ROLE OF INTERLEUKIN-8 IN ODONTOGENEIC DIFFERENTIATION OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH CULTURED ON HUMAN AMNIOTIC MEMBRANE

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by

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LIST OF ABBREAVIATIONS

%	Percentage
°C	Degree Celcius
3D	Three-dimensional
4E-BP1	4E-binding protein 1
AGI	Axon growth inhibitors
AIDS	Acquired immunodeficiency disease
AKT	Protein kinase b
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BGN	Bioactive glass nanoparticles
BMMSCs	Bone marrow-mesenchymal stem cells
BMPR-IA	Bone morphogenetic protein receptor, type 1A
BMPR-IB	Bone morphogenetic protein receptor, type 1A
BMPR-II	Bone morphogenetic protein receptor type II
BMPs	Bone morphogenetic proteins
BMSCs	Bone mesenchymal stem cells
bp	Base pair
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CBFA1	Core-binding factor subunit alpha-1
CD	Cluster of differentiation
cm	Centimetre
CO_2	Carbon dioxide
COL-1	Collagen type 1

C_T	Cycle threshold
CXCR1	C-X-C chemokine receptor type 1
CXCR2	C-X-C chemokine receptor type 2
DEPC	Diethyl pyrocarbonate
DEX	Dexamethasone
DFSCs	Dental follicle stem cells
DMP1	Dentin matrix protein 1
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPCs	Dental pulp cells
DPP	Dentin phosphoprotein
DPSCs	Dental pulp stem cells
DSP	Dentin sialoprotein
DSPP	Dentin sioalophosphoprotein
ECM	Extracellular matrix
EG	Embryonic germ
ELISA	Enzyme-linked immunosorbent assay
ESCs	Embryonic stem cells
et al.	and others
FBS	Fetal bovine serum
FGF-2	Fibroblast growth factor-2
FGFR	Fibroblast growth factor receptors
g	Gram
GDF11	Growth differentiation factor 11
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GPCRs	G-protein-coupled receptors
HA/TCP	Hydroxyapatite/tricalcium phosphate
HAM	Human amniotic membrane
hDPCs	Human dental pulp cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC_{50}	Inhibitory concentration 50%
IFN- γ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
IL-1β	Interleukin-1 beta
JAK2	Janus kinase 2
JNK	C-jun n-terminal kinases
kGy	Kilogray
L	Litre
LB	Lithium borate
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage-colony stimulating factor
MEM	Minimum essential medium
MEPE	Matrix extracellular phosphoglycoprotein
MIF	Macrophage inhibitory factor
min	Minutes
mm	Millimetre
mM	Milimolar
mRNA	Messenger ribonucleic acid

MSCs	Mesenchymal stem cells
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NFM	Neurofilament
NF-Y	Transcription factor Y
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated b cells
ng	Nanogram
NH_4OH	Ammonia hydroxide
NTC	Non-template control
OCN	Osteocalcin
OCT4	Octamer-binding transcription factor 4
OD	Optical density
OPN	Osteopontin
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PDLSCs	Periodontal ligament stem cells
pH	Potential hydrogen
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PRF	Platelet-rich fibrin
QR	Relative quantity
R^2	Correlation coefficient
RHD	Rel homology domain

RHIL-8	Recombinant human interleukin-8
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
rpm	Round per minute
RT-PCR	Reverse transcriptase - polymerase chain reaction
RUNX2	Runt-related transcription factor 2
s	Second
S	SHED
SA	SHED + amnion
SAB	SHED + amnion + bone morphogenetic protein 2
SAB8	SHED + amnion + bone morphogenetic protein 2 + rhil-8
SABR	SHED + amnion + bone morphogenetic protein 2 + reparixin
SABR8	SHED + amnion + bone morphogenetic protein 2 + reparixin + rhil-8
SB	SHED + bone morphogenetic protein 2
SCAP	Stem cells from the apical papilla
SCID	Severe combined immunodeficiency
SEM	Standard error mean
SHED	Stem cells from human exfoliated deciduous teeth
SOX2	Sex determining region Y
STAT	Signal transducers and activators of transcription
STAT3	Signal transducer and activator of transcription 3
TCP	Tricalcium phosphate
TEM	Transmission electron microscope
TGF-β	Transforming growth factor beta
Th17	T helper 17

TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TORC1	Target of rapamycin complex 1
TORC2	Target of rapamycin complex 2
V	Voltage
WNT	Wingless/Integrated
wt	Weight
α	Alpha
β	Beta
β-TCP	Beta-tricalcium phosphate
γ	Gamma
κ	Kappa
μg	Microgram
μl	Microlitre
μm	Micrometre
μM	Micromolar

PERANAN INTERLEUKIN-8 TERHADAP PEMBEZAAN ODONTOGENIK SEL TUNJANG DARIPADA GIGI SUSU MANUSIA YANG TERKELUPAS YANG DIKULTUR DI ATAS MEMBRAN AMNIOTIK MANUSIA

ABSTRAK

Interleukin-8 (IL-8), sitokin radang dengan kesan biologi pleiotropik, telah dilaporkan terlibat dalam odontogenesis dan terlibat dalam tindak balas imun berperantara odontoblast. Walau bagaimanapun, mekanisme tepat mengenai bagaimana pengaruh IL-8 terhadap odontogenesis masih tidak jelas. Oleh itu, kajian ini dijalankan untuk menyiasat peranan dan mekanisme tapak jalan imunomodulator IL-8 dalam pembezaan sel-sel stem dari gigi susu manusia yang dicabut (SHED) kepada sel-sel serupa odontoblast. SHED dibiakkan di atas membran amniotik manusia (HAM) dan dirawat dengan protein morfogenetik tulang-2 (BMP-2). Empat kumpulan telah ditentukan: SHED sahaja (S), SHED dengan BMP-2 (SB), SHED di atas membran amniotik tanpa BMP-2 (SA), SHED di atas membran amniotik dengan BMP-2 (SAB). Pasca rawatan, SHED telah dituai pada hari 1, 7, 10 dan 14, dan potensi pembezaan odontogenik SHED dinilai oleh ekspresi penanda odontogenik menggunakan tindak balas rantai polimerase transkriptase berbalik dan pemendapan kalsium oleh pewarnaan alizarin merah. Kepekatan optimum reparixin dan rhIL-8 ditentukan menggunakan Real Time RT-PCR. Untuk kajian kesan IL-8, empat lagi kumpulan telah ditentukan; SHED di atas membran amniotik dengan BMP-2 (SAB), SHED di atas membran amniotik dengan BMP-2 dan reparixin (SABR), SHED di atas membran amniotik dengan BMP-2 dan rhIL-8 (SAB8), SHED di atas membran amniotik dengan BMP-2, reparixin dan rhIL-8 (SABR8). SHED dirawat dengan

reparixin sebagai perencat IL-8, manakala rekombinan IL-8 manusia (rhIL-8) sebagai agen aruhan, dan analisis hiliran IL-8 dijalankan menggunakan Real Time RT-PCR. Tahap rembesan protein IL-8 SHED dengan dan tanpa aruhan dan perencatan IL-8 semasa pembezaan odontogenik dianalisis menggunakan ujian ELISA. Kesan IL-8 dalam pemendapan kalsium SHED ditentukan dengan menggunakan pewarnaan merah alizarin. Keputusan kajian ini menunjukkan bahawa penanda gen odontoblast khusus *DSPP*, *DMP1*, *OPN* sangat tinggi pada hari ke-7 dan hari-hari seterusnya kerana perbezaan odontogenik telah berlaku. Walau bagaimanapun, kedua-dua ekspresi *BMP-2* dan *COL-1* kekal rendah. Hasil kajian menunjukkan kepekatan optimum untuk reparixin dan rhIL-8, masing-masing adalah 50 nM dan 0.01 ng/ml. Untuk analisis tapak jalan hiliran IL-8, tapak jalan isyarat PI3K/AKT/mTOR dan JAK2/STAT3 dicadangkan untuk terlibat dalam pembezaan odontogenik kerana ekspresi kesemua penanda ini adalah sangat tinggi, sedangkan perencatan IL-8 menggunakan reparixin menyebabkan pengurangan ekspresi kesemua gen tersebut dengan ketara. Manakala ekspresi *NF-κB* secara relatifnya menunjukkan ekspresi yang rendah dalam semua sampel apabila dibandingkan dengan gen lain sekaligus menyarankan bahawa isyarat NF-κB memainkan peranan kecil dalam pembezaan odontogenik. Di samping itu, pengawalaturan ekspresi *DSPP*, *DMP1* dan *OPN*, dan intensiti alizarin yang lebih tinggi dilihat pada kumpulan SHED yang direncat dengan reparixin (SABR) menunjukkan pembezaan odontogenik telah berlaku. Walau bagaimanapun, tiada perubahan ketara yang diperhatikan dalam ekspresi penanda odontoblast dan mineralisasi SHED apabila SHED diaruh oleh rhIL-8. Kesimpulan daripada kajian ini mencadangkan bahawa perencatan reseptor IL-8 oleh reparixin menggalakkan pembezaan odontogenik SHED yang dikultur di atas permukaan HAM dengan kehadiran BMP-2.

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ABSTRACT

Interleukin-8 (IL-8), an inflammatory cytokine with pleiotropic biological effects, has been reported to be implicated in odontogenesis and involved in odontoblast-mediated immune responses. However, the exact mechanism on how IL-8 influences odontogenesis remains unclear. Hence, this study was conducted to investigate the role and mechanism of IL-8-immunomodulatory pathway in the differentiation of stem cells from human exfoliated deciduous teeth (SHED) into odontoblast-like cells. SHED were seeded on human amniotic membrane (HAM) and treated with bone morphogenetic protein-2 (BMP-2). Four groups were assigned: SHED only (S), SHED with BMP-2 (SB), SHED on amniotic membrane without BMP-2 (SA), SHED on amniotic membrane with BMP-2 (SAB). Following treatment, SHED were harvested on day 1, 7, 10 and 14, and odontogeneic differentiation potential was assessed by the expression of odontogeneic markers using reverse transcriptase polymerase chain reaction and calcium deposition by Alizarin Red S staining. Thereafter, the optimal concentration of reparixin and rhIL-8 were determined using Real Time RT-PCR. For IL-8 study, another four groups were assigned; SHED on amniotic membrane with BMP-2 (SAB), SHED on amniotic membrane with BMP-2 and reparixin (SABR), SHED on amniotic membrane with BMP-2 and rhIL-8 (SAB8), SHED on amniotic membrane with BMP-2, reparixin and rhIL-8 (SABR8). SHED were treated with reparixin as IL-8 inhibitor, while

recombinant human IL-8 (rhIL-8) as IL-8 inductor, and downstream analysis were conducted using Real Time RT-PCR. The levels of IL-8 protein secretion of SHED with and without IL-8 induction and inhibition during odontogeneic differentiation was analysed using ELISA. The effect of IL-8 in calcium deposition of SHED was determined using Alizarin Red S staining. Results of the present study showed that odontoblast specific gene markers *DSPP*, *DMP1*, *OPN* were highly expressed on day 7 onwards as odontogeneic differentiation occurred. On the other hand, the *BMP-2* and *COL-1* expressions remain lowly expressed. The optimal concentration for reparixin and rhIL-8 were found to be 50 nM and 0.01 ng/ml, respectively. For IL-8 downstream pathway analysis, PI3K/AKT/mTOR and JAK2/STAT3 signalling pathways were suggested to be involved in odontogeneic differentiation as the expression of all the markers were high, whereas inhibition of IL-8 using reparixin caused significant reduction of their expression. *NF-κB* expression showed relatively low expression in all samples when compared to other genes suggesting that NF-κB signalling plays a minor role in odontogeneic differentiation. In addition, up-regulation of *DSPP*, *DMP1* and *OPN* expression, and higher intensity of alizarin staining were observed when SHED were inhibited with reparixin (SABR) suggested odontogeneic differentiation has occurred. However, no significant changes were observed in the expression of odontoblast markers and SHED mineralisation when SHED were induced with rhIL-8. In conclusion, this study suggests that inhibition of IL-8 receptor by reparixin promotes odontogeneic differentiation of SHED when cultured on HAM treated with BMP-2.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Dental cavities and tooth decay are most common worldwide health issues with dental cavities and periodontal diseases posing the major causes of tooth loss. These oral problems affect the quality of life of patients of all ages and results in an economic burden on healthcare systems around the world (Baelum *et al*., 2007). The present common treatment is to remove the lesions and to replace the dental tissues with synthetic materials, such as amalgam, resin and gutta-percha. However, these non-biological substitutes are incapable of replacing the biological function of the lost natural tissue causing the reduction of mechanical properties and vitality of the teeth (Dietschi, 2008).

Latest advancement in tissue engineering technology has prompted a developing interest for the improvement of regenerative dentin-pulp structure, which could give natural replacement or repair the damaged tooth tissues (Murray *et al*., 2007). In tissue engineering, a scaffold, stem cells and growth factors are used as essential components, and their interactions have been regarded to be important for regeneration of tissues. Tooth regeneration using tissue engineering concept is a promising biological approach which aims to regenerate natural tooth-like mineral tissues in terms of histology, morphology and function (Andrea and Paul, 2009).

The combination of stem cells with osteo/odonto-inductive agent and a natural scaffold that develops into a three-dimensional (3D) construct and become one of the most standout among approaches for dental tissue engineering. Stem cells from human exfoliated deciduous teeth (SHED) are a type of mesenchymal stem cells (MSCs) that are easily accessible, with extensive proliferative potential and have high potency to differentiate into multiple lineages. Bone morphogenetic protein-2 (BMP-2) is a growth factor which has been shown to induce odontogeneic differentiation of SHED (Iohara *et al*., 2004), while human amniotic membrane (HAM) has unique characteristics that make it potentially attractive as a biomaterial scaffold. These three elements, SHED/BMP-2/HAM could be an ideal 3D model for tooth tissue engineering, hence chosen as the 3D construct for this current study.

The mitotic, metabolic and development activities of MSCs are regulated by components in the extracellular environment including autocrine and paracrine factors synthesised by the MSCs themselves (Sumanasinghe *et al*., 2009). Also, the signals that modulate the growth and differentiation capacity of MSCs provide valuable information on the cascades involved in their terminal differentiation. The existence of, and the changes in these signals during *in vitro* MSC differentiation need to be investigated in order to identify the key factors, and to understand the mechanisms involved especially in the differentiation of MSCs into odontoblast-like cells.

Stimulation of MSCs with various types of pro-inflammatory cytokines to induce odontogenesis has been recently studied. Pro-inflammatory cytokines such as interleukin-8 (IL-8), interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and

interferon gamma (IFN-γ) are involved in the differentiation of dental pulp stem cell (Chang *et al*., 2005; Yang *et al*., 2011; Ueda *et al*., 2014; He *et al*., 2017). These studiess have primarily focused on determining appropriate modulators required to achieve successful odontogeneic differentiation of MSC.

IL-8 commonly recognised as pro-inflammatory cytokine with wide range of biological effects especially its function as a chemoattractant besides being activating factor for neutrophils. IL-8 has been demonstrated for its involvement in the tumor progression due to its ability to enhance the invasion, proliferation, adhesion, angiogenesis and metastatic potential of tumor cells (Kim *et al*., 2001). IL-8 has also been reported to have a direct stimulatory effect on osteoclastogenesis and bone resorption (Bendre *et al*., 2003). Virve *et al*., (2007) reported that over 20-fold induction of IL-8 was detected in odontoblasts in response to the TGF-β1 treatment, and IL-8 was the only interleukin to change markedly after the TGF-β1 treatment in odontoblasts.

Previous study has demonstrated that dentin matrix protein 1 (DMP1) was involved in intra-pulpal mineralisation and stimulates the production of IL-6 and IL-8 from pulp fibroblasts (Abd-Elmeguid *et al*., 2012). Also, a study reported that IL-8 was up-regulated during fibroblast growth factor-2 (FGF-2)-induced odontogeneic differentiation of human dental pulp cells (hDPCs) (Kim *et al*., 2010a). Another study has shown that IL-8 expression has immunomodulatory properties of MSCs in periapical lesions (Dokic *et al*., 2013).

All these data suggest that IL-8 may be a key cytokine in odontoblast-mediated immune responses. However, the exact mechanism on how IL-8 influences odontogenesis remains unclear. Hence, before SHED/BMP-2/HAM could be used in clinical therapy, it is critical to understand their biological properties in response to extrinsic and intrinsic stimuli. Also, the effects of pro-inflammatory cytokines should be especially clarified.

1.2 Research justification

Tooth loss or absence is a common condition that can be caused by various pathological circumstances. The replacement of the missing tooth is important for medical and aesthetic reasons. Recently, scientists focus on tooth tissue engineering, as a potential treatment, beyond the existing prosthetic methods. Tooth engineering is a promising new therapeutic approach that seeks to replace the missing tooth with a bioengineered one or to restore the damaged dental tissue.

Numerous experiments have been carried out exhibiting the capacity of MSCs to differentiate into odontoblast-like cells with different results and success rate. However, to date, to our best knowledge, no published studies have addressed the effect of IL-8 in odontogeneic differentiation, where IL-8 is believed to play a role when SHED are cultured on HAM with the addition of BMP-2. Moreover, investigation of the interaction between SHED and the inflammatory environment would lead to better understanding in MSC cross talk and intercellular signalling in odontogenesis. Also, this study could shed light on the possibilities of regenerative therapies in dental field.

1.3 Research objective

1.3.1 General objective

This study was conducted to investigate the role and mechanism of IL-8-immunomodulatory pathway in the differentiation of SHED into odontoblast-like cells when cultured on human amniotic membrane in the presence of bone morphogenetic protein-2.

1.3.2 Specific objectives

1. To determine the gene expression level of odontoblast specific markers in SHED when differentiated into odontoblast-like cells using RT-PCR.
2. To determine the optimum concentration of recombinant human IL-8 as an IL-8 inducer and reparixin as an IL-8 inhibitor using Real Time RT-PCR.
3. To investigate IL-8 protein expression level in SHED under IL-8 induction and inhibition using ELISA.
4. To investigate the effects of IL-8 on PI3K/AKT/mTOR pathway under IL-8 induction and inhibition during odontogeneic differentiation using Real Time RT-PCR.

CHAPTER 2

LITERATURE REVIEW

2.1 Human Teeth

Human teeth are a group of hard tissue which are small, whitish, calcareous, bony structures found in the mouth. Teeth are basically generate forces that break down items of food by tearing, grinding and crushing them to a finer particles for swallowing and chemical digestion. Humans have two sets of teeth which are classified into four basic types: incisors, canines, premolars, and molars which each of them does a different job. The incisors at the front of the mouth used for cutting food or object, the canines used for tearing and ripping food, both premolars and molars chew, crush and grind food.

The human dentition is composed of two sets of teeth which are primary and permanent. Primary teeth also known as deciduous teeth, or milk teeth, are the first set of teeth, in humans and other diphyodont mammals. They are developed during the embryonic stage of development and appear during infancy (Peterkova *et al*., 2014). Primary teeth are usually lost and replaced by permanent teeth.

The visible permanent teeth can cause root resorption, where the permanent teeth push on the roots of the primary teeth, which causes the roots to be dissolved by odontoclasts (as well as surrounding alveolar bone by osteoclasts) and become absorbed by the upcoming permanent teeth. This process of replacing the primary

teeth with permanent teeth is called exfoliation (Sahara *et al*., 1993). This may last from age six to age twelve. By age twelve there usually are only permanent teeth remaining.

Primary teeth are different from permanent teeth as the enamel of the deciduous teeth is thinner than the enamel on permanent teeth (De Menezes Oliveira *et al*., 2010). As a result, the primary teeth usually look a lot whiter than the permanent teeth. Because the primary teeth are designed to fall out, they have differently shaped roots than permanent teeth. The roots of the deciduous teeth are thinner and shorter than the roots of the permanent teeth (George *et al*., 2016).

Furthermore, the short length of the tooth's roots makes it easier for them to dissolve when it's time for the tooth to fall out and ensures that the permanent teeth have space to form beneath them. Another big difference between the primary teeth and the permanent teeth is the number of them. According to the American Dental Association, human beings typically have 20 primary teeth and 32 permanent teeth, including four wisdom teeth (American Dental Association, 2006).

2.1.1 Tooth structure

The tooth has two anatomical parts. The portion of a tooth exposed to the oral cavity is known as the dental crown, and the portion below the dental crown is known as the tooth root. Each tooth consists of the four parts; enamel, dentine, cementum, and dental pulp. The first three are hard tissues while the pulp is soft tissue. The tooth is

supported by the tissue consisting of the alveolar bone, gums and the periodontal ligament (Palumbo, 2011). Cross-sectional image of tooth is shown in Figure 2.1.

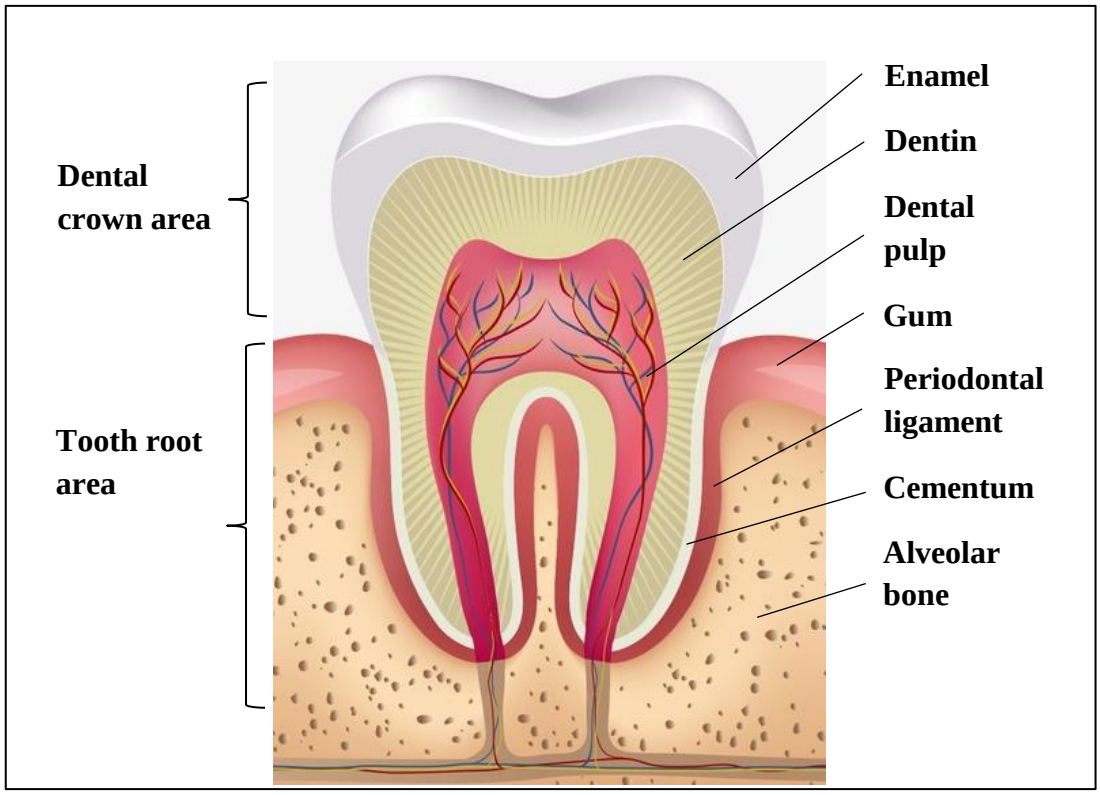

Figure 2.1: The cross-sectional image of tooth structure.

(Adapted from : https://pngtree.com/freepng/teeth-cross-section_701417.html)

Enamel is the hardest substance and most highly mineralised substances in the human body and contains the highest percentage of minerals (96% in weight) inorganic hydroxyapatite mineral along with small amount of water (4% in weight) (Gutierrez-Salazara and Reyes-Gasga, 2003). Enamel is formed by ameloblasts, in which covers the anatomical crown of a tooth and meets the cementum covering the root at the line called cemento-enamel junction. Once fully formed, it does not contain blood vessels or nerves. Enamel is mainly responsible for aesthetic, colour, translucency and texture of the tooth. It primarily composed of minerals that made up

of millions of rods or prisms, in a tighly packed and highly organised mass of hydroxyapatite crystal (Garg and Garg, 2013).

Dentine is calcified tissue made up of calcium phosphate and some other insoluble calcium compounds that is protected by the tooth's enamel. It is formed by odontoblasts, the most peripheral columnar shape cells arranged along the interface between dentine and pulp. Dentine can be divided into several types according to the site, function and origin of the dentine such as mantle dentine, circumpulpal dentine (intertubular dentine, peritubular dentine, primary, secondary and tertiary dentine), root dentine and apical dentine (Goldberg *et al*., 2011; Tjaderhane *et al*., 2012).

Cementum is a specialised bone like substance covering the root of a tooth. It is approximately 45% inorganic material (mainly hydroxyapatite), 33% organic material (mainly collagen), and 22% water (Ross *et al*., 2002). Cementum is excreted by cementoblasts at the root surface of the tooth and is the thickest at the root apex. It is yellowish and softer than dentine and enamel (Chen and Jin, 2010). The principal role of cementum is to serve as a medium by which the periodontal ligaments can attach to the tooth for stability. At the cemento-enamel junction, the cementum is acellular due to its lack of cellular components, and this acellular type covers at least two third of the root. The more permeable form of cementum, which is cellular cementum, covers about a third of the root apex (Ten Cate, 1998).

Dental pulp is a specialised loose connective tissue located at the centre of the tooth. Dental pulp communicates with the periodontal ligament through apical foramina and other accessory canals that are found at every level. Dental pulp contains cells,

neurovascular and ground substance elements (Pashley *et al*., 1994). The cellular elements include undifferentiated MSCs, odontoblasts, fibroblasts and defensive cells such as lymphocytes, mast cells, basophils, macrophages, eosinophils, neutrophils and plasma cells (Yu and Abbott, 2007).

2.2 Odontoblast

Odontoblasts are a type of post-mitotic cells and originally differentiated from mesenchymal papilla cells during development of human craniofacial by migration of neural crest cells (Arana-Chavez *et al*., 2004). Odontoblasts generally differentiate according to subunit-specific temporal and spatial patterns of tooth, and secrete predentin-dentine components (Goldberg and Smith, 2004). These post-mitotic polarised cells are aligned as monolayer at the periphery of the dental pulp, and their cytoplasmic processes extend into dentinal tubules. Odontoblasts are connected by junctional complexes; however, these sealing materials presumably do not totally seal the inter-odontoblastic spaces. Thereby making a palisade of cells that provide a permeability barrier; gap junctions are also responsible for intra-cellular communication, which may be involved in regulating the pulp healing process (Magloir *et al*., 2004).

The presence of all the elements of the secretory and mineralisation machinery in the cells confirms their intense activity, notably during primary dentinogenesis. At a later stage during secondary dentinogenesis, the cells return to a quiescent state, with a reduced number of cytoplasmic organelles (Jones and Boyde, 1984). Unlike osteocytes, odontoblasts do not become incorporated in the matrix, except for their

processes that are embedded in the tubules. This is why dentine must not be considered as an individual tissue but rather as the dentine-pulp complex (Ferretti *et al*., 2006).

The odontoblast processes contain limited organelles (which are believed to be responsible for the later secretion of intra-tubular dentine), but are mostly filled by a dense network of micro-filaments and other micro-tubules. Increasingly, coronal odontoblasts are regarded as being different from those found in the root (Nanci, 2003). The coronal odontoblasts are elongated and pyramidal with an apical nucleus, whereas radicular odontoblasts are more cuboidal, which is potentially an indication of significantly lower cellular activity (Simon *et al*., 2009b).

2.2.1 Odontogenesis

Odontogenesis or tooth development is an intricate process that involves interactions by which tooth form from embryonic cells, grow, and erupt into the mouth. For human teeth to have a healthy oral environment, all parts of the tooth must develop during appropriate stages of fetal development. Primary teeth start to form between the sixth and eighth week of prenatal development, and permanent teeth begin to form in the twentieth week (Bei, 2009). Tooth development is commonly divided into the following stages: the initiation stage, the bud stage, the cap stage, the bell stage, and finally maturation.

The initiation of tooth begins at the end of the fifth week of human gestation. A localised thickening or placodes within the primary epithelial bands, formed after

about 37 days of development, initiate tooth development (Nanci, 2008). In a subdivision of the primary epithelial band, the dental lamina, localised proliferative activity leads epithelial outgrowths into the ectomesenchyme. Since the underlying ectomesenchyme is more active than the epithelial cells, these ectomesenchymal cells accumulate the epithelial outgrowths soon afterwards. As those cells fold, the forming structure proceeds as per the following descriptive morphological stages of tooth development: bud, cap, and bell. Folding and growth of the epithelium give the final shape of the tooth crown (Tucker and Sharpe, 2004)

The bud stage is characterised by the appearance of a tooth bud without a clear arrangement of cells. The stage technically begins once epithelial cells proliferate into the ectomesenchyme of the jaw (Mammoto *et al*., 2011). Typically, this occurs when the fetus is around 8 weeks old. The tooth bud itself is the group of cells at the periphery of the dental lamina. Along with the formation of the dental lamina, 10 round epithelial structures, each referred to as a bud, develop at the distal aspect of the dental lamina of each arch. These correspond to the 10 primary teeth of each dental arch, and they signify the bud stage of tooth development. Each bud is separated from the ectomesenchyme by a basement membrane. Ectomesenchymal cells congregate deep to the bud, forming a cluster of cells, which is the initiation of the condensation of the ectomesenchyme (Han *et al*., 2003).

Cap stage occur when the tooth bud grows around the ectomesenchymal aggregation, taking on the appearance of a cap, and becomes the enamel organ covering the dental papilla (Krivanek *et al*., 2017). A condensation of ectomesenchymal cells called the dental sac or follicle surrounds the enamel organ and limits the dental papilla.

Eventually, the enamel organ will produce enamel, the dental papilla will produce dentine and pulp, and the dental sac will produce all the supporting structures of a tooth, the periodontium (Nanci, 2008).

The bell stage is known for the differentiation of enamel organ into bell-shaped with four cell types and dental papilla into two cell types. The mesenchymal cells in the dental papilla, adjacent to the inner enamel epithelium, differentiate into odontoblasts. The odontoblasts produce predentine, and deposit it adjacent to the inner enamel epithelium. The predentin later calcifies to form dentine. Cells of the inner enamel epithelium near the dentine form ameloblasts, which produce enamel in the form of prisms or rods over the dentine layer, thus help form the outer layer of the tooth or the crown (D'Souza, 2012).

Maturation stage is characterised by the completion of calcification. Hard tissues including enamel and dentine develop during this stage. Formation of dentine, known as dentinogenesis, is the first identifiable feature of this stage. The formation of dentine must always occur before the formation of enamel. The different stages of formation of dentine results in different types of dentin: mantle dentine, primary dentine, secondary dentine and tertiary dentine (Chiego, 2014).

2.2.2 Odontoblast specific markers

Cell surface marker is an antigenic determinant molecule found on the surface of cells that often function to characterise cell types (Cartwright, 2007). These markers enable cells to be characterised based on what molecules are present on their surface.

There are several cell surface markers expressed by odontoblasts, namely DSPP (dentine sialophosphoprotein), DMP1 (dentine matrix protein 1), OPN (osteopontin), ALP (alkaline phosphatase), and COL-1 (collagen 1).

Dentine sialophosphoprotein (DSPP) is a secreted precursor protein that is rapidly cleaved post-translationally into two dentine products: dentine sialoprotein (DSP) and dentine phosphoprotein (DPP) (MacDougall *et al*., 1997). DSP is poly ionic macromolecules that contain large amounts of carbohydrate and sialic acid. Meanwhile, DPP contain large amount of aspartic acid and phosphoserine. This feature proposed an important role of dentine ECM in dentine mineralisation and mineral nucleation (George *et al*., 1996). Several experimental data have demonstrated that high expression of DSPP in odontoblasts, transiently in preameloblasts, and low levels expression detected in bone (D'souza *et al*., 1997; Qin *et al*., 2002; Baba *et al*., 2004). Taken together, these data suggest that the key role of DSPP in tooth formation and mineralisation.

Dentine matrix protein 1 (DMP1) is an acidic phosphoprotein that regulates many important cellular processing including differentiation and ECM production which is normally expressed by both osteoblasts and odontoblasts (George *et al*., 1993). This non-collagenous protein highly composed of aspartic acid, glutamic acid, and serine residues (George *et al*., 1994; MacDougall *et al*., 1998). DMP1 constitutes a crucial component of bone and dentine matrix, and therefore is suggested to play a key role in the initiation of mineralisation through the regulation of mineral crystallinity (Linde and Goldberg, 1993). Moreover, early expression *DMP1* can be observed

during the commitment of neural crest-derived cells into odontoblasts (Narayanan *et al*., 2006).

Osteopontin (OPN) is an acidic, rich phosphorylated glycoprotein, and highly expressed by cells resident in the bone, teeth, and its expression often present in several pathologic calcification sites of soft tissues (Rathinam *et al*., 2015). In bone, OPN is a major component found in cell-matrix interfacial structures called cement lines and lamina limitans (McKee and Nanci, 1996). In normal bone remodelling, OPN is responsible for the regulation of bone cell adhesion, migration, osteoclast function, cell survival and deposition of bone matrix (Sodek *et al*., 2000). In teeth, OPN induce pulp mineralisation in the early stages of matrix formation and helps the initiation of reparative dentinogeneic process. Besides, function of OPN is implicated in diverse biological and molecular events taking place during odontogenic process of progenitor cells (Kuratate *et al*., 2008).

Alkaline phosphatase (ALP) is an endogenous enzyme present in many organs, particularly high detectable in liver, bile duct, kidney, bone, intestinal mucosa (Rosalki *et al*., 1999). ALP activity is most often involved in the beginning of matrix mineralisation and has been proposed as an early marker in the cascade of osteo/odontoblast differentiation (Min *et al*., 2010). Pang *et al*., (2006) reported that the activity of ALP in odontoblast-like cells is higher than undifferentiated cells. Therefore, ALP is believed to stimulate the formation of mineralised nodule and calcium deposition in most cells differentiated into odontoblasts, as it is considered as an early marker of odontoblastic differentiation.

Collagen type I (COL-1) is the dominant fibrous protein in hard tissues, including bone and dentin. In dentin, collagen comprises about 80 to 90% of the organic matrix and is present as nano-sized fibers. COL-1 provides the framework for the deposition of apatite crystal (Linde and Goldberg, 1993; Butler, 1995; Butler, 1998). Previous study by Bleicher *et al*., (1999) reported that, at the late stage of odontogeneic differentiation, COL-1 synthesis was dramatically increased. According to Nakashima *et al*., (1994), bone morphogenetic protein-4 (BMP-4) caused stimulation of the expression of *COL-1* transcription in dental pulp cells and also, *COL-1* has been shown to further regulate the expression of *DMP1* and *OCN* in rat DPCs (Mizuno *et al*., 2003).

2.3 Tissue engineering and regenerative endodontics

Tissue engineering evolved from the field of biomaterials development and can be defined as the involvement of stem cells, scaffolds, and signalling factors, alone or in combination aim to improve or replace biological tissues. Tissue engineering was also defined by Langer and Vacanti as an interdisciplinary field that applies the principle of engineering and life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ (Langer and Vacanti, 1993). Apart from that, tissue engineering aims to stimulate the body to heal itself. Tissue engineering also promotes the growth of tissue outside the body which can then be implanted as natural tissue (Nadig, 2009).

Regenerative endodontics is based on the application of tissue engineering concept. Regenerative endodontics can be defined as biologically based procedures designed

to replace damaged structures, including dentine and root structures, as well as cells of the pulp-dentine complex (Murray *et al*., 2007). Clearly, it is a field of active research that is dynamically growing in both basic research and clinical applications. It has been reported that the scope of regenerative endodontics may be increased to include the replacement of periapical tissues, periodontal ligaments, gingiva and even whole teeth. This treatment would give patients a clear alternative to the artificial tooth implants that are currently available (Murray and Garcia-Godoy, 2006).

The ingredients for regeneration consists of the following interactive triad; i) an appropriate cell source ii) a supportive matrix (scaffold) and iii) inductive biological factors or signals. Often these disciplines are combined rather than used individually to create regenerative therapies (Murray *et al*., 2007). Efficient regenerative-based treatment must include the optimal cell source, scaffold design, bioreactors and the use of microfabrication technology.

2.3.1 Stem cells

In order to generate engineered tissue, the cells chosen will have to be able to respond to their environment, differentiate, form new tissue and integrate with native tissue. Stem cells are commonly defined as cells that have the innate ability to continuously divide either to maintain the stem cells pool (self-replication), or generate another cells (progenitors) capable of differentiating into various other types of cells (multi-lineage differentiation) (Rao, 2004). Stem cells can be classified as either embryonic or postnatal stem cells (Fortier, 2005).

Embryonic stem cells possess the ability to differentiate into all types of somatic cells and divide at an unlimited number of times. However, ethical concerns on the use of these kind of cells remain a highly contentious matter, because harvesting them requires the destruction of human embryos early in development (Ulmer *et al*., 2010). To date, isolation of these embryonic stem cells has been obtained only from animal species (Kang *et al*., 2008; Zhu *et al*., 2008).

Similar to embryonic stem cells, postnatal stem cells are capable of self-renewal. However, postnatal cells are multi-potent; that is, they have a more limited capacity for differentiating into other cell types than the totipotent embryonic stem cells. Bone marrow, umbilical cord, adipose tissues, placenta and skin have been considered as the most common source of postnatal MSCs used for clinical applications (Annibali *et al*., 2014). Postnatal multi-potent stem cells were also found in dental tissues, such as dental pulp stem cells (DPSCs) (Gronthos *et al*., 2000), SHED (Miura *et al*., 2003), periodontal ligament stem cells (PDLSCs) (Seo *et al*., 2004), dental follicle stem cells (DFSCs) (Morsczeck *et al*., 2005a) and stem cells from apical papillae (SCAP) (Huang *et al*., 2008).

2.3.1(a) Dental stem cells

Recent stem cell studies in the dental field have identified many adult stem cell sources in the oral and maxillofacial region. These cells are believed to reside in a specific area of each tissue (Figure 2.2). Current studies have indicated that the dental stem cells may have the potential to regenerate bone, periodontal ligament,

and possibly, teeth (Miura *et al*., 2003; Seo *et al*., 2004; Morsczeck *et al*., 2005b; Yokoi *et al*., 2007).

One of the first tooth-related stem cell types was found in the pulp of permanent teeth and was named as dental pulp stem cells (DPSCs). The description of DPSCs inside the pulp parenchyma demonstrated that the dental organ provides a "niche" environment for replacement cells (Gronthos *et al*., 2000). Another population of stem cells has also been reported in the pulp of deciduous teeth. These cells, as mentioned earlier known as SHED (Miura *et al*., 2003), are particularly interesting because they are relatively easy to collect when the deciduous tooth is shed, and replaced by the permanent successor.

Moreover, a further group of MSCs has been reported in the apical papilla of human immature teeth. These multi-potent cells have been termed as stem cells of apical papilla (SCAP) (Huang *et al*., 2008). Since they may be bone marrow-derived cells, SCAP have potential for osteogeneic and dentinogeneic differentiation. Furthermore, another multi-potent cell named as periodontal ligament stem cells (PDLSCs) successfully isolated from periodontal ligament of the periodontium. PDLSCs represent a promising cell-based therapy because they are able to differentiate into periodontal tissues such as cementoblast-like cells and collagen-forming cells (Seo *et al*., 2004).

Finally, dental follicle progenitor stem cells (DFPSCs) obtained from mesenchymal tissue that surrounds the developing tooth germ also another type multi-potent stem cells derived from dental tissue. DFPSCs have immunomodulatory properties, high

proliferation potential and capacity to differentiate into odontoblasts, cementoblasts, osteoblasts and other cells implicated in the teeth (Morsczeck *et al*., 2005b). *In vivo* DFPSCs transplantation can lead to the development of periodontal ligament-like tissues (Yokoi *et al*., 2007).

Most research is directed toward regeneration of damaged dentin, pulp, resorbed root, periodontal regeneration and repair perforations. Whole tooth regeneration to replace the traditional dental implants is also in the pipeline. Other than that, tissue engineering applications using dental stem cells that may promote more rapid healing of oral wounds and ulcers as well as the use of gene-transfer methods to manipulate salivary proteins and oral microbial colonisation patterns are now promising and possible (Jain and Bansal, 2013).

Also, dental stem cells appear to be a promising reservoir of multi-potent cells, and as such, offer significant potential for use in various non-dental biotechnological applications. The presence of a population of stem or progenitor cells in the dental pulp provides a local source of cells for generating new "odontoblast-like" cells for both natural pulp wound healing or regeneration and direct pulp capping after injury to the tooth (Gandhi *et al*., 2011). An important focus of future studies will be a more precise characterisation of these stem cells and their potentials to allow their most effective application in new regenerative therapies.

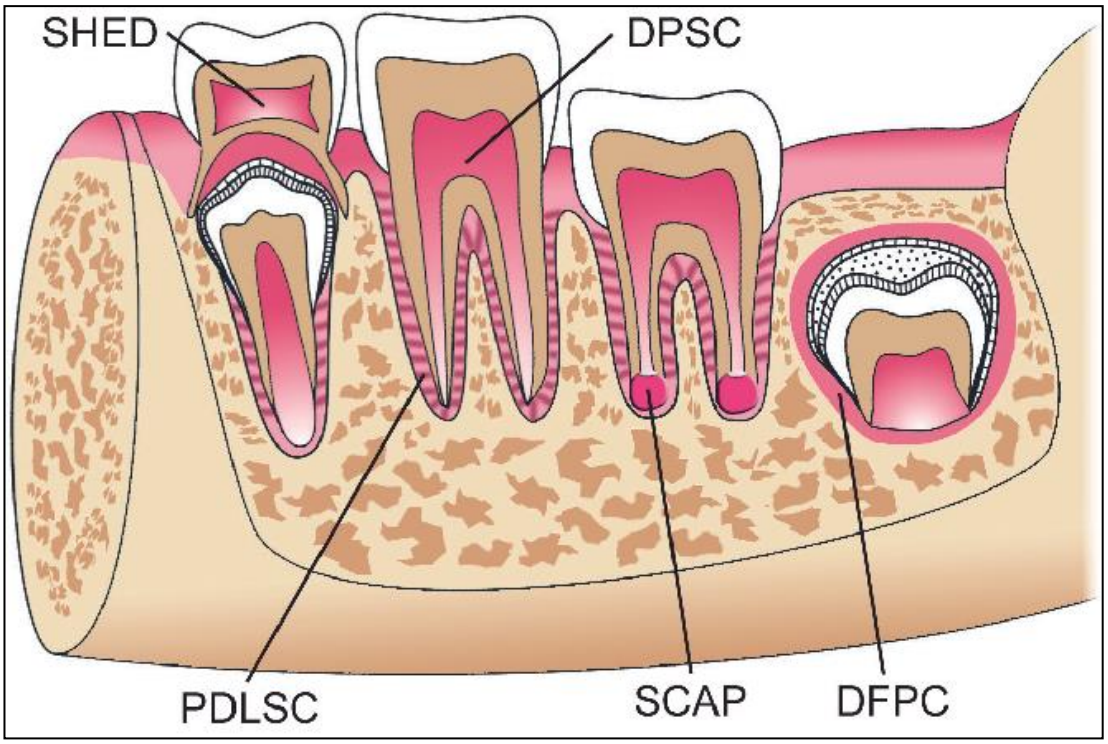

Figure 2.2: Sources of dental stem cells. SHED: Stem cells from Human Exfoliated Deciduous teeth, DPSC: Dental Pulp Stem Cells, PDLSC: Periodontal Ligament Stem Cells, SCAP: Stem Cells of the Apical Papilla, DFSC: Dental Follicle Stem Cells (Adapted from Bojic *et al*., 2014).

2.3.1(b) Stem cells from human exfoliated deciduous teeth (SHED)

SHED were identified for the first time in 2003 by Miura *et al*., as a novel population of multi-potent postnatal stem cells. They can be easily acquired as a by-product of routine tooth extraction treatment without ethical concerns. The pulp can be easily dissected and stored for a long-term in liquid nitrogen in stem cell banks, to be used in future for transplantation without the risk of immunologic reaction (Bakopoulou *et al*., 2011).

Compared with dental pulp isolated from permanent teeth, SHED provides a better source of stem cells because a significant number of stem cells decreased with aging,

and stem cell quality within niche could be affected by genetic and microenvironment factors (Annibali *et al*., 2014). Moreover, it has been suggested that exfoliated deciduous teeth can be stored for SHED banking as MSC sources (Arora *et al*., 2009). SHED can be cryopreserved for an extended period of time when needed, they can be carefully thawed to maintain their viability (Suchanek *et al*., 2007).

Like other types of MSCs, SHED can be characterised by spindle of fibroblast-like appearance, high proliferation ability and expression of mesenchymal markers, such as CD105, CD29, CD90, CD146, and CD44 (Wang *et al*., 2012). It has been reported that more than 90% of dental pulp MSCs population was positive for mesenchymal-related antigens CD29, CD44, CD73, CD90, CD105 and CD166 (Trubiani *et al*., 2012). SHED showed higher proliferation rate and cell population doublings when compared with DPSCs and bone marrow derived mesenchymal stem cells (BMMSCs), suggesting that they represent a more immature population of multi-potent stem cells (Miura *et al*., 2003).

2.3.1(c) Multi-potent differentiation capability of SHED

Among the dental pulp stem cells, SHED have recently attracted attention as novel multi-potential stem cell sources. SHED distinctively showed multi-potential differentiation capability into a variety of cell types. They can differentiate odontogeneically, osteogeneically, adipogeneically, chondrogeneically both *in vitro* and *in vivo* (Daltoe *et al*., 2014).

SHED can undergo osteogenesis under *in vitro* condition by the expression of bone specific markers such as bone sialoprotein (BSP), osteocalcin (OCN), alkaline phosphatase (ALP), matrix extracellular phosphoglycoprotein (MEPE), core-binding factor alpha 1 (CBFA1), and osterix. Osterix is a transcription regulator required for osteoblast differentiation and bone formation (Annibali *et al*., 2014). Few studies have investigated the capability of SHED in bone tissue repair and/or regeneration when SHED were cultured on a scaffold of hydroxyapatite (HA)/tricalcium phosphate (TCP) (Yamaza *et al*., 2010).

Under *in vivo* condition, SHED were reported to be able to repair critical-size parietal defects in immunocompromised mice after 6 months of transplantation. SHED were able to form bone-like tissue on the surface of HA/TCP by the positive immunohistochemical staining of ALP, COL-1, and BSP. These results suggest that deciduous teeth can provide guidelines for the eruption of permanent teeth as well as can promote induction in bone formation during eruption of permanent teeth (Huang *et al*., 2009).

SHED also can be successfully differentiated into chondrogeneic lineages *in vitro* and *in vivo*. The potential of SHED to develop into chondrocyte-like cells can be achieved using several inductive reagents such as insulin-transferrin-selenium, L-ascorbic acid 2-phosphate, sodium pyruvate, L-proline, TGF-β, dexamethasone, HEPES, BMP-6, FBS, bovine serum albumin, penicillin and streptomycin (Annibali *et al*., 2014). A study by Koyama *et al*., (2009) reported that SHED treated with BMP-2 was capable to induce the expression of chondrogeneic markers including SOX9, type 2 collagen and type X collagen.

In 2014, Chen and co-workers demonstrated the chondrogeneic potential of SHED *in vitro* by culturing these cells for 14 days in chondrogeneic differentiation medium containing dexamethasone, insulin, ascorbate phosphate, TGF-β_3 and basic fibroblast growth factor. For their *in vivo* study, SHED were seeded onto beta-tricalcium phosphate (β-TCP) scaffolds and transplanted into the subcutaneous space on the back of nude mice. Interestingly, they found that SHED showed colony-forming capacity, and the combination of SHED and β-TCP was able to generate new cartilage-like tissues (Chen *et al*., 2014). Taken together, these results demonstrate that successfully induced chondrogenesis of SHED *in vitro* and *in vivo* may bring new therapeutic potential application of SHED in cartilage tissue engineering.

The adipogeneic differentiation capability of SHED has been reported by Miura *et al*., (2003) where they showed that, using an adipogeneic inductive mixture, SHED possess the potential to develop into oil red O-positive lipid-laden fat cells. They also reported that, there was an up-regulation in the expression of two adipocyte-specific transcription factors: peroxisome proliferator-activated receptor (PPAR)-γ 2 and lipoprotein lipase. These findings were consistent with a study by Koyama *et al*., (2009) who demonstrated SHED have higher capability to differentiate into adipocyte-like cells compared to DPSCs.

However, some studies reported that SHED have less capability in differentiating into adipocyte-like cells. According to Yamaza *et al*., (2010), SHED suffered remarkable inability in adipogenesis as induced SHED showed decreased numbers of lipid-specific oil red O-positive cells and reduced expression of adipocyte-specific protein by Western blot analysis. The similar scenarios were described by Pivoriunas