SIGNALLING PATHWAYS INVOLVED IN IL-8 MEDIATED ODONTOGENIC DIFFERENTIATION OF SHED CULTURED ON HUMAN AMNIOTIC MEMBRANE WITH BMP-2

By

ZUL FAIZUDDIN BIN OSMAN

Thesis submitted in the fulfilment of the requirements for the degree of

Doctor of Philosophy

December 2021

ACKNOWLEDGMENT

I owe my deepest gratitude to Dr Khairul Bariah Ahmad Amin Noordin, my principle supervisor, whose sincerity and encouragement I will never forget. I will be forever grateful for the opportunity given to me. Her mentorship, advice and unsurpassed knowledge have been an inspiration to me throughout these years.

I am sincerely thankful to my co-supervisors, Associate Professor Dr Azlina Ahmad and Associate Professor TP Kannan, whose encouragement and supervision enabled me to develop a deeper understanding of cell biology. I would like extend my huge, warm thanks to Fuad, Wafa', Siti Nurnasihah and Hamshawagini for the informal support and encouragement. This project was carried out in the Craniofacial Laboratory, School of Dental Sciences between 2015 and 2020. Special thanks to Universiti Sains Malaysia (USM) for the financial support through Graduate Assistant Scheme and USM Research University Grant 1001/PPSG/812127 for the research funding.

I am thankful for the everlasting love from my parents and my siblings. To my late father, I hope that this work makes you proud. I miss you ayah.

And last, but not least, I would like to dedicate my biggest appreciation to my wife, Farizan Ahmad and my beloved children, Mikael and Nessa who have been my emotional base during my study. I wrote my thesis with them by my side. To them, thank you very much for always believing in me. Words can never say enough, how much I do love you all. Thank you.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
µg	Microgram
µl	Microliter
µm	Micrometer
µM	Micromolar
ABMSCs	Alveolar bone-derived MSCs
AIDS	Acquired immunodeficiency syndrom
AKT	Protein kinase B
ALP	Alkaline phosphatase
AM	Amniotic membrane
APC	Allophycocyanin
APCs	Apical papilla cells
APS	Ammonium persulfate
BM	Bone marrow
BMMSCs	Bone marrow mesenchymal stem cells
BMP-2	Bone morphogenetic protein-2
BMPs	Bone morphogenetic proteins
BSP	Bone sialoprotein
CCL5	CC chemokine ligand-5
CD	Cluster of differentiation
cDNA	Complimentary DNA
CFU-F	Colony-forming unit-fibroblast

CNTF	Ciliary neurotrophic factor
Co-Smad	Common mediator Smad
Col 1	Collagen type 1 alpha 1 gene
CREB	cAMP response element-binding protein
CSCs	Cancer stem cells
CXCL8	C-X-C motif chemokine ligand 8
CXCR1	C-X-C chemokine receptor type 1
CXCR2	C-X-C chemokine receptor type 2
DEAE	Diethylaminoethyl cellulose
DEPC	Diethyl pyrocarbonate
Dex	Dexamethasone
DFPCs	Dental follicle progenitor stem cells
DMP1	Dentin matrix protein 1
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DPSCs	Dental pulp stem cells
DPP	Dentin phosphoprotein
dsDNA	Double-stranded DNA
DSP	Dentin sialoprotein
DSPP	Dentin sialophosphoprotein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular receptor kinase
et al.	and others

FAK	Focal adhesion kinase
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FGFs	Fibroblast growth factors
gDNA	Genomic DNA
GDF11	Growth differentiation factor 11
GMSCs	Gingival mesenchymal stem cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
g	Gram
HDPCs	Human dental pulp cells
HESCs	Human embryonic stem cells
HLA-DR	Human leukocyte antigen-DR
HMDS	Hexamethyldisilazane
hMSC	Human mesenchymal stem cell
HRP	Horseradish peroxidase
HYA	Hyaluronic acid
IFNs	Interferons
IAEA	International Atomic Energy Agency
IGFs	Insulin-like growth factors
IgG	Immunoglobin G
IL-1	Interleukin-1
IL-1α	Interleukin-1 alpha
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
IL-8	Interleukin-8

IL-10	Interleukin-10
ISCT	International Society for Cellular Therapy
JAK2	Janus kinase 2
JePEM	Human Research Ethics Committee
JNK	c-Jun N terminal kinase
KN-3	Rat odontoblast-like cells
kb	Kilobase
kGy	Kilogray
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MDPC-23	Mouse dental papilla cell-23
MEPE	Matrix extracellular phosphoglycoprotein
min	Minute
ml	Millilitre
mM	Millimolar
mm	Millimetre
MNA	Malaysia Nuclear Agency
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
Na_2HPO_4	Di-sodium hydrogen phosphate
NaH_2PO_4	Sodium phosphate monobasic
ng	Nanogram
NF-κB	Nuclear factor-κB

Nm	Nanometre
nM	Nanomolar
OCN	Osteocalcin
OPN	Osteopontin
PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem cells
PEG	Polyethylene glycol
pH	Potential hydrogen
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PLGA	poly lactic-*co*-glycolic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pen-Strep	Penicillin-Streptomycin
PerCP-Cy5.5	Peridinin-chlorophyll-protein-CY5.5
PIAS	Protein inhibitors of activated STAT
PLLA	Poly-L-lactic acid
PVDF	Polyvinylidine fluoride
qPCR	Quantitative PCR
RANKL	Receptor activator of nuclear factor kappa-B ligand
RGD	Arginine-glycine-aspartate
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
R-Pe	Phycoerythrin

rpm	Round per minute
Runx2	Runt-related transcription factor 2
SCAPs	Stem cells from apical papilla
Ser/Thr	Serine/Threonine
TGPCs	Tooth germ progenitor cells
V	Voltage
Wnt	Wingless-integration pathways
A	Alpha
B	Beta
γ	Gamma

TAPAK JALAN PENGISYARATAN YANG TERLIBAT DALAM PEMBEZAAN ODONTOGENIK SHED BERPERANTARA IL-8 YANG DIKULTUR DI ATAS MEMBRAN AMNIOTIK MANUSIA DENGAN BMP-2

ABSTRAK

Pengetahuan semasa mengenai kaedah perubatan menggunakan membran amnion (AM) sebagai perancah dalam bidang regeneratif pergigian adalah masih terhad. Kajian terdahulu menunjukkan bahawa faktor pertumbuhan protein tulang morfogenesis-2 (BMP-2) membantu pembezaan sel tunjang daripada gigi susu manusia yang terkelupas (SHED) melalui pengaktifan sitokin interleukin-8 (IL-8). Walau bagaimanapun, pengetahuan biologi asas mengenai perkara ini masih belum dapat difahami. Kajian ini bertujuan untuk mencari fungsi dan mekanisma pengisyaratan imunomodulatori IL-8 sewaktu pembezaan sel odontoblas dari SHED. Dalam kajian ini, SHED telah dikultur di atas AM dan dirawat dengan BMP-2. Analisis aliran sitometri, tindak balas berantai polimerase multipleks dan pemedapan Western telah dijalankan bagi menentukan kadar ekspresi penanda-penanda sel stem, ekspresi gen-gen sitokin yang terlibat dalam inflamasi, ekspresi protin penanda-penanda sel odontoblas, dan molekul yang terlibat dalam signal pengisyaratan imunomodulatori IL-8. Akhirnya, struktur ultra SHED dan sel seperti-odontoblas juga disiasat menggunakan mikroskop elektron penskanan (SEM). Hasil kajian menunjukkan potensi SHED untuk membeza kepada sel seperti-odontoblas apabila dikultur di atas perancah AM. SHED telah diperincikan melalui analisis aliran sitometri dan menunjukkan profil ekspresi pelbagai penanda sel stem seperti kluster pembezaan CD44, CD73, CD90, dan CD105 tetapi tiada ekspresi penanda hematopoietik CD34 dan CD45. Keputusan juga menunjukkan SHED mempunyai

ekspresi yang kuat bagi penanda-penanda permukaan sel stem mesenkima (MSC) CD90, CD44, CD105 dan CD73 dengan 100% ekspresi manakala hanya 84.1% bagi CD105. Di bawah stimulasi BMP-2, ia telah menunjukkan bahawa berlaku ekspresi sitokin pro-inflamatori seperti IL-8, IL-6, IL-1β, TGF-β dan TNF-α. Kejayaan pembezaan SHED kepada sel seperti-odontoblas telah ditunjukkan melalui ekspresi penanda-penanda odontoblas seperti dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), alkaline phosphatase (ALP) dan osteopontin (OPN). Penambahanan IL-8 secara eksogenus menunjukkan peningkatan ekspresi penanda-penanda odontoblas seperti DSPP, DMP1 dan ALP. Sewaktu pembezaan SHED, ekspresi IL-8 yang tinggi telah menggalakkan tapak jalan pengisyaratan melibatkan PI3K/Akt/NF-κB. Tapak jalan pengisyaratan ini diketahui bertanggungjawab dalam regulasi proses biologi seperti kemandirian, proliferasi, dan pembezaan sel. Pemerhatian terhadap struktur ultra SHED telah selanjutanya meyakinkan proses pembezaan SHED kepada sel seperti-odontoblas. Kehadiran ciri-ciri fenotip sel seperti-odontoblas, badan sel berlajur dengan beberapa titik, dan kewujudan pemineralan teraruh sel pada badan sel mencadangkan morfologi odontoblas matang. Kesimpulannya, kajian ini telah mengesahkan buat pertama kali proses pembezaan lengkap SHED kepada sel seperti-odontoblas melalui stimulasi BMP-2 dalam masa 14 hari melalui signal pengisyaratan IL-8.

SIGNALLING PATHWAYS INVOLVED IN IL-8 MEDIATED ODONTOGENIC DIFFERENTIATION OF SHED CULTURED ON HUMAN AMNIOTIC MEMBRANE WITH BMP-2

ABSTRACT

Current knowledge about the treatment modalities using amniotic membrane (AM) scaffold for dental regeneration is still limited. It has been previously shown that bone morphogenetic protein-2 (BMP-2) growth factor assisted stem cells from human exfoliated deciduous teeth (SHED) differentiation into odontoblast-like cells via activation of interleukin-8 (IL-8) cytokine. Nevertheless, the fundamental biology of IL-8 mediation in the process is yet to be understood. This study aims at finding the roles and mechanisms of IL-8 immunomodulatory pathway during odontoblast differentiation of SHED. In this study, SHED was cultured on AM scaffold with BMP-2 treatment. Flow cytometry analysis, multiplex polymerase chain reaction (PCR), and Western blot were conducted to determine the expression of stem cell markers, the expression of inflammatory cytokines genes, the protein expression of odontoblast markers, and molecules involved in the IL-8 immunomodulatory pathways. Finally, the ultrastructure of SHED and odontoblast-like cells were also investigated via scanning electron microscopy (SEM) imaging. The results presented in this thesis showed the potential of SHED to differentiate into odontoblast-like cells on AM scaffold. SHED was characterised, and exhibited expression profiles of various stem cell markers for the presence of mesenchymal stem cell (MSC) markers (cluster of differentiation) CD44, CD73, CD90, CD105 and the absence of hematopoietic markers CD34 and CD45 using flow cytometry

analysis. The results also indicated that SHED revealed strong expression of MSC surface markers CD90, CD44 and CD73 with 100% expression while CD105 has 84.1% expression. Under stimulation with BMP-2, it was demonstrated that pro-inflammatory cytokines namely IL-8, IL-6, IL-1β, TGF-β and TNF-α were expressed. Successful differentiation of SHED into odontoblast-like cells was demonstrated by the expression of odontoblast markers such as dentin sialophosphoprotein (DSPP), dentin matrix protein 1 (DMP1), alkaline phosphatase (ALP) and osteopontin (OPN). Exogenous IL-8 showed to enhance the expression of odontogenic markers of DSPP, DMP1 and ALP. During the differentiation of SHED, high expression of IL-8 protein stimulated the cascades of signalling pathway of PI3K/Akt/NF-κB. This pathway is known to be responsible for regulating the biological process of cell survival, proliferation and differentiation. Visualisation of SHED ultrastructure further confirmed the differentiation process of SHED into odontoblast-like cells. Emerging of phenotypic characteristics of odontoblast-like cell with a columnar cell body and several processes as well as strong cell induced mineralisation on the cell body suggesting morphology of mature odontoblast. In conclusion, this study confirmed for the first time that complete differentiation process of SHED into odontoblast-like cells occur with BMP-2 stimulation within 14 days via IL-8 signalling pathways mediation.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Many remarkable developments have been achieved in the oral health of Malaysians over the past 50 years. However, there still exist areas with major oral health problems and inadequate resources availability. Tooth decay remains to be a significant public health issue in Malaysia with an estimate of 80% caries problem in adults (Umer and Umer, 2011). Bone and dental loss is detrimental in affecting the quality of health and life of the people and poses a serious financial implication on the whole population (Righolt *et al*., 2018). Periodontal treatment, dental implants and dental prostheses remain the mainstream therapies for oral diseases. Nevertheless, current therapies for oral diseases are limited to halt disease progression and advance clinical diagnostic parameters. However, present therapies fail to regenerate lost tissue. Hence, new approaches are required to achieve bone and dental tissues regeneration (Zheng *et al*., 2019).

The prospect of utilising stem cells acquired from dental tissue for cell therapy in regenerative medicine has been indicated by emerging research in recent years. Stem cells from human exfoliated deciduous teeth (SHED) was discovered by Miura *et al*. (2003) where SHED was recognised as a population of postnatal stem cells with the high proliferative capability and pluripotent differentiation. On top of that, SHED was demonstrated in several studies to differentiate into the odontoblasts-like cell

(Sakai *et al*., 2010, Kim *et al*., 2011, Gosau *et al*., 2013). This fact demonstrates the potential of SHED to be beneficial and useful in dental pulp regeneration.

Interleukin-8 (IL-8), a potent chemokine that is mainly responsible for the recruitment of immune cells to sites of inflammation or infection (He *et al*., 2012b). Recent data have indicated that besides being a major neutrophil chemoattractant, IL-8 also plays important roles as a growth factor in promoting differentiation of cells, including adipocyte-like cells (Yamasaki *et al*., 2004), neuroendocrine cells (Huang *et al*., 2005) and chondrocytes (Yang *et al*., 2018).

Inflammatory cytokines such as IL-8 and bone morphogenetic proteins (BMPs) such as bone morphogenetic protein-2 are separately studied for their contributions in dental regeneration. Interestingly, there is a study indicating cross-talk between these two components in promoting rapid initiation of osteogenic differentiation (Lin *et al*., 2019). van der Kraan and Davidson (2015) also previously reported synergistic functions of BMP-2 and IL-8 in rheumatoid arthritis synoviocytes. Nevertheless, the detailed mechanism underlying BMP-2 and IL-8 actions in cell differentiation is yet to be elucidated.

Despite the fact that understanding the molecular mechanisms governing cellular developments are very challenging, it is noteworthy that considerable progress has been made in several aspects of cell differentiation and development by elucidation of the underlying signalling pathways. Advancement in knowledge of the signalling pathways and their regulation are essential for interpretation as well as possible manipulation of the developmental signals in tissue engineering. It helps scientists to

carefully design *in vitro* and *in vivo* experiments, and ultimately clinical applications of new therapeutic approaches. In view of IL-8 involvement in odontoblast differentiation, understanding the underlying signalling pathways are crucial in terms of providing assistance for experimental design where higher differentiation capacity can be obtained, and highly functional odontoblasts can be created for clinical application. Moreover, morphological assessment via scanning electron microscopy (SEM) analysis of odontoblast-like cells differentiated from SHED will provide comprehensive understanding of the effects of growth factors and cytokines during the process. As much as the importance of molecular analysis, information at the ultrastructural level gives ultimate understanding of the cell differentiation process.

Pertaining to SHED differentiation into odontoblast-like cells, there has yet to be any published study carried out to investigate the involvement of IL-8 signalling pathway during the differentiation process following BMP-2 growth factor stimulation. Therefore, this study will provide a fundamental understanding concerning the involvement of IL-8 and its mediated pathways during odontogenic differentiation of SHED into odontoblast-like cells.

A previous study has shown that odontoblast-like cells differentiated from SHED cultured on amniotic membrane (AM) expressed high level of interleukin-6 (IL-6) and IL-8 cytokines following BMP-2 growth factor treatment (Alshehadat, 2014). However, to date, only few publications reported IL-8 involvement in SHED differentiation. Therefore, this study aimed to see if IL-8 gene expression was increased during the differentiation process. Secondly, it was aimed to see if the IL-8 promoted the increase in the levels of odontoblast-specific protein expressions and

also to elucidate the IL-8 pathway in SHED differentiated into odontoblast-like cells. Furthermore, the enhanced odontogenic differentiation of SHED due to the presence of IL-8 was also analysed by the presence of IL-8 receptor inhibitor reparixin.

1.2 Justification of study

Naturally derived scaffolds such as AM offer a promising possibility in dental pulp regeneration. It is biologically active and typically promote excellent cell adhesion and growth with an elastic feature and is able to support stem cells growth and differentiation Furthermore, AM is also biodegradable and so allow host cells, over time, to produce their own ECM and replace the degraded scaffold (O'brien, 2011). It's low immunogenicity makes AM suitable for dental implantation, and, therefore, making it applicable for tissue engineering application (Mohan *et al*., 2017).

With the aid of suitable scaffold such as AM and BMP-2 growth factor, as well as understanding the molecular network to assist the process, SHED has great potential to achieve functional odontogenesis stem cell-mediated tissue repair. BMP-2 is an important growth factor in odontoblast differentiation. It was produced by the odontoblasts and promote differentiation and involves in dentin formation (Yang *et al*., 2012). BMP-2 also modulates the expression of odontogenic markers such as DMP1, DSPP, ALP and Nestin (Casagrande *et al*., 2010). It was demonstrated that severe defects in odontogenesis and dentin formation with the removal of the BMP-2 gene in early-polarizing odontoblasts (Yang *et al*., 2012).

It has been shown that during SHED differentiation into odontoblast-like cells on the AM scaffold, overexpression of IL-8 was detected (Alshehadat, 2014). Despite the biological importance of various inflammatory cytokines and chemokines during dental pulp regeneration, there has yet to be a study investigating the roles of IL-8 and its underlying mechanism in SHED differentiation into odontoblast-like cells. Interestingly, a preliminary study conducted by our group has shown that SHED seeded on AM and treated with BMP-2 expressed significantly high expression levels of IL-6 and IL-8 during the differentiation process into odontoblast-like cells (Alshehadat, 2014). While over-expression of IL-6 in SHED has been reported before (Govitvattana *et al*., 2013, Wang *et al*., 2012a), no report on IL-8 has yet to be found.

Several involvements of IL-8 in mediating cell differentiation have been previously documented in inducing chondrogenic differentiation of human bone marrow mesenchymal stem cells (hBMSCs) (Yang *et al.*, 2018) and mature hepatocytes differentiation into cholangiocyte phenotype (Sasaki *et al.*, 2019). The processes occur by elevated functions of IL-8 mediated signalling pathways via its receptors (Yang *et al* 2018, (Jia *et al.*, 2017). Moreover, oncology research has also documented inhibition of breast cancer stem cells differentiation by introducing CXCR1 receptor inhibitor thereby limiting the activation of IL-8/CXCR1 (Jia *et al.*, 2017).

As shown by the previous evidence (Yang *et al* 2018, (Jia *et al.*, 2017), CXCR1 and CRCX2 are the two receptors involved in IL-8 activation during cell differentiation. Inhibition of IL-8 functions can be achieved via several ways such as introduction of

IL-8 neutralising antibody or inhibition of IL-8 receptors CXCR1/2 with reparixin (Jia *et al*., 2017). Reparixin, a powerful inhibitor to study the IL-8 signalling pathways, has been shown in various cancers such as breast, melanoma and colon to reduce cancer stem cells (CSCs) number in patients (Goldstein *et al.*, 2020, Fu *et al.*, 2018). Meanwhile, little is known about the effects of reparixin in IL-8 mediating odontoblast differentiation of SHED on AM scaffold. Therefore, understanding the signalling pathways involved in the process will provide information, not only to understand the underlying mechanisms but also to help design the best possible way for a successful dental regeneration approach.

In order to investigate the underlying mechanism of SHED differentiation involving IL-8, several important markers from the IL-8 signalling pathway were investigated in this study. They are phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), mammalian target of rapamycin (mTOR), janus kinase 2 (JAK2), STAT3 and NF-κB. Meanwhile, to study the functional significance of these molecules, IL-8 receptor inhibitor, reparixin, was used. The inhibitor mechanism of action determines which molecules are crucial in the SHED process of differentiation, therefore highlighting the possible pathway involved.

To further evaluate the potential use of SHED in dental pulp regeneration, ultrastructure imaging and composition of the cells and scaffold were determined using (scanning electron microscope) SEM. *In vitro* behaviour such as adhesion, proliferation, and odontoblastic differentiation of SHED seeded on AM were visualised at certain time points. SEM imaging information helped in determining the feasibility and compatibility of SHED cultured on AM in the hope of developing

new endodontic treatments that will supplant the conventional pulpectomy/root canal filling procedure (Ferri and Hunziker, 2011).

This study not only provides valuable information in understanding the roles of IL-8 and its underlying pathway during SHED differentiation into odontoblast-like cells, but also enlightens the potential of SHED and AM as valuable materials to repair the impaired dentin-pulp tissues. In the end, the knowledge obtained from this study helps in paving the path for successful dental tissue regeneration.

1.3 General Objective

To investigate the role and mechanism of IL-8 immunomodulatory pathway in the odontoblast differentiation of SHED cultured on AM with BMP-2 treatment.

1.4 Specific objectives

1. To determine the expression of inflammatory cytokines and the protein expression of odontoblast markers in SHED differentiated into odontoblast-like cells.

2. To determine the protein expressions of IL-8 in SHED differentiated into odontoblast-like cells with or without IL-8 receptor inhibitor, reparixin.

3. To determine the protein expression of molecules in the IL-8-immunomodulatory pathway, in SHED differentiated into odontoblast-like cells with or without IL-8 receptor inhibitor, reparixin.

4. To visualise the ultrastructure of SHED cultured on AM and differentiated into odontoblast-like cells.

1.5 Research hypotheses

1. SHED differentiated into odontoblast-like cells expressed inflammatory cytokines and odontoblast markers.

2. SHED differentiated into odontoblast-like cells in the presence of IL-8.

3. SHED differentiated into odontoblast-like cells mediated by IL-8-immunomodulatory pathway involving PI3K, Akt, mTOR, NF-κB, JAK2 and STAT3.

4. SHED differentiated into odontoblast-like cells and expressed gradual morphological changes with the presentation of odontoblast features.

CHAPTER 2

LITERATURE REVIEW

2.1 Background

One of the growing fields in medicine involves tissue engineering and regenerative approach with the concept of stem cells application, biocompatible scaffolds, and growth factors to construct functional tissues (Yamauchi *et al*., 2011). There are several important aspects in tooth repair: re-mineralisation of the enamel, dentin repair, and engineering of the whole tooth (Angelova Volponi *et al*., 2018). Over time, dentin becomes exposed to the external environment and increases its risk of injury and bacterial infection. In addition, the location of the dental pulp's sensory centre right under the dentin will destroy the integrity of a tooth and deprive structural support to the pulp (Tang and Saito, 2018). In tackling difficult dental-related clinical situations such as teeth loss and periodontal diseases, stem cell-based tissue engineering appears to offer great potential because postnatal stem cells isolated from teeth are readily accessible and brings a non-invasive way of harvesting especially from teeth that are meant to be extracted. Therefore, application of stem cells is very beneficial in tissue regeneration and development.

2.2 Teeth structure

Teeth have a unique structure that contains the hardest biological substance known, the enamel. Exclusively found in vertebrates, the structure of enamel is believed to

originate from the neural crest cells (Mantesso and Sharpe, 2009). Known as a complex biological organ, the basic structure of the tooth consists of three different layers, outer enamel, a middle dentin, and an inner dental pulp layer (Luo *et al*., 2018). Together, a tooth was formed by dental pulp, cementum, dentin and enamel (Yuan *et al*., 2011).

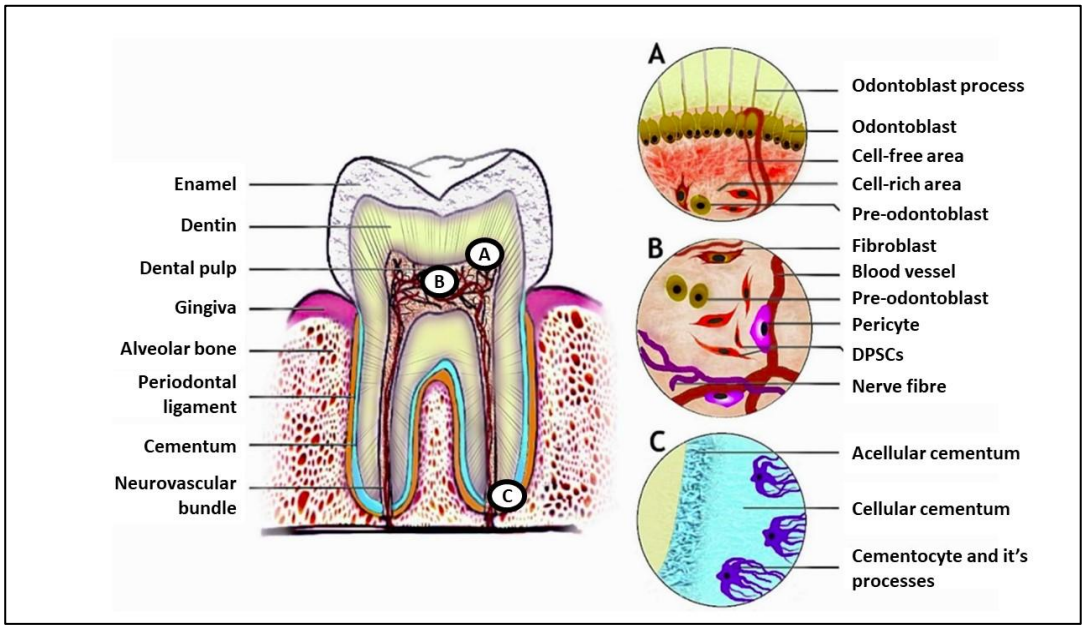

Figure 2.1: Structure of a tooth, dental tissue and distribution of stem cell populations. (A) Odontoblast, cells that line up the dental pulp, with odontoblast processes extending into enamel. (B) Various types of cell populations in dental pulp, DPSCs is one example, which can differentiate into odontoblasts. (C) Cementocytes, active cells responsible for cementum secretion, help keep the tooth stable within the structure. Its cellular processes are arranged towards the periodontal ligament (Modified from (Baranova *et al.*, 2020).

2.2.1 Dental pulp

Dental pulp is a soft oral tissue, which is composed of several types of cell populations, and is encased in a thick, absorbent mineralised chamber (Angelova Volponi *et al*., 2018). It also contains mesenchymal cells, neural fibres, blood vessels and lymphatic vessels and plays a role in producing dentin and serves to support the

biological and physiological strength of the dentin (Tatullo *et al*., 2015). Originate from a similar region; pulp tissue and dentin tissue closely interact with each other by making up a complex of pulp-dentin structure. Enamel and dentin are hard tissue structures that cover the soft dental pulp.

The pulp tissue itself is also composed of Type I and III collagen and a selection of non-collagenous proteins, containing a major component of proteoglycan proteins. There is a layer of odontoblasts, the primary cells of the dental pulp structure, inside the pulp chamber (Colombo *et al*., 2014). Odontoblasts are non-proliferative terminally differentiated cells (Lin *et al*., 2013b) but are replaceable by native multipotent stem cells found in both primary and permanent teeth. Odontoblasts are also able to generate new tubular dentin (Piva *et al*., 2014). Odontoblasts originate from mesenchyme of neural crest, which appears during the early craniofacial development. The cells play important roles during dentinogenesis, the formation of dentin.

2.2.2 Dentin

Dentin is a major part of mineralised ECM of the tooth, which forms the majority of teeth. Dentinogenesis occurs during the primary and secondary dentin formation (Arana-Chavez and Massa, 2004). The major components of dentin is Type I collagen (90%) and the remaining are 10% non-collagenous proteins, which happen to be components of the bone too (MacDougall *et al*., 1997). Dentin originated from odontoblasts, which form a layer at the edge of the dental pulp (Figure 2.1). Tubular structure is formed by dentin. The tubules are more pervasive at the surface of the

pulp than at the enamel–dentin joint. Each tubule is consistently filled with the structure of odontoblast. The presence of different numbers of tubules as well as the thickness of the dentin gives a diffusion gradient across the dentin.

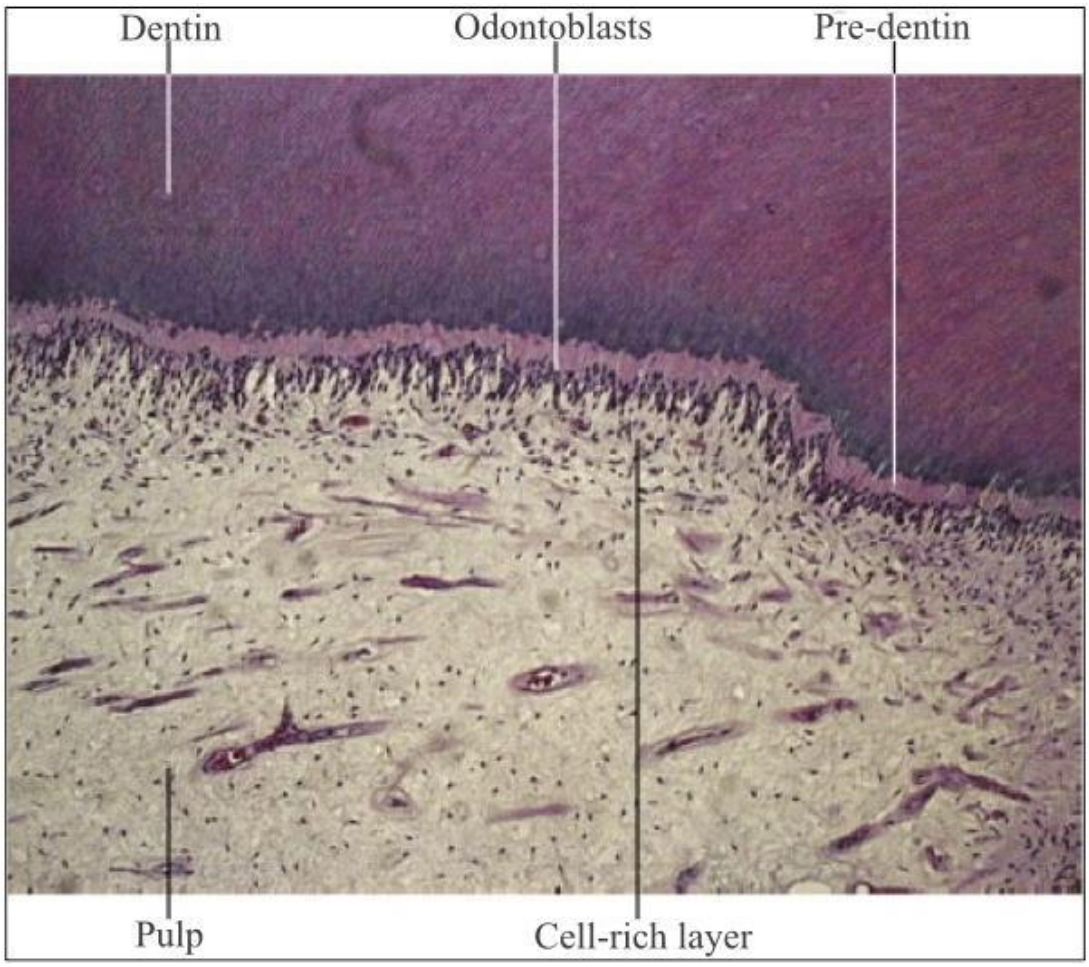

Figure 2.2: Dentin-pulp complex consisting of a soft pulp chamber with odontoblasts and hard dentin structure (Saunders, 2011).

In a condition of less severe dentin decay, the pulp structure should be able to naturally generate new dentin for the repair. Pulp responses to injury by utilising two strategies, which are reactionary dentin by secretion of the original odontoblasts or reparative dentin by secretion of the odontoblast-like cells (Aguiar and Arana-Chavez, 2007). Pulp also mineralises within the dentinal tubules to reduce peritubular dentin diameter and to reduce their ability to permit transmission across

them. During restorative procedures, odontoblasts can be damaged and will be replaced by pulp stem cells. Stem cells will undergo differentiation into odontoblast-like cells and orchestrate tissue mineralisation at the site of injury (Murray *et al*., 2003). In dentin regeneration, growth factors are involved in promoting stem cell differentiation into odontoblasts; stimulate the secretion of matrix and mineralisation; and assist in angiogenesis and neurogenesis. Among growth factors with significant roles in regeneration are the TGF-β family, BMP family, FGFs, and non-collagenous proteins such as DMP1, DSP, DPP and VEGF (Sharma *et al*., 2010).

2.2.3 Odontoblast

As depicted in Figure 2.2, odontoblasts are located at the periphery of the dental pulp (Saunders, 2011). These cells undergo several differentiation stages from ecto-mesenchymal cells of the dental papilla (Zhang *et al.*, 2018). The formation of odontoblast occurs via epithelial-mesenchymal interactions during tooth development and solely responsible for dentin secretion (Kawashima and Okiji, 2016). The cells appear in large columnar or fibroblastic shape and arranged in a palisading pattern at the periphery of the pulp (Arana-Chavez and Massa, 2004).

As previously mentioned, odontoblast differentiation originates from the cells of the dental papilla to initially produce short, columnar-shaped pre-odontoblast cells (Zhang *et al.*, 2018). These cells elongate and subject their cellular processes towards the basement membrane where dental epithelium and ecto-mesenchyme interface. When the cells are fully differentiated, they are called secretory odontoblasts with

polarised columnar cells containing numerous organelles in their supranuclear area (Hosseinpour *et al.*, 2021). These highly polarised cells begin to extend their cytoplasmic processes into the dentinal tubules in dentin to form a sheet of cells interconnected by gap and tight junctions (Abbass *et al.*, 2020).

As odontoblasts are formed from post-mitotic cells, they are unable to further divide to produce new lines of dentin secreting cells (Balzano *et al.*, 2021). Therefore, if odontoblasts died or damaged, no further dentin could be secreted unless new odontoblast-like cells differentiated into the area. Mature odontoblasts synthesise various proteins for dentinogenesis, and the major secretory product is type I collagen, which comprises about 85% of the organic materials in dentin (Linde and Goldberg, 1993). Once primary dentin is secreted, secretory odontoblasts become smaller in size, less polarised with reduced organelles and autophagic vacuoles (Couve *et al.*, 2013). Primary dentin is a structure made up of type I collagen fibers that are mineralised due to non-collagenous proteins (Linde and Goldberg, 1993). Meanwhile, maintenance of tooth structure is carried out by secondary dentin synthesis throughout life (Goldberg *et al.*, 2011). In the case of tooth damage or injury, tertiary dentin is secreted by odontoblasts as reactionary and reparative dentin (Aguiar and Arana-Chavez, 2007).

Odontoblasts differentiation or odontogenesis is considered a highly complex process while its precise mechanisms remain unclear. Several factors that have been associated with induction of odontoblasts differentiation include bone morphogenetic protein (BMP), transforming growth factor proteins, fibroblast growth factor (FGF), cytokines and chemokines (Kawashima and Okiji, 2016). Among these, the members

of the BMP family are the most extensively studied for differentiation of odontoblast and osteoblast (Chen *et al.*, 2008).

Besides being secretory cells, odontoblasts also play the roles of host defence by detecting exogenous stimuli such as bacterial or bacterial products. Odontoblasts possess a specialized innate immune system to fight oral pathogens. Generally, the rapid initial sensing of microbial pathogens, especially pathogen-associated molecular patterns (PAMPs) shared by microorganisms, are mediated by pattern recognition receptors (PRRs), such as Toll-like receptor and the nucleotide-binding oligomerization domain (NOD). The innate immune responses in odontoblasts initiated by sensing oral pathogens provide host protective events, such as inflammatory reactions, to produce a variety of pro-inflammatory mediators, including chemokines and cytokines. These attract various inflammatory cells and cause antibacterial reactions, such as the production of defensins, to kill microorganisms in the proximal region of the odontoblast layer (Yumoto *et al.*, 2018). Besides, increasing evidence also demonstrated odontoblasts function as sensory cells to detect nociceptive signals such as voltage-gated sodium channels, thermal, mechanical and chemical stimuli. This evidence support the notion that odontoblasts not only play role in dentin formation, but also function as nociceptors and defensive cells in the dental pulp (Kawashima and Okiji, 2016).

2.2.4 Teeth regeneration

Tooth losses often occur due to dental caries, periodontal disease, and genetic disorders. One of the potential areas for development in order to tackle this issue is

based on the cellular approach of tissue regeneration. (Angelova Volponi *et al*., 2018). Current methods for teeth replacement include tooth implantation using synthetic materials, auto transplantation and subsequent reshaping. These techniques are commonly used in dentistry years, but yet to meet complete satisfaction for cosmetic or physiological reasons (Mantesso and Sharpe, 2009). The regeneration of teeth involves regrowth of a complete tooth as an organ as well as the restoration of biological functions of tooth's individual components such as dental pulp, cementum, dentin and enamel (Yuan *et al*., 2011). Teeth regeneration can be categorised into seven categories as follows; i. regeneration of the entire structure of anatomically correct tooth; ii. regeneration of dental pulp; iii. regeneration of the root; iv. synthesis of dentin to seal off and restore exposed pulp chamber or to replace current synthetic materials; v. regeneration of cementum for repairing periodontium or for making up loss of dentin or cementum due to orthodontic tooth movement; vi. periodontal regeneration including cementum, ligament (PDL), periodontal and alveolar bone; vii. enamel-like structures regeneration as natural substitute for enamel remineralisation of enamel and dentin.

Human dental pulp capability to react to injury by forming tertiary dentin has been well recognised for decades. Replacement of odontoblast involves multiple deoxyribonucleic acid (DNA) processes such as replications, as well as movement of pulpal cells from the deeper pulp to the exposure site. Cells involved in the replacement process are derived from cells in the deeper area of pulp that can migrate after induction, differentiated as odontoblasts (Mantesso and Sharpe, 2009). For example, odontoblastic differentiation is demonstrable by increased mineralised nodule formation and messenger ribonucleic acid (mRNA) presence of several

odontoblastic markers after being regulated by lipopolysaccharide in a dose-dependent manner (He *et al*., 2015).

Dental stem cell technologies are currently emerging as an alternative for teeth regeneration ascribed for their high potential in multipotency, self-renewal and ability to differentiate into specific cell types. Integration of stem cells and scaffold technology highlights the potential of simultaneous regeneration of tissues needed to form the pulp-dentin complex. A small subpopulation of stem cells in dental pulp responds to the progression of caries in the pulp and these stem cells differentiate to odontoblast-like cells replacing dead odontoblasts from cariogenic disease (Rosa *et al*., 2011).

Pulp implantation is one of the techniques for dental pulp regeneration. The implantation was performed by growing single monolayer pulp cells on biodegradable membrane filters. The biodegradable filters will then be rolled together forming three dimensional (3D) pulp tissues, and finally, the tissues can be implanted into disinfected root canal systems (Murray *et al*., 2007). In another study, Xuan *et al*. (2018) suggested that dental stem cells are involved in assisting the recovery of injured teeth by *in vitro* expansion of autologous tooth stem cells from deciduous teeth and implanting the cells in animal models and human patients. It was demonstrated the implanted teeth were able to regenerate complete and functional dental pulp, which consist of an odontoblast layer, nerves, and blood vessels and reinstate sensation to stimuli such as temperature.

The important aspects of pulp regeneration are vascularisation loaded tissues, neuron formation, and dentin deposition (Yang *et al*., 2015b). Appropriate dental material for dental pulp regeneration should have close contact with the pulp besides meeting three criteria: first, the material is biocompatible; second, the structure is resistant to repeated masticatory pressure; and third, the material is not susceptible to any leakage which might allow oral microorganisms penetration into the pulp space (Cao *et al*., 2015). Odontoblasts maintain the mineralised tissues while other cell types actively support odontoblasts activity in a dynamic and complex balance. Mesenchymal progenitor cells for example assist in tissue maintenance and repair function, besides providing a reservoir of multipotent cells with the capability to differentiate into various types of cells. Furthermore, mesenchymal progenitor cells are indispensable to teeth long-term function and to the design of the scaffold for dental regeneration (Colombo *et al*., 2014).

2.3 Mesenchymal stem cells

The essential elements of tissue engineering are the stem cells, growth factors, and a carrier or scaffold of the ECM (Nakashima and Akamine, 2005). Hematopoietic, endothelial, mesenchymal, intestinal, mammary, muscle, neural, skin and hair follicle stem cells are the type of stem cells and, as the name implies, these cells principally arise from the tissue of origin (Ledesma-Martínez *et al*., 2015). The intense debate surrounding the application of embryonic stem cells has shifted the focus to using adult's stem cells in tissue engineering with the support of scaffolds and growth factors (Mozaffari *et al*., 2019). MSCs are generally derived from various connective tissues and serve important roles in immunotherapy and tissue

regeneration due to the cells capacity for self-renewal, multilineage differentiation and immunosuppressive potential (Wang *et al*., 2018a) and suitable for regenerative medicine due to its high proliferation capabilities (Verma *et al*., 2014).

By definition, MSCs are referring to adult multipotent stem cells with high differentiation capability into various tissues, such as bone, cartilage, fat and neural cells (Verma *et al*., 2014, Otabe *et al*., 2012). In the beginning, MSCs were known as fibroblast-like cells isolated from bone marrow (BM) and capable of adhering to plastic in culture (Friedenstein *et al*., 1976). From BM, adherent CFU-F with high replicative ability *in vitro* was isolated for the first time. It can develop into multiple specialised cell types such as adipocytes, osteoblasts, chondrocytes and hematopoietic supporting stroma when following a re-transplantation *in vivo* (Friedenstein *et al*., 1976). MSCs exist in the majority type of tissues and involve significantly in tissue repair and regeneration (Wang *et al*., 2018b). Moreover, with extensive tissue distribution, MSCs are capable to induce tissue repair in both preclinical and clinical models (Ledesma-Martínez *et al*., 2015).

2.3.1 Dental mesenchymal stem cells

Dental tissues provide abundant sources of MSCs, which can be beneficial for the application of tissue engineering (Estrela *et al*., 2011). There are various parts of the oral cavity whereby dental stem cells reside, such as dental pulp, alveolar bone, apical papilla, PDL, oral mucosa, dental follicle and gingiva. All of the stem cells within this cavity originated from the cranial neural crest (Wang *et al*., 2018b).

MSCs derived from postnatal dental pulp and PDL tissues are able for multipotential *in vitro* differentiation and *in vivo* tissue generation (Estrela *et al*., 2011).

Despite great potential for tissue engineering approach, the understanding of dental stem cells properties in terms of its functional applications following *in vivo* implantation remain poorly unrecognised (Lei *et al*., 2014). Their potential use remains to be challenging in the dental field in years to come (Mantesso and Sharpe, 2009). The MSCs of DPSCs were first isolated and classified as highly clonogenic cells with the ability to produce a highly calcified cell population (Ledesma-Martínez *et al*., 2015).

Previous evidence demonstrated that various subgroups of MSCs can be isolated from human dental tissues (Liu *et al*., 2015). These include; DPSCs (Gronthos *et al*., 2000), SHED (Miura *et al*., 2003), PDLSCs (Seo *et al*., 2004), dental follicle progenitor stem cells (DFPCs) (Morsczeck *et al*., 2005), alveolar bone-derived MSCs (ABMSCs) (Matsubara *et al*., 2005), tooth germ progenitor cells (TGPCs) (Ikeda *et al*., 2008), stem cells from apical papilla (SCAPs) (Sonoyama *et al*., 2008) and gingival MSCs (GMSCs) (Zhang *et al*., 2009). Various *in vitro* and animal studies prove that dental stem cells can differentiate into osseous, odontogenic, adipose, endothelial, and neural-like tissues (Sedgley and Botero, 2012, Ravindran *et al*., 2014, Yamada *et al*., 2011). DPSCs are considered as a valuable source of stem cells for orthopaedic and oral maxillofacial reconstruction. Moreover, DPSCs application may go beyond the scope of the stomatognathic system. It has been shown that DPSCs are able to synthesis mineralised tissue; an ECM and various

parts such as dentin, dental pulp and PDL in xenograft models (Ledesma-Martínez *et al*., 2015).

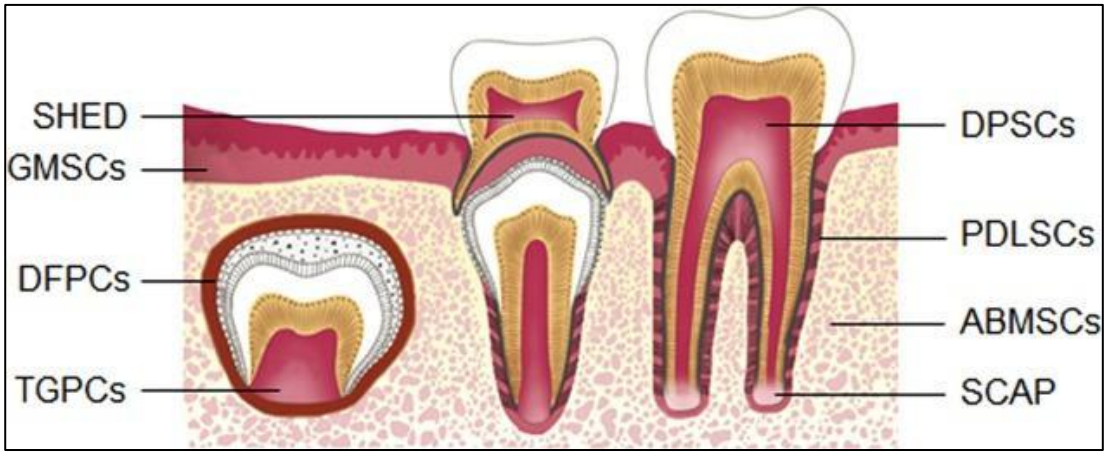

Figure 2.3: A schematic representation of several types of MSCs derived from teeth including stem cells from exfoliated deciduous teeth (SHED), dental pulp stem cells (DPSCs), gingiva-derived MSCs (GMSCs), dental follicle progenitor cells (DFPCs), periodontal ligament stem cell (PDLSCs), alveolar bone-derived mesenchymal stem cells (ABMSCs), tooth germ progenitor cells (TGPCs), and stem cells from the apical papilla (SCAPs) (Liu *et al*., 2015).

In general, human dental tissue-derived MSCs such as SHED, DPSCs, SCAPs, PDLSCs and DFPCs share several common surface markers such as CD13, CD29, CD44, CD73, CD90, CD105, CD146, CD166, CD271 (Liu *et al*., 2015). Yamada *et al*. (2006) used flow cytometry analysis to detect and characterise DPSCs. They found DPSCs as positive for mesenchymal lineage markers (CD13, CD29, CD44, CD73, and CD105), but negative for monocytic marker (CD14), and hematopoietic lineage markers (CD34, and CD45).

2.4 Stem cells from human exfoliated deciduous teeth (SHED)

SHED can be derived non-invasively from primary teeth, or even carious ones, meant for disposable and readily accessible postnatal human tissue (Werle *et al*.,

2016). With limited ethical concern and easy harvesting technique, SHED becomes an attractive and useful source for bone regeneration approach (Kunimatsu *et al*., 2018, Rosa *et al*., 2011). Under controlled culture conditions SHED can be differentiated (Yamaza *et al*., 2010, Liu *et al*., 2015) into six lineages namely dentinogenic (Minguell and Erices, 2006), chondrogenic, myogenic (Sakaguchi *et al*., 2005), neurogenic, adipogenic and osteogenic (Miura *et al*., 2003). Under stimulation of neurogenic growth conditions, SHED express a number of neural cell markers, develop sphere-like clusters, and produce structure of processes. Moreover, SHED is also able to display myogenic and chondrogenic properties. Upon culture on a dentin's slice, SHED demonstrated capability to differentiate into endothelial cells. In hepatic differentiation medium, SHED displayed morphological and functional characteristics of hepatocytes besides producing specific hepatic proteins, (Liu *et al*., 2015).

Human SHED generally express STRO-1, CD13, CD29, CD 44, CD73, and osteogenic marker genes such as alkaline phosphatase (*ALP*) gene, runt-related transcription factor 2 (*Runx2*) gene, and osteocalcin (*OCN*) gene *in vitro* (Yamada *et al*., 2010). SHED often display the highest differentiation capacity compared to DPSCs, PDLSCs, and SCAPs (Yusoff *et al*., 2015). SHED showed osteogenic potency similar to BMMSCs, with high ALP activity following 1 week of induction. Besides, SHED demonstrated higher proliferation rates and gene expression levels of FGF and BMP-2 compared to BMMSCs and DPSCs (Kunimatsu *et al*., 2018). SHED has also been demonstrated to differentiate into osteogenic-like cells via expression of prominent osteogenic markers such as Runx2, DSP and OCN (Yamaza *et al*., 2010). SHED is often known for their active proliferation rate (Kunimatsu *et*

al., 2018), high cell population-doubling time, and ability to form sphere-like clusters. As proposed by International Society for Cellular Therapy (ISCT), SHED expresses significant surface markers to meet the minimal criterion for MSCs besides expressing the embryonic stem cell markers such as Oct4 and Nanog, the neural stem cell marker nestin, and the stage-specific embryonic antigens SSEA-3 and SSEA-4 (Liu *et al*., 2015).

Cordeiro *et al*. (2008) suggested that SHED could be an ideal source of stem cells to repair damaged dental tissues and promote bone regeneration. To date, culturing SHED on AM scaffold has yet to be studied. AM scaffold has been mainly used in corneal implantation (Gomes *et al*., 2010). Most tissue engineering studies involving SHED were conducted on chitosan (Farea *et al*., 2014, Guan *et al*., 2015, Su *et al*., 2014), focusing mainly on bone regeneration. Moreover, SHED culture on synthetic granular hydroxyapatite bone graft and coral scaffolds had also been introduced in promoting stem cells differentiation and proliferation (Nawi *et al*., 2013).

The proliferation of SHED increased when cultured on a cell-matrix with bone-like stiffness, but the proliferation rate is significantly decreased when cultured on a connective tissue-like surface. Meanwhile, a combination of dexamethasone (Dex) increased the presence of osteogenic markers in SHED on a rigid surface (Viale-Bouroncle *et al*., 2012) pointing to the ability of firm matrix surface supports SHED osteogenic differentiation. Osteogenic differentiation improves in hard surface compared to soft matrix surface. A number of parameters must be considered for a suitable scaffold, such as the mechanical strength, porosity, and support of surface morphology for cell anchorage and survival (Zhang *et al*., 2013b).

A previous study by Coyac *et al*. (2013) has shown the superiority of 3D scaffolds in assisting SHED in osteogenic differentiation. A 3D dense collagen scaffold, made by plastic compression of preformed, highly hydrated collagen gels, was used in an *in vitro* study of SHED culture model. The increase in calcium and phosphate concentration, under osteogenic conditions, within the cell-seeded scaffolds compared with acellular scaffolds demonstrated that 3D scaffolds promote osteogenic and odontogenic cell differentiation and mineralisation (Coyac *et al*., 2013). A study by Alshehadat (2014) suggests that deciduous teeth stem cells successfully differentiated into odontoblast-like cells when cultured on AM and stimulated with BMP-2 growth factor.

2.4.1 Differentiation of SHED into odontoblasts

Odontoblast differentiation is a necessary step for tooth formation and is also controlled by a complex gene regulatory network (Tao *et al*., 2019). Previous evidence shows that SHED is capable of differentiating into odontoblast *in vitro* and *in vivo*. A study by Miura and colleagues in 2003 had transplanted SHED from culture into mice with reduced immunity resulting in the differentiation of the cells into odontoblasts with a dentin-like structure. This was proved by positive staining of the dentin towards dentin sialophosphoprotein (DSPP) antibody. These findings indicated for the first time, SHED ability to differentiate into odontoblast-like cells *in vivo* (Miura *et al*., 2003).

In the layer year, Sakai *et al*. (2010) investigated SHED growth on tooth slice/scaffolds, which was subcutaneously implanted into immunodeficient mice.