seeding density?
2. Can SHED maintain the gene expression of stem cell markers and express angiogenic and migratory markers following the angiogenic differentiation protocol?

1.5 Hypothesis

1. SHED migratory rate is proportional to high/low seeding density.
2. SHED potentially maintained the gene expression of stem cell markers and expressed angiogenic and cell migratory markers following the angiogenic differentiation protocol.

CHAPTER 2

LITERATURE REVIEW

2.1 The cellular biology of wound repair

Wound healing begins immediately at the moment of injury following surface lesions or just after exposure to radiation, chemical agents or extreme temperatures (Değim et al., 2002). Repair, in the context of healing of damaged tissue, can be defined as the restoration of tissue architecture and function after an injury which consists of two separate processes: regeneration and replacement (Krafts, 2010). A natural wound healing process is a complex and continuous activity involving blood coagulation, acute inflammation, cell migration, proliferation and differentiation, angiogenesis, re-epithelialisation, synthesis and remodelling of extracellular matrix (ECM) (Maxson *et al.*, 2012). In general, wound healing occurs in three phases: the inflammatory phase, the reparative phase, and the maturation phase, as shown in Figure 2.1.

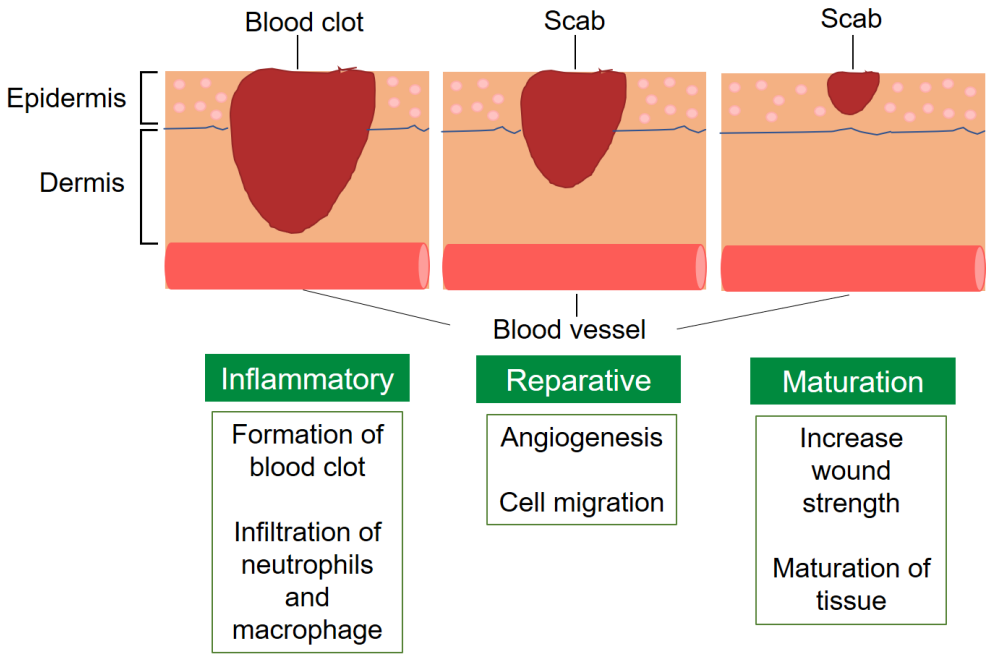

Figure 2. 1 Stages of wound healing.

2.1.1 The inflammatory phase

Inflammatory is the first phase that takes place immediately after the injury, which consists of coagulation and hemostasis. Platelet aggregation and haemostasis cascade help to prevent local haemorrhage, and the blood clot contributes to the stopping of the bleeding and serves as a provisional matrix for the wound healing (Laurens *et al.*, 2006). Blood clot formation also enhances platelets adhesion and aggregation, which later form a procoagulant surface that produces thrombin and fibrin to help prevent blood loss at sites of vascular injury (Nurden *et al.*, 2008). Arterial thrombus formation was initiated when the platelet adhesion on the reactive subendothelial matrix proteins (von Willebrand factor (VWF), collagens type I, III and VI) take place through the specific platelet receptors glycoproteins (GP) Ib-IX-V or GPVI (Savage *et al.*, 1998).

Next, sequential infiltration of neutrophils, macrophages and lymphocytes will take place to further supports an inflammatory reaction (Nishio *et al.*, 2008). Neutrophils will carry out the antimicrobial activity to prevent wounds from infections (Wilgus *et al.*, 2013). Another essential aspect of inflammatory phase is the presence of macrophage, a phagocytic cell that defence the host, promote and resolute the inflammation, as well as removing the apoptotic cells to support further cell proliferation and tissue restoration (Koh and DiPietro, 2011). The depletion of macrophage may result in increased levels of tumour necrosis factor-α (TNF-α) and reduced levels of both transforming growth factor-β1 (TGF-β1) and vascular endothelial growth factor (VEGF) in the wound, leading to delayed re-epithelialisation, reduced collagen deposition, impaired angiogenesis, and decreased cell proliferation in the healing wounds (Mirza *et al.*, 2009).

2.1.2 The reparative phase

The reparative phase or also known as proliferative phase is marked by the initiation of fibroblast migration, followed by the deposition of newly synthesised ECM, and an abundant formation of granulation tissue (Velnar *et al.*, 2009). Briefly, the diverse process of healing takes place during the reparative phase, starting with the proliferation and migration of keratinocytes at the wound edge through a process termed epithelialisation (Pastar *et al.*, 2014; Werner and Grose, 2003). Re-epithelialisation can be enhanced by the increase of TGF-β1 and microRNA-21 together with the increased of dermal fibroblast infiltration into the wound (Reynolds *et al.*, 2005; Yang *et al.*, 2011).

Following the migration of endothelial cells to the injury sites, angiogenesis will take place and further proliferate and differentiate to restore or create blood supply to the growing tissues (Crowther *et al.*, 2001). The most potent angiogenic cytokines in wound angiogenesis include VEGF, TGF-β, angiopoietin and fibroblast growth factor (FGF) (Li *et al.*, 2003b). Fibroblasts migrate into the wound tissue while producing the collagen-based ECM that ultimately replaces the provisional fibrin-based matrix and helps to reapproximate wound edges through their contractile properties (Baum and Arpey, 2005). Fibroblast gradually switches its dominant function to collagen production once they have migrated into the wound (Welch *et al.*, 1990).

The end of the proliferative phase is identified by the formation of granulation tissue (Sinno and Prakash, 2013). Within a few days, capillary sprouts begin to invade the fibrin/fibronectin-rich wound clot to form the organisation of microvascular network throughout the granulation tissue, leading to accumulation of collagen and production of immature ECM (Tonnesen *et al.*, 2000). The newly formed ECM (provisional matrix) provides a scaffold or conduit for cell migration, and once an abundant

collagen matrix has been deposited in the wound, collagen production by the fibroblasts will be terminated, and the fibroblast-rich granulation tissue is replaced by a relatively acellular scar (Clark *et al.*, 1982; Singer and Clark, 1999).

2.1.3 The maturation phase

Maturation or remodelling is the final phase of wound healing. This process will increase the wound strength for up to 2 years, with progressive organisation and development of the tissues (Steed, 1997). The remodelling of an acute wound maintains a balance between degradation and synthesis, increasing collagen bundles diameter, as well as the degradation of hyaluronic acid and fibronectin (Maxson *et al.*, 2012). Wound contraction occurs when abundant of ECM accumulation is reached by day 7, whereas fibroblasts transform into myofibroblast phenotype supplied with actin bundles that gather in the wound ECM by extending pseudopodia, attaching to ECM molecules, such as fibronectin and collagen (Clark, 1993). As the wound heals, apoptosis will further reduce the density of fibroblasts and macrophages (Greenhalgh, 1998). As time goes by, the growth of capillaries will stop, followed by reduced blood flow to the area and subsequent lower metabolic activity at the wound site (Falanga, 1998).

2.2 Tissue repair and regeneration

Tissue engineering is a form of tissue regeneration using an approach consisting of a biocompatible scaffold, appropriate growth factors and suitable type of stem cells. Cell-based tissue engineering that has been widely applied in regenerative medicine such as neurology, orthopaedic and dentistry (Chen *et al.*, 2015; Liu *et al.*, 2016; Tatara and Mikos, 2016; Yamauchi *et al.*, 2011). It is a relatively young field that combines engineering, clinical sciences and life sciences with the aim to repair or regrow tissues. A recent discovery in tissue reparative research provides a new era for therapeutic

medicine due to its rapid progress and the tremendous potential benefit involving all tissues in our body (Neel *et al.*, 2014; Patrick, 2001). Tissue repair and regeneration following injury demand a precise orchestration of complex signalling cascades to coordinate the growth of structures that are spatially proximate but physiologically distinct (Rennert *et al.*, 2012). The development of stem cells technologies and biomaterials can further design the tissue architecture where loss or damaged tissues will not be treated using only materials tolerated by the body but congregating biomaterials and biological principles to deliver to the body its original construction (Rosa, 2013). Hence, it is undeniably crucial for researchers to understand the role of each supporting elements in tissue engineering to ensure a successful outcome from the procedure.

2.2.1 Stem cells in tissue repair

Stem cell is one of the vital components of tissue repair. Stem cells are undifferentiated cells, which undergo asymmetrical cell division either to produce more stem cells or to differentiate to form specialised cells. The use of stem cells, which are often called 'master cells' enable the clinical practitioner to achieve bone repair, as well as reconstruction of injured or pathologically damaged dental structure with predictability without compromising on donor site morbidity (Sreenivas *et al.*, 2011). The cells are collected by isolating them from a patient or donor, followed by meticulous *in vitro* culture under appropriate conditions, and re-implantation into the defective sites of the patient to recover the previously normal function (Vishwakarma *et al.*, 2015).

2.2.1(a) Characterisation of Stem Cell

Characterisation of stem cells is crucial, as it will provide the essential information on the cells' niche, proliferation pattern, differentiation lineage and capacity. A defined

set of populations may provide a better understanding of the identity of the cells and reduce the risk of culturing contaminated cells. According to the guideline proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy, the three minimum criteria for characterisation of stem cells involves the plastic-adherent in culture condition, positive expression of CD73, CD90 and CD105 surface molecules, and the confirmation on the self-renewal capacity and ability to differentiate into specific lineages (Baghaei *et al.*, 2017).

Since many changes observed in the protein level are not proportionate to the level of the corresponding mRNA, the gene expression data can be coupled with the protein analysis to have a better understanding of how the cell behaviour is regulated (Lu *et al.*, 2009; van Hoof *et al.*, 2012). For example, a study on human MSC from multiple lineages reveals the involvement of *actin filament-associated protein, frizzled 7, dickkopf 3, protein tyrosine phosphatase receptor F,* and *RAB3B* genes in promoting cell survival and influencing the commitment of MSC (Song *et al.*, 2006). The results were confirmed on both gene and protein levels using RT-PCR and western blot.

2.2.1(a)(i) Stem cell markers expression

Human embryonic stem cells (hESCs) expressed several surface markers including various glycolipids and glycoproteins that were initially identified on human embryonal carcinoma cells or in human preimplantation embryos and the expression of specified surface markers is maintained in hES cells following prolonged periods of cultures (Hoffman and Carpenter, 2005). Pluripotent ESCs express markers including the nuclear transcription factors Oct4, Nanog and Sox2; the keratin sulfate antigens Tra-1-60 and Tra-1-81; and the glycolipid antigens SSEA3 and SSEA4 in human (Martí et al., 2013). Other than that, hESCs were also reported to expressed CD90, CD133, CD117 and CD135 (Carpenter *et al.*, 2004).

Meanwhile, in adult stem cell, the commonly identified markers that are expressed in mesenchymal stem cells (MSCs) are CD105, CD73, CD44, CD45, CD90 (Thy-1), CD71, as well as the ganglioside GD2, CD271 and STRO-1 (Dissanayaka et al., 2012; Uccelli et al., 2008). One of the minimum characteristics of MSCs is a positive expression of CD105, CD73 and CD90, with lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules (Dominici *et al.*, 2006).

Ecto-5'-nucleotidase as a glycosylphosphatidylinositol-anchored membrane protein which is defined as the lymphocyte differentiation antigen (CD73). The primary function of CD73 is to catalyse the final step in the hydrolysis of ATP to adenosine and proved to be a positive marker for human MSCs (Ramos *et al.*, 2016; Zhou *et al.*, 2007). MSC also expressed CD90 (Thy-1), a useful differentiation marker following the development of osteoblast (Wiesmann *et al.*, 2006). The reduction in CD90 expression enhances the osteogenic and adipogenic differentiation of MSCs *in vitro* which shows that CD90 controls the differentiation of MSCs by acting as an obstacle in the pathway of differentiation commitment (Moraes *et al.*, 2016). Meanwhile, CD105 (endoglin) is known as the accessory receptor for TGF-β and positively expressed as MSCs-specific cell surface markers (Kays *et al.*, 2014; Maleki *et al.*, 2014).

2.2.1(a)(ii) Auto-renewal capacity and differentiation lineage

Stem cells are well known for their ability to self-renew. This ability is essential in living organisms to be carried throughout the lifetime to repair damage tissues due to injury or illness. Self-renewal is the process by which a stem cell divides asymmetrically to generate one or two daughter stem cells that have a developmental potential similar to the mother cell (Shenghui *et al.*, 2009). However, stem cells do not have an endless capacity to divide or can undergo constant self-renewal, but the self-

renewal divisions are tightly regulated within the tissue to ensure lifelong maintenance (Fuchs and Chen, 2013). In fact, accumulation of DNA damage accompanies physiological stem cells ageing in human (Rübe *et al.*, 2011). Plus, under stress condition, stem cells functional capacity was severely affected leading to the loss of proliferative potential, diminished self-renewal, increased apoptosis and functional exhaustion (Rossi *et al.*, 2007).

Another important characteristic of stem cells is the ability to differentiate into other types of cells. Pluripotent stem cells are stem cells that can differentiate into all types of lineage or the three embryonic germ layers; endoderm, mesoderm and ectoderm (Pera and Tam, 2010; Thomson *et al.*, 1998). Two types of pluripotent stem cells include embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Evans and Kaufman, 1981; Romito and Cobellis, 2016; Takahashi and Yamanaka, 2006). *In vitro,* human pluripotent stem cells have been successfully cultured in a thermoresponsive hydrogel culture system with the ability to retain the long-term, serial expansion and to differentiate into multiple cell lineages (Lei and Schaffer, 2013). Meanwhile, multipotent stem cells are stem cells that can differentiate into all types of cells within one particular lineage (Khanlarkhani *et al.*, 2016). Adult stem cells can be classified as multipotent stem cells owing to the limited ability to differentiate into one or more cell lines (Sobhani *et al.*, 2017). Adipose-derived stem cell is one example of a multipotent adult stem cell. It can give rise to neuronal, osteogenic and adipogenic lineage (Huang *et al.*, 2007; Kakudo *et al.*, 2007; Lv *et al.*, 2015; Morandi *et al.*, 2016).

2.2.1(b) The different types of stem cell

Stem cells can be divided into three main types: ESCs that are derived from embryos; adult stem cells that are derived from adult tissues; and iPSCs that are generated artificially by reprogramming adult somatic cells into ESCs-like cells (Otsu *et al.*, 2014). In comparison to embryonic stem cell, adult stem cells are more preferred to be used clinically due to less ethical issues and low immunological responses (Deepika *et al.*, 2015). Adult stem cells have been purified from various adult tissues such as bone, blood and dental origin (De Wynter *et al.*, 1995; Simpson *et al.*, 2012; Wang *et al.*, 2017; Zhao *et al.*, 2017). The two most common types of adult stem cells, with distinct identified differentiation lineage, are the hematopoietic stem cell (HSC) and mesenchymal stem cells (MSCs) (Ulloa-Montoya *et al.*, 2007).

HSC is defined as cells that can undergo self-renewal and generate differentiated progeny of multiple blood cell lineages (myeloid and lymphoid) (Keller, 1992). HSCs occupy multiple niches, including sinusoidal endothelium such as spleen and bone marrow as well as endosteum (Kiel *et al.*, 2005). Immature HSC, located in the bone marrow after birth maintains the adequate production of blood cells besides being able to reconstitute the hematopoietic system in disease-related bone marrow failure and bone marrow aplasia (Gunsilius *et al.*, 2001). Other than that, HSC that reside primarily in the bone marrow do circulate in the peripheral blood and can replenish damaged or missing components of the hematopoietic and immunologic system (Trigg, 2004).

MSCs, a type of multipotent stem cell that is found within the bone marrow microenvironment, defined by its ability to differentiate into the osteogenic, chondrogenic, tendonogenic, adipogenic, myogenic and endothelial cell lineages (Majumdar *et al.*, 1998; Oswald *et al.*, 2004). Compared to other types of adult stem

cell, MSC and MSC-like cells offer better therapeutic potential as they can differentiate not only into mesenchymal lineages but also into ectodermal and endodermal derivatives, as well as regeneration of other connective tissues such as dentin, cementum and periodontal ligament (Chanda *et al.*, 2010; Shi *et al.*, 2005). Bone marrow, umbilical cord blood and adipose tissue are among the known sources of MSCs (Dharmasaroja, 2009; Erices *et al.*, 2000; Kern *et al.*, 2006; Zuk *et al.*, 2002).

On top of that, brain, spleen, liver, kidney, lung, bone marrow, muscle, thymus, pancreas are the different organs and tissues reported to be the source of cells with mesenchymal stem characteristics (da Silva Meirelles *et al.*, 2006). *In vivo*, MSCs stain positive by flow cytometry for haematopoietic markers CD29, CD73, CD90, CD105 and CD166. MSCs also demonstrate prolonged skin allograft survival and possess several immunomodulatory effects (Le Blanc and Ringden, 2007). Interactions of human MSCs with the various immune cells are shown to inhibit the inflammatory responses and promote the anti-inflammatory pathways (Aggarwal and Pittenger, 2005). Among other sources of stem cells, dental tissue has been identified as a rich source for the adult stem cells with mesenchymal stem cell characteristics.

2.2.1(c) Stem cells from the oral and maxillofacial region

Dental stem cells were successfully isolated from various sources within the oral and maxilla facial region, which includes the dental pulp, exfoliated deciduous teeth (SHED), the periodontal ligament, the dental follicle and the dental papilla and many more (Chadipiralla *et al.*, 2010; Mangano *et al.*, 2010; Morsczeck *et al.*, 2010; Yagyuu *et al.*, 2010). This mesenchymal-stem-cell-like population exhibits the capacity for self-renewal and multilineage differentiation potential (Huang *et al.*, 2009; Ulmer *et al.*, 2009). The next section will briefly introduce the different types of dental stem

cells. These stem cells have been purified and characterised for their potential role in tissue repair and regeneration. Figure 2.2 displays the different sources of stem cells from the oral and maxillofacial region.

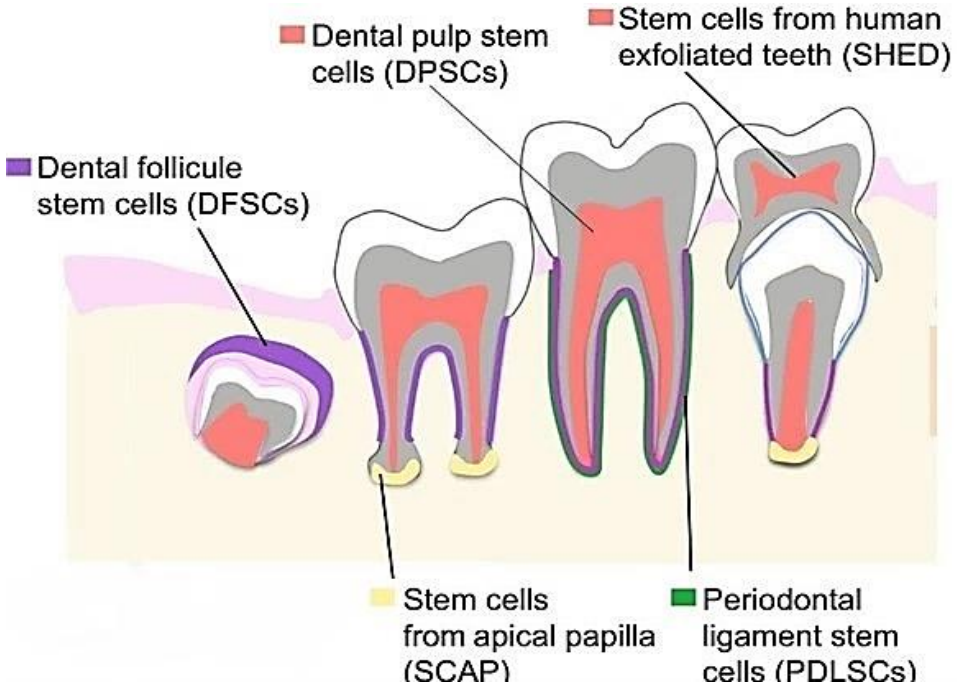

Figure 2. 2 Sources of dental stem cells. Reproduced from Sharpe (2016).

2.2.1(c)(i) Stem cell from human exfoliated deciduous teeth (SHED)

SHED possess mesenchymal-like characteristics with potential benefit for clinical applications, owing to the feasibility of isolation and less ethical concern. SHED are highly proliferative multipotent cells derived from primary teeth that usually being discarded (Rosa *et al.*, 2016). SHED express two early mesenchymal stem-cell markers, the cell surface molecules STRO-1 and CD146 (MUC18) (Miura *et al.*, 2003b). SHED offer massive potential for development, owing to their rapid maturation as opposed to other types of adult stem cells (Yin *et al.*, 2016). Besides, SHED can also be preserved for up to 3 years using cryopreservation techniques, without affecting the biological, immunological and therapeutic function of the cells

(Ma *et al.*, 2012). Reports indicated no significant differences between characteristics and population doubling time of cryopreserved SHED and fresh SHED (Lee *et al.*, 2015; Lee *et al.*, 2011b).

SHED is an ideal candidate to repair damaged tooth structures, induce bone regeneration and possibly treat neuronal tissue injury or degenerative diseases. SHED have high proliferation ability and can differentiate *in vitro* and *in vivo*, forming neurons, adipocytes, odontoblasts, osteoblast and chondrocytes (Silva, 2015; Yu *et al.*, 2014). Moreover, SHED has been proven to be able to differentiate into angiogenic endothelial cells, odontoblasts and smooth muscle cells for vascular tissue engineering (Sakai *et al.*, 2010; Xu *et al.*, 2017). A clearly defined characteristics and biological activity of SHED may enable their application in cell-based repair treatment, pretty much sooner than expected.

2.2.1(c)(ii) Dental Pulp Stem Cell (DPSC)

Dental pulp stem cell which resides within the perivascular niche of the dental pulp is thought to arrive from migrating cranial neural crest (CNC) cells. DPSC provides a readily accessible source of exogenous stem/precursor cells for therapeutic paradigms to treat neurological disease (Arthur *et al.*, 2008). DPSC remain detectable in humans up to the age of 30, by producing sporadic calcified nodules *in vitro* and forming a mineralised tissue after transplantation *in vivo* (Laino *et al.*, 2005). Under chemically defined culture condition, DPSCs can be induced to undergo normal differentiation into smooth and skeletal muscles, neurons, cartilage and bone *in vitro* while showing dense engraftment in various tissues after *in vivo* transplantation of these cells into immunocompromised mice (Kerkis *et al.*, 2006). DPSCs are ideal for tissue reconstruction as they possess easy access to the collection site, produces very low morbidity, highly efficient extraction of stem cells from pulp tissue, extensive

differentiation ability and the desirable interactivity with biomaterials (d'Aquino *et al.*, 2008).

2.2.1(c)(iii) Stem cells from apical papilla (SCAP)

Stem cells from apical papilla is a population of stem cells isolated from the apical root papilla of human teeth. SCAP expressed MSC markers such as STRO-1, ALP, CD24, CD29, CD73, CD90, CD105, CD106, CD146, CD166 and ALP. The population of SCAP appear to have a greater capacity for dentin regeneration than DPSC since they can form odontoblast-like cells, are likely to be the cell source of primary odontoblasts for the root dentin formation as it produced dentin *in vivo* (Sonoyama *et al.*, 2006; Sonoyama *et al.*, 2008). SCAP is the primary source of odontoblasts that are responsible for the formation of root dentin (Huang *et al.*, 2008). SCAP cultures also showed a significantly higher proliferation rate and mineralisation potential compared to DPSC which might be of significance for their use in bone/dental tissue engineering (Bakopoulou *et al.*, 2011).

2.2.1(c)(iv) Periodontal ligament stem cell (PDLSC)

The periodontal ligament stem cell is another type of stem cell from the dental origin. PDLSC represent a unique mesenchymal stem cell population as demonstrated by their capacity to differentiate into a cementoblast-like cells/periodontal ligament-like tissue, adipocytes and collagen-forming cells *in vivo*, contribute to periodontal tissue repair while displaying cell surface marker characteristics (STRO-1 and CD146/MUC18) and differentiation potential similar to bone marrow stromal stem cells (BMSSCs) and DPSC (Seo *et al.*, 2004; Wada *et al.*, 2009). PDLSC are capable of regenerating periodontal tissues, leading to favourable treatment for periodontitis (Gay *et al.*, 2007; Liu *et al.*, 2008). PDLSC can also repair allogeneic bone defects in an experimental model of periodontitis without causing immunological rejections, likely due to the low

immunogenicity and immunosuppressive function possessed by PDLSCs (Ding *et al.*, 2010).

2.2.1(c)(v) Dental follicle precursor cells (DFPC)

Dental follicle precursor cells can differentiate and form healthy connective tissues and produce clusters of mineralised tissue (Morsczeck *et al.*, 2009). DFPC derived from neural crest cells is a type of transient cell population in developing vertebrates and progenitors for the peripheral nervous system. These type of cells are favourable for neural differentiation and neural tissue regeneration due to the ability to perform transdifferentiation into epithelial-like cells (Beck *et al.*, 2011). DFPC represent cells from a developing tissue which is in common with SCAP and might be more plasticity than other dental stem cells, owing to the ability to form the periodontal ligament (PDL) by differentiating into PDL fibroblasts that secrete collagen and interact with fibres on the surfaces of adjacent bone and cementum (Estrela *et al.*, 2011). There are no ethical issues regarding DFPC isolation since dental follicle is present in impacted teeth which are commonly extracted and disposed of as medical waste (Bojic *et al.*, 2014).

2.2.2 Growth factors in tissue repair

Cytokines are small proteins that are released by cells that specifically affected the interactions and communications between cells (Zhang and An, 2007). Growth factors are cytokines that stimulate cell growth and are widely used in clinical practice, such as treatment of nervous system disease, wound healing, revascularisation, and bone repair (Zhao *et al.*, 2015). The delivery of growth factors can regulate the provision of environment for cell development (Lee *et al.*, 2011a). In some cases, growth factors that are combined with scaffold demonstrate an increase new bone formation by promoting the infiltration of the cell into the scaffold as well as the differentiation of

progenitor cells into a specific cell and tissue type (Blackwood *et al.*, 2012). Growth factor also plays a vital role in tissue repair as the exogenous growth factor may further support the proliferation of cellular components that involved in tissue development and the healing process (Chen *et al.*, 2010).

Several examples of cytokines involved in angiogenesis of tissue repair include Angiopoietin-1, Interleukin-8 and VE-Cadherin. Angiopoietin-1 (Ang-1) is an angiogenic factor that signals through the endothelial cell-specific Tie2 receptor, a tyrosine kinase that is essential for normal vascular development in the mouse similar to VEGF (Maisonpierre *et al.*, 1997). Ang-1 also prevent apoptosis, support vascular sprouting or branching and stabilise the blood vessels (Babaei *et al.*, 2003; Taura *et al.*, 2008). Other than that, Ang-1 requisitely collaborate with VEGF during blood vessel development; thus *Ang-1* mRNA was expressed in tumour cells (Gale *et al.*, 2002; Stratmann *et al.*, 1998).

Interleukin-8 (IL-8) promotes angiogenic responses in endothelial cells, infiltrates neutrophils at the tumour site and also enhances the proliferation, survival and apoptosis inhibitor of CXCR1- and CXCR2-expressing endothelial cells (Li *et al.*, 2003a; Waugh and Wilson, 2008). Besides, *IL-8* is an important pro-angiogenic mediator which increase endothelial permeability during the early stage of angiogenesis and induces the migration and proliferation of endothelial cells and smooth muscle cells (Petreaca *et al.*, 2007; Simonini *et al.*, 2000).

Vascular Endothelial (VE)-Cadherin mediates specific intracellular signalling in the endothelium that modulate the functional reactivity (Lampugnani and Dejana, 1997). VE-Cadherin is an essential adhesive molecule at the inter-endothelial adherent junction as it controls vascular integrity and normal organ function (Corada *et al.*,

1999). In addition, the control of vascular integrity and vascular permeability requires the expression of VE-Cadherin since it can transfer information intracellularly through interaction with a complex network of cytoskeletal and signalling molecules (Dejana *et al.*, 1999).

Whereas, CCR1, CXCR4 and CCL28 are several examples of cytokines involved in the cell migration of tissue repair. CCR1 can promote the migration of monocytes, macrophages and T cells (Schall and Proudfoot, 2011). Overexpression of CCR1 increased the migration of murine MSC, protected it from apoptosis and also enhanced the survival and engraftment of MSC (Huang *et al.*, 2010a). Other than that, CCR1 was proven to be correlated to structural changes in the airway suggests that CCR1-positive cells have an active role in tissue remodelling and together with CCR4, they form functional receptors on human cord-blast derived mast cells with the capacity to mediate migration towards CCL5 (Amin et al., 2005; Juremalm et al., 2002).

CXCR4 is important in the migration, homing, survival and development of multiple cell types including in the nucleus of the migrating hematopoietic stem cell, and have a dynamic expression that regulated by both autocrine and paracrine mechanisms to induce multiple cell responses (Bhakta *et al.*, 2006; Dar *et al.*, 2006). *In vivo*, intravenous infusion of MSC with CXCR4 overexpression leads to increased in the number of MSC homing in the infarct region, decreased in anterior wall thinning and improved chamber compliance following coronary occlusion in rats (Cheng *et al.*, 2008).

CCL28 was expressed in the large and small intestine as it is important in maintaining gut immunity and promoting immune cell migration (Nagafusa and Sayama, 2020). CCL28 production that was regulated by NF-kB pathway plays a crucial role following

spinal cord injury by recruiting CCR10-expressing and immunosuppressive Treg cells (Nagafusa and Sayama, 2020). Besides, CCL28 signalling via its primary receptor, CCR10 promotes wound angiogenesis in endothelial cells through endothelial Nitric Oxide Synthase-dependent Src, p13K and MAPK signalling (Chen *et al.*, 2020).

2.2.3 Scaffold in tissue repair

Scaffolds for tissue repair and regeneration are designed to influence the physical, chemical and biological environment surrounding a cell population (Howard *et al.*, 2008). An ideal biomaterial scaffold will provide mechanical support to an injured site and also deliver growth factors and cells into a defect to encourage tissue growth besides degrading in a controlled manner without causing a significant inflammatory response (Mistry and Mikos, 2005). There are two types of scaffold used in the reparative process; either originated from natural resources or synthetically created (Dhandayuthapani *et al.*, 2011). Some example of biological scaffold includes human amniotic membrane (HAM) that were used in cartilage repair and regeneration studies (Díaz-Prado *et al.*, 2010; Jin *et al.*, 2007). Chitosan is also a type of natural scaffold used in melanocyte transplantation and biocompatibility study in mice (Lin *et al.*, 2005; VandeVord *et al.*, 2002).

Other than that, extracellular matrix (ECM) was also successfully used as a natural scaffold in facilitating the recruitment of marrow-derived cells into remodelling sites in mice and restoration of the musculotendinous junction in canine (Badylak *et al.*, 2001; Turner *et al.*, 2010). As for synthetic scaffold, peptide, polycaprolactone, hydrogel, composite silk and nano/micro-fibre-combined scaffolds had been used in various *in vitro* and *in vivo* tissue repair studies (Rustad *et al.*, 2012; Santos *et al.*, 2008; Schantz *et al.*, 2007; Seo *et al.*, 2009; Wang *et al.*, 2008). Positive remodelling, vascular formation, and tissue repair depend significantly on the ability of cells to

migrate into or through the injected scaffold material *in vivo* (Singelyn and Christman, 2011).

2.3 Angiogenesis study in tissue repair

Angiogenesis is new capillaries sprouting from a pre-existing blood vessel that includes complex multistep processes involving remodelling of the extracellular matrix, migration and proliferation of endothelial cells, lumen formation and functional maturation of blood vessels (Breier and Risau, 1996). Studies of angiogenesis in stem cell-based tissue repair mainly focus on ensuring the survival and functional capacity of transplanted tissues in the treatment of angiogenic-related diseases, such as stroke, cardiovascular diseases and wound healing by using stem cells (Fatimah *et al.*, 2013). The increased of angiogenesis following transplantation can help to relieve the symptoms of ischemia through the supply of oxygen and nutrients which lead to the restoration of damaged tissues (Reddy *et al.*, 2020).

2.3.1 *In vitro* monolayer and 3D culture

As the importance of the angiogenesis process in tissue repair has been well known, various studies had been performed to investigate the mechanism and other relevant factors influencing the process. The vast majority of earlier angiogenesis studies were conducted using the approach of culturing monolayer cells directly on the plastic surface (Gajdusek *et al.*, 1993; Merwin *et al.*, 1990; O'Connor *et al.*, 2000). MSC monolayer studies have been used to show the angiogenic function of BMSCs in direct and indirect co-cultures and to prove that BMSCs are able to maintain their lineage-specific angiogenic differentiation (Böhrnsen and Schliephake, 2016). However, as more information is successfully gathered on the characteristics of the cells, researchers started to use scaffold or 3D culture to study the process in a more complex

environment. MSCs significantly demonstrated lower expression of VEGF, SDF, and HGF, compared to spheroid-cultured-MSCs (Lee *et al.*, 2016).

In order to mimic the complex extracellular matrix structure, scaffold, one of the key components in tissue engineering, has been introduced to *in vitro* stem cell studies. In previous years, Matrigel scaffold has been successfully described in detail for their application in 3D angiogenesis studies using cells of various origins, including human umbilical vein endothelial cell (HUVEC), MSC, adipose-derived stem cell (ADSC), muscle-derived satellite cells and other types of adult stem cells from human and rat (Cai *et al.*, 2015; Liang *et al.*, 2007b; Mohammadi *et al.*, 2015; Oswald *et al.*, 2004; Tapon-Bretaudière *et al.*, 2002). From time to time, various types of scaffolds have been tested for angiogenic induction protocol. For instance, peptide-amphiphile (PA) and growth factor infused transparent 3D hydrogel was found to exhibit a significant increase of angiogenesis around the injected site, at the back of experimental mice (Hosseinkhani *et al.*, 2006).

2.3.2 *In vitro* angiogenic induction protocol

Diverse methods have been used over the years to induce angiogenesis within the *in vitro* culture. VEGF is the classic inducer of angiogenesis that is used widely in the angiogenic study. It has been proved that VEGF actively enhances angiogenesis of endothelial cell (Ferrara *et al.*, 2003). Magnetic resonance imaging (MRI) was used to measure the effects of VEGF on angiogenesis of embolic ischemic rats, and it was found that VEGF accelerated the angiogenesis in the ischemic brain and lowered neurological deficits during stroke recovery (Zhang *et al.*, 2000). EGM2 medium is another inducer used in the induction of angiogenesis (Arnaoutova and Kleinman, 2010; Souza *et al.*, 2018). A study on angiogenic differentiation of human MSC