THE EFFECTS OF SIRNA-TARGETING IL-17A RECEPTOR IN REGULATING THE OSTEOGENIC DIFFERENTIATION IN STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

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by

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LIST OF SYMBOLS

ng/mL	Nanogram per mililitre
nM	Nanomolar
nmol	Nanomol
µg/mL	Microgram per mililitre
µL	Microlitre
µm	Micrometre
µM	Micromolar
V	Volt
w/v	Weight per volume
ng/mL	Nanogram per mililitre
nM	Nanomolar
nmol	Nanomol
µg/mL	Microgram per mililitre
µL	Microlitre
µm	Micrometre
µM	Micromolar
V	Volt
w/v	Weight per volume

LIST OF ABBREVIATIONS

α-mem	Alpha minimum essential medium
AA-FLS	Fibroblast-like synoviocytes of adjuvant induced-arthritis
ACTB	Beta (β)-actin
AGO-2	Argonaute 2
ALP	Alkaline phosphatase
ANOVA	Analysis of Variance
ARS	Alizarin Red S
BMP	Bone morphogenetic
BMSC	Bone marrow-derived stem cells
BSA	Bovine serum albumin
BSC 2	Biosafety cabinet level 2
BTE	Bone tissue engineering
CBAD	C/EBP-b activation domain
CCL20	Chemokine ligand 20
CD	Cluster of Differentiation
cDNA	Complementary deoxyribonucleic acid
C/EBP	CCAAT/enhancer-binding protein
CFU-M	Macrophage colony-forming unit
CO2	Carbon dioxide
COL1A1	Collagen type I Alpha I
COPD	Chronic obstructive pulmonary disease
CTLA8	Cytotoxic T-lymphocyte-associated antigen 8
CXCL1	C-X-C Motif Ligand 1
Cyr61	Cysteine-rich angiogenic inducer 61
DEPC	Diethyl pyrocarbonate
DFSC	Dental follicle stem cells
DMSO	Dimethyl sulfoxide
DPSC	Dental pulp stem cells
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid

ESC	Embryonic stem cells
EST	Expressed sequence tag
ETBR	Ethidium bromide
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCL	Hydrochloric acid
IgE	Immunoglobulin E
IGF-1	Insulin-like growth factor 1
IL-17RA	Interleukin-17A
IPSC	Induced pluripotent stem cells
LB Buffer	Lithium borate buffer
LCN2	Lipocalin-2
MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony-stimulating factor
miRNA	Micro ribonucleic acid
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MSC	Mesenchymal stem cells
NaCL	Sodium chloride
NF-kB	Nuclear factor kappa B
OCN	Osteocalcin
OM	Osteogenic medium
OPG	Osteoprotegerin
OPN	Osteopontin
ORF 13	Open reading frame 13
PBS	Phosphate buffer saline
PE	Phycoerythrin
PES	Polyethersulfone
PRP	Platelet-rich plasma
PGE2	Prostaglandin E2
qRT-PCR	Quantitative real-time polymerase chain reaction

RA	Rheumatoid arthritis
RACEPCR	Rapid amplification of cDNA ends polymerase chain reaction
RANKL	Receptor activator of nuclear factor kappa beta
rIL-17A	Recombinant Interleukin-17A
RISC	RNA-induced silencing complex
ROX	6-carboxyl-X-Rhodamine
rpm	Revolutions per minute
RUNX2	Runt-related transcription factor 2
SEFIR	Similar expression of fibroblast growth factor and IL-17R
SHED	Stem cells from human exfoliated deciduous teeth
siRNA	Small interfering ribonucleic acid
SPSS	Statistical Package for Social Sciences
STAT3	Signal transducer and activator of transcription 3
TGF-β	Transforming growth factor beta
Th 1	T helper type 1
TNF-α	Tumor necrosis factor alpha
TRBP	TAR-RNA-binding protein
Tregs	Regulatory T cells
UV	Ultraviolet

LIST OF APPENDICES

KESAN SIRNA-TERSASAR-RESEPTOR A INTERLEUKIN-17 DALAM MENGAWAL ATUR PENGEKSPRESIAN GEN OSTEOGENIK DALAMSEL STEM DARIPADA GIGI SUSU MANUSIA YANG TERLUPAS

ABSTRAK

Interleukin 17-A memainkan peranan penting dalam mekanisme pembezaan osteogenik serta dalam pembentukan semula tulang. Sehingga kini, terdapat kajian yang terhad dalam menerangkan potensi siRNA terhadap pengekspresian *IL-17RA* dan bagaimana modulasinya mempengaruhi proses pembezaan osteogenik. Oleh itu, kajian ini bertujuan untuk menilai kesan siRNA tersasar terhadap *IL-17RA* dalam mengawal pembezaan osteogenik dan pengekspresian penanda osteogenik dalam sel stem daripada gigi susu manusia yang terlupas (SHED). SHED dikulturkan dalam medium lengkap yang dibekalkan dengan medium osteogenik yang mengandungi 50 µg/mL L-ascorbic acid, 10 mM β-glycerophosphate, dan 100 nM dexamethasone untuk mengaruh pembezaan osteogenik selama 7 dan 14 hari. SHED yang dibezakan telah dikulturkan dalam dua keadaan: Kumpulan 1 dirawat dengan kepekatan optimum IL- 17A (50 ng/mL) dan Kumpulan 2 dirawat dengan IL-17A dan ditransfeksi dengan kepekatan optimum siRNA-targeting-*IL-17RA* (50 nM) selama 48 jam. Aktiviti pemineralan oleh pewarnaan merah Alizarin dalam semua kumpulan juga dilakukan pada hari ke-14 dan hari ke-21 pembezaan. Kesan siRNA dinilai dengan mengukur tahap pengekspresian penanda osteogenik seperti *ALP, OPG, RANKL, COL1A1*, dan *RUNX2* oleh qPCR selepas 7 dan 14 hari. SHED yang tidak dirawat dicirikan oleh pewarnaan positif untuk penanda sel stem seperti CD90, CD73, dan CD105 dan diwarnakan secara negatif untuk penanda sel hematopoietik CD14. SHED yang dibezakan menunjukkan pengekspresian *ALP, COL1A1*, dan *RUNX2* yang signifikan

pada hari ke-7 dan hari ke-14 pembezaan. Pewarnaan kumpulan yang dirawat IL-17A oleh Alizarin merah menunjukkan peningkatan aktiviti pemendapan kalsium berbanding kumpulan SHED yang tidak dirawat. Begitu juga, pengekspresian *ALP, OPG, COL1A1*, dan *RUNX2* dikawal atur menaik dengan signifikan dalam SHED yang dirawat IL-17A. Walau bagaimanapun, pengekspresian *RANKL* telah dikawal atur menurun. Menariknya, SHED yang ditransfeksi siRNA menunjukkan pengurangan pengawal aturan *ALP, OPG, COL1A1*, dan *RUNX2* yang signifikan manakala *RANKL* dikawal atur menaik. Penemuan ini menunjukkan bahawa IL-17A meningkatkan osteogenesis dengan menggalakkan pembezaan osteogenik dan siRNA tersasar-*IL-17RA* telah mengganggu fungsi IL-17A/IL-17RA, dengan itu mencadangkan kepentingan IL-17A dalam pengantaraan mekanisme fisiologi tulang.

THE EFFECTS OF SIRNA-TARGETING IL-17A RECEPTOR IN REGULATING THE EXPRESSION OF OSTEOGENIC GENES IN STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

ABSTRACT

Interleukin-17-A holds significant roles in osteogenic differentiation and bone remodelling mechanism. To date, limited studies describe the effects of small interfering RNA (siRNA) on the expression of IL-17A receptor (*IL-17RA*) and how the modulation influences the process of osteogenic differentiation. Thus, the present study was to evaluate the effects of siRNA-targeting-*IL-17RA* on the osteogenic differentiation and the expression levels of osteogenic markers in stem cells from human exfoliated deciduous teeth (SHED). SHED were cultured in complete Minimum Essential Medium α supplemented with osteogenic medium which consists of 50 µg/mL L-ascorbic acid, 10 mM β-glycerophosphate, and 100 nM dexamethasone to induce osteogenic differentiation in SHED for 7 and 14 days. Differentiated SHED were cultured into two conditions: Group 1was treated with optimized concentration of IL- 17A (50 ng/mL) and Group 2 was treated with IL-17A and transfected with optimized concentration of siRNA-targeting-*IL-17RA* (50 nM) for 48 hours. Mineralisation activity by Alizarin red staining was performed on day 14 and day 21. The effects of siRNA were evaluated by measuring the expression levels of osteogenic markers such as *ALP, OPG, RANKL, COL1A1*, and *RUNX2* by qPCR after 7 and 14 days. Untreated SHED were characterised by positively stained for stem cell markers such as CD90, CD73, and CD105 and were negatively stained for hematopoietic cell marker CD14. Differentiated-SHED showed significant expressions of *ALP, COL1A1*, and *RUNX2* on day 7 and day 14 of differentiation. Staining of IL-17A-treated-SHED by Alizarin red demonstrated an increased calcium deposition compared to untreated

SHED. Similarly, the expressions of *ALP, OPG, COL1A1*, and *RUNX2* were significantly upregulated in IL-17A-treated SHED. However, RANKL expression was downregulated. Interestingly, siRNA- transfected SHED showed significant downregulation of *ALP, OPG, COL1A1*, and *RUNX2* while *RANKL* was upregulated. These findings demonstrate that IL-17A enhances osteogenesis by promoting osteogenic differentiation and that siRNA- targeting-*IL-17RA* had interfered with the functions of IL-17A/IL-17RA, thus suggesting the importance of IL-17A in mediating the physiological mechanism of bone metabolism.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Bone possesses intrinsic capacity, involving a complex integration of cells, growth factors, and extracellular matrix for significance mechanism of bone regeneration as part of bone repair, as well as in bone remodelling. Old or damaged bone undergoes resorption by osteoclasts, followed by the deposition of new bone materials by osteoblasts. The activities of these two major players (osteoclast and osteoblast) in bone remodelling are influenced directly or indirectly by hormonal signals. When bone is injured, repair and regeneration mechanisms are involved. Repair plays the role in fixing the injured tissue without increasing bone volume whereas regeneration involves the differentiation of new cells, the formation of new bone tissue, and eventually results in an increase in the volume of new skeletal cells (AI-Aql et al., 2008). It is known that inflammatory response is also initiated by the recruitment of immune cells to the site of injury. The secretion of multiple factors, inflammatory mediators, and others also takes place. These intense inflammatory events are important to ensure normal fracture healing through angiogenesis vessels, repair injured tissue, and finally bone remodelling (Baht et al., 2018).

Osteogenesis and ossification are the processes in bone formation that involve the transformation of pre-existing mesenchymal tissue into bone tissue. Osteogenesis consists of two major mechanisms such as intramembranous ossification, the direct conversion of mesenchymal tissue into bone, and endochondral ossification which is cartilage as an intermediate form before being replaced by bone cells. The process of osteogenesis is involved and highly controlled by several signalling pathways and is regulated by two key regulators which are osteoblast and osteocytes. Osteoblasts

originated from mesenchymal origin, are responsible for the synthesis and mineralisation of bone during both initial bone formation and bone remodelling. Osteoblast and osteocytes are bone cells that produce synergistic relationship in regulating osteogenic differentiation in vitro (Birmingham et al., 2012). Besides that, the involvement of cytokines is crucial in bone remodelling process. However, most of the cytokines involved in bone regulations are of favourable to bone resorption. A review in 2021 described the significant effects of some inflammatory cytokines in bone remodelling process such as tumour necrosis factor alpha (TNF-α), interleukin 1(IL-1), and interleukin 6 (IL-6) (Epsley et al., 2021). TNF-α is a pro-inflammatory cytokine that directly promotes the productions of receptor activator of nuclear factor κB ligand (RANKL) in osteocytes, directing to bone resorption (osteocytes-related cytokine). Apart from that, IL-1 acts on osteoblast to induce prostaglandin E (PGE) 2 which is a powerful inducer for bone resorption. Miyaura et al. in their study showed that IL-1 induced the synthesis of PGE2 with the help of cytosolic phospholipase A2α and might potentially induce *RANKL* expressions (Miyaura et al., 2003). Instead of favouring the bone resorption, some cytokines also play crucial roles in bone formation. For example, interleukin 10 (IL-10) has been proven to have dual effects on the osteogenesis of human bone marrow stem cells in vitro. At lower concentrations, IL-10 through the activation of p38 mitogen-activated protein kinase (p38/MAPK) signalling pathway could increase the expressions of osteo-specific mRNAs and proteins and vice versa (E. Chen et al., 2018). Interestingly, previous studies have proven the role of T-cell derived cytokine, interleukin 17A (IL-17A) that inhibit adipogenesis but promote osteogenesis in human bone marrow mesenchymal stem cells (BMSCs) (Noh, 2012).

MSCs are multipotent stromal cells which can differentiate into variety of cell types and have the ability to undergo osteogenic and chondrogenic differentiation. Stem

cells from human exfoliated deciduous teeth (SHED) are one of the multipotent stem cells. As the name, the stem cells are derived from deciduous teeth that exfoliate as layers. An initial study by Miura et al., on SHED in which they described the isolation and characterisation of SHED (Miura et al., 2003). They characterised SHED through the expression of the mesenchymal stem cell markers such as CD73, CD90, and CD44 but not the hematopoietic stem cell markers such as CD34. Apart from that, they also demonstrated that SHED also expressed osteogenic genes alkaline phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX2*), collagen type 1 (*Col1A1*), adipogenic genes such as peroxisome proliferator-activated receptor gamma 2 (*PPARγ2*) and lipoprotein lipase (*LPL*), chondrogenic genes (*Col1A1* and aggrecan (*Acan*)), and neurogenic gene *Nestin*. These diversities of markers expressed by SHED indicate the cells to have multilineage differentiation ability (N. Zhang et al., 2016). This multipotential of SHED makes them a good candidate to renew degenerating tissues and a good source for bone regeneration studies.

SHED are often being used in osteogenic differentiation research as they are highly proliferative and clonogenic cells that have the ability to differentiate into variety of cell types including neural cells, adipocytes, and odontoblast (Miura et al., 2003). As for its appearance, SHED appear more rounded without long processes but some reported that SHED were spindle-like, and some may appear as stellate-shaped or as polygonal appearance (Yasser et al., 2012).

IL-17 is the cytokine derived from activated T-helper 17 cells. IL-17 cytokine family consists of six members including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A and IL-17F are the most studied and well-described of their biological functions and regulations. IL-17 mediates its biological function via its

surface receptor on target cells, which is known as IL-17 receptor (IL-17R) (Jin and Dong, 2013). IL-17A receptor (IL-17RA) is the first binding protein to be identified (Maitra et al., 2007). IL-17A exerts its pro- inflammatory reactions through the receptor, IL-17RA and its interactions with several mediators such as interferon-γ, TNF-α, interleukin 11 (IL-11), and interleukin 1-β (IL-1β). It plays a crucial role in immunity and exerts powerful protective effects against infections caused by bacteria, fungi, virus, and parasites. Moreover, IL-17A holds a vital role in immunoregulatory of the respiratory tract by vigorously stimulating the production of some humoral components in innate immunity (Ge et al., 2020). Apart from that, few studies have also proven the potential of IL-17A in bone regulation. A previous study in 2014 by Osta et. al. carried out an investigation on the role of IL-17A in potentiating the role of TNF-α to positively modulate osteogenic differentiation in human MSCs. The study demonstrated the synergistic effects of IL-17A and TNF-α increased matrix mineralisation and *ALP* activity in human MSCs (Osta et al., 2014). Furthermore, IL-17A itself could enhance the proliferation, osteogenic differentiation, and increased in matrix mineralisation of SHED (Sebastian et al., 2018).

1.2 Problem statement

Bone metabolism is a complex process which involves a devoted cooperation between bone cells such as osteoblasts, osteoclasts, and osteocytes through the process of bone formation and bone resorption to maintain bone homeostasis. The imbalance of bone homeostasis may significantly induce bone-related diseases such as osteoporosis, which affects mostly women over the age of 50 years old worldwide (Sozen et al., 2017). Bone-related diseases and bone defects combined with tumour or infections have resulted in decreased quality of life, increased morbidity, and increased disability-

adjusted life span. Clinicians came with traditional procedures such as autologous and allogeneic grafts. Bone grafts are usually used to augment bone repair and bone regeneration. However, those treatments may cause a series of complications and are challenging in clinical practice. Autologous bone-grafting requires high cost and demands second operation on patients. Besides that, patients may suffer from surgical risks including bleeding, scarring, inflammation, infection, and chronic pain. Meanwhile, allogeneic bone grafting can result in the risk of an immunologic reaction and transmission of infection. Plus, allografts have reduced osteoinductive properties and have no cellular components (Amini et al., 2012). In order to resolve these challenges in bone defect treatments, cell-based therapies become promising alternatives to promote bone regeneration and fracture healing. Numerous studies have revealed that MSCs are ideal candidates for use in bone tissue engineering. However, novel strategy to overcome current obstacles in bone regenerative medicine is needed. Bone regeneration is a highly regulated multistep process that also involves post-transcriptional regulation by small interfering RNA (siRNA). The proposed study is trying to clarify the possible role of siRNA in osteogenic differentiation of MSCs and thus could offer a new strategy for bone regeneration.

1.3 Significance of study

Investigations on the role of IL-17A in the process of bone remodeling have been extensively implemented to prove vital functional role of the cytokine. IL-17A has the ability to promote osteogenic differentiation in SHED (Sebastian et al., 2018). This has been reported by many studies which investigated on the ability of IL-17A in promoting osteogenic differentiation (Jeong et al., 2020; Jo et al., 2018; Lin et al., 2022; Shah et al., 2020; Sritharan et al., 2018). Furthermore, they discovered more convincing

synergistic effects of IL-6 and IL-17A in promoting osteogenic differentiation. IL-17A synergize with IL-6 to induce osteogenic differentiation of MC3T3-E1 seeded on hydroxyapatite by increasing the expression of *OPG* and reducing the expression of *RANKL*, thus increasing *OPG/RANKL* ratio and lessen the osteoclastogenic response (Sritharan et al., 2018). In addition, the interaction between IL-17A and TNF-α may induce osteogenic differentiation of human MSCs (Osta et al., 2014). As demonstrated previously, IL-17A-treated SHED may become a perfect source for bone regeneration, consequently, this can provide a good platform for bone tissue engineering in the future (Sebastian et al., 2018). The importance of IL- 17A effects on osteogenesis may provide a massive understanding of some bone defect diseases.

RNA interference has gained attention in the therapeutic application of gene silencing in human by promoting the degradation of mRNA which plays an important role in gene regulation. siRNA and microRNA (miRNA) are non-coding RNA, known for their roles in gene regulation, and have attracted attention for their potential roles in drug discovery and development (Lam et al., 2015). siRNA are able to efficiently and specifically silence genes. siRNA-targeting-*IL-17RA* have been used especially in autoimmune and inflammatory diseases studies to investigate the role of pro-inflammatory cytokines IL-17A and the interactions with its receptor as therapeutic potential in the diseases (Kurte et al., 2018). Some siRNA may have immunoregulatory effects and this knowledge could provide an insight into the roles of IL-17RA in mediating the physiological mechanism of bone. The effect of siRNA-targeting IL-17RA towards osteogenic differentiation of MSCs is not described elsewhere. The physiological role of siRNA-targeting *IL-17RA* is also still unknown.

1.4 Research hypothesis

siRNA-targeting *IL-17RA* downregulated the expression of *IL-17RA* and regulated the osteogenic differentiation activity in SHED.

1.5 Research Question

1. Does siRNA-targeting *IL-17RA* enhance osteogenic differentiation of SHED?

2. What is the effect of siRNA-targeting *IL-17RA* on mineralisation activity of SHED-derived osteoblast cells?

3. What is the effect of siRNA-targeting *IL-17RA* on selected osteogenic genes expression of SHED-derived osteoblast cells?

1.6 Research objectives

1.6.1 General objectives

To study the effect of siRNA-targeting *IL-17RA* on the osteogenic differentiation of the stem cells from human exfoliated deciduous teeth (SHED).

1.6.2 Specific objectives

a) To optimize the concentration of siRNA-targeting *IL-17RA* by transient transfection of siRNA onto SHED

b) To determine the effects of siRNA-targeting *IL-17RA* on mineralisation activity of SHED

c) To evaluate the effects of siRNA-targeting *IL-17RA* on the expression of osteogenic markers such as *ALP, COL1A1, RUNX2, OPG*, and *RANKL*

1.7 Flow chart of study

The flow chart of study is illustrated in Figure 1.1.

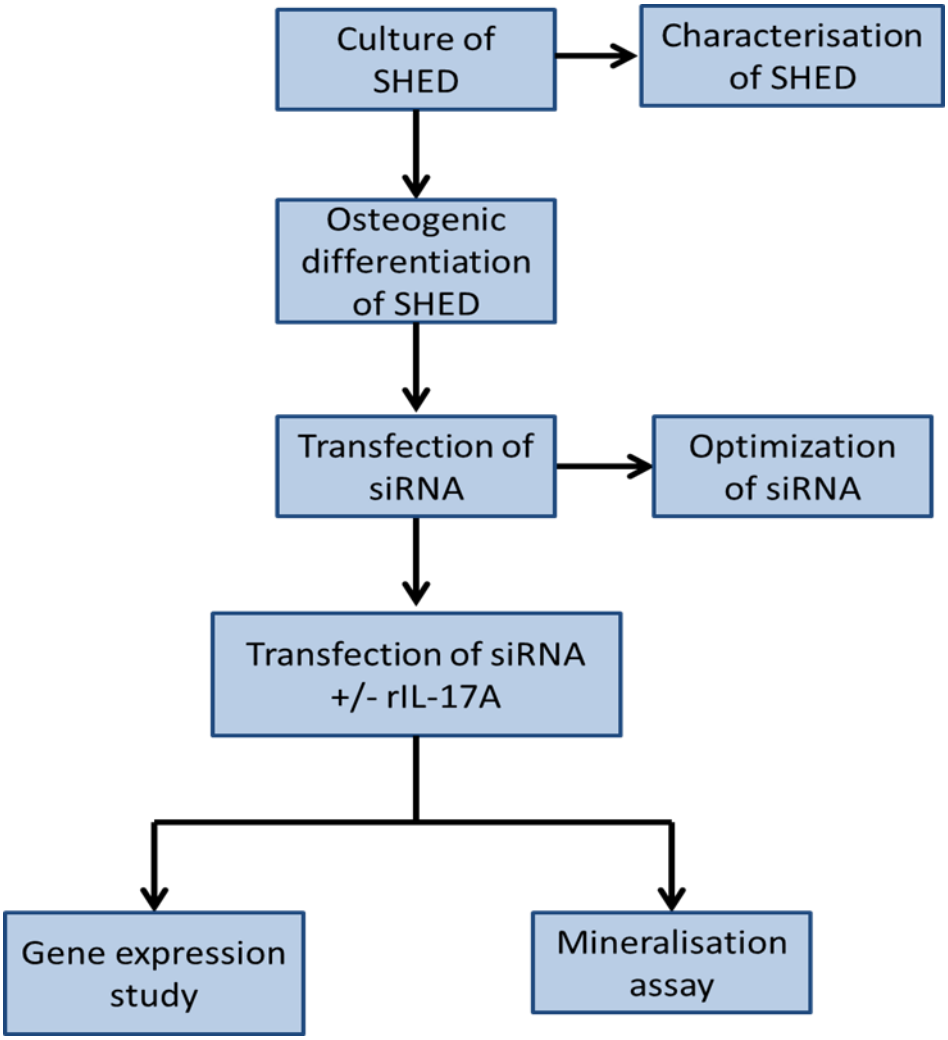

Figure 1.1 The flow chart of study

The study was started with the culture of SHED followed by osteogenic differentiation of SHED. Differentiated and treated SHED were transfected by optimised siRNa for gene expression study and mineralisation.

CHAPTER 2

LITERATURE REVIEW

2.1 Bone

Bone is a rigid and firm tissue that constitutes part of the skeletal system in most vertebrae. The skeleton is comprised mainly of two types of bone which are spongy bone that has lower density and denser corticol bone which makes up to 80% of the skeleton. Bone develops embryologically through two main processes of intramembranous ossification and endochondrial ossification. Intramembranous ossification involves the differentiation of mesenchymal stem cells directly into bone. Meanwhile, endochondorial ossification involves complex processes which begin with cartilage templates replaced by bone. The process occurs in a few zones starting with proliferative zone where the division of cartilage cells or chondrocytes takes place, the enlargement of chondrocytes in hypertrophic zone, calcified zone, and finally grows into mature bone. Bone consists of mainly collagen fibres and small crystal forms of inorganic bone minerals.

Bone tissues are made up of different types of bone cells such as osteoblasts, osteoclast, and osteocytes. Bones are crucial in protecting various organs and soft tissue, provide a marrow for red and white blood cells production, storing minerals, providing structure and support to the body as well as enabling motility of vertebrae. Bone metabolism is the balancing of both osteoblast and osteoclast in bone formation and bone resorption respectively in maintaining bone homeostasis. The imbalance in bone metabolism may result in skeletal diseases such as rheumatoid arthritis.

2.2 Bone remodelling

Bone undergoes modelling and remodelling to change shape and size. Bone modelling is the process where bone changes shape or size in response to physiological changes or mechanical forces encountered by the skeleton. The majority of the processes are completed by skeletal maturity. Besides that, formation and resorption during bone modelling must be uncoupled, which is through the independent action of osteoblast and osteoclast Meanwhile, bone remodelling is the process for the bones to maintain its strength and mineral homeostasis. Bone remodelling happens to remove old, damaged bones and replace them with new bone matrix which they undertake mineralisation to form new bone. It occurs throughout the life until death to prevent accumulation of old and damaged bones in skeleton but repeatedly occurs in aging women and men.

Bone remodelling is generally consisted of 4 phases that occur continually (Allen and Burr, 2019) (Figure 2.1). It starts with the activation phase in which osteoclast progenitors are engaged to damaged or defect bone surface. Osteoclast progenitors become mature and resorb the damaged bone during the resorption phase. Then, it is followed by reversal phase where osteoclast dies, and osteoblast progenitors are recruited. Finally, the formation phase occurs when osteoblasts become mature and produce new mineralised bone matrix (Allen and Burr, 2019).

The main characters in bone remodelling are osteoblasts and osteoclasts. The function of these two cells is tightly coupled to maintain bone mineral homeostasis. The failure in balancing these two processes will cause some mineral diseases such as osteoporosis or osteopetrosis which are the excessive bone loss or bone formation. Other cells that are important in bone homeostasis include osteocyte, which is derived from cytoblasts.

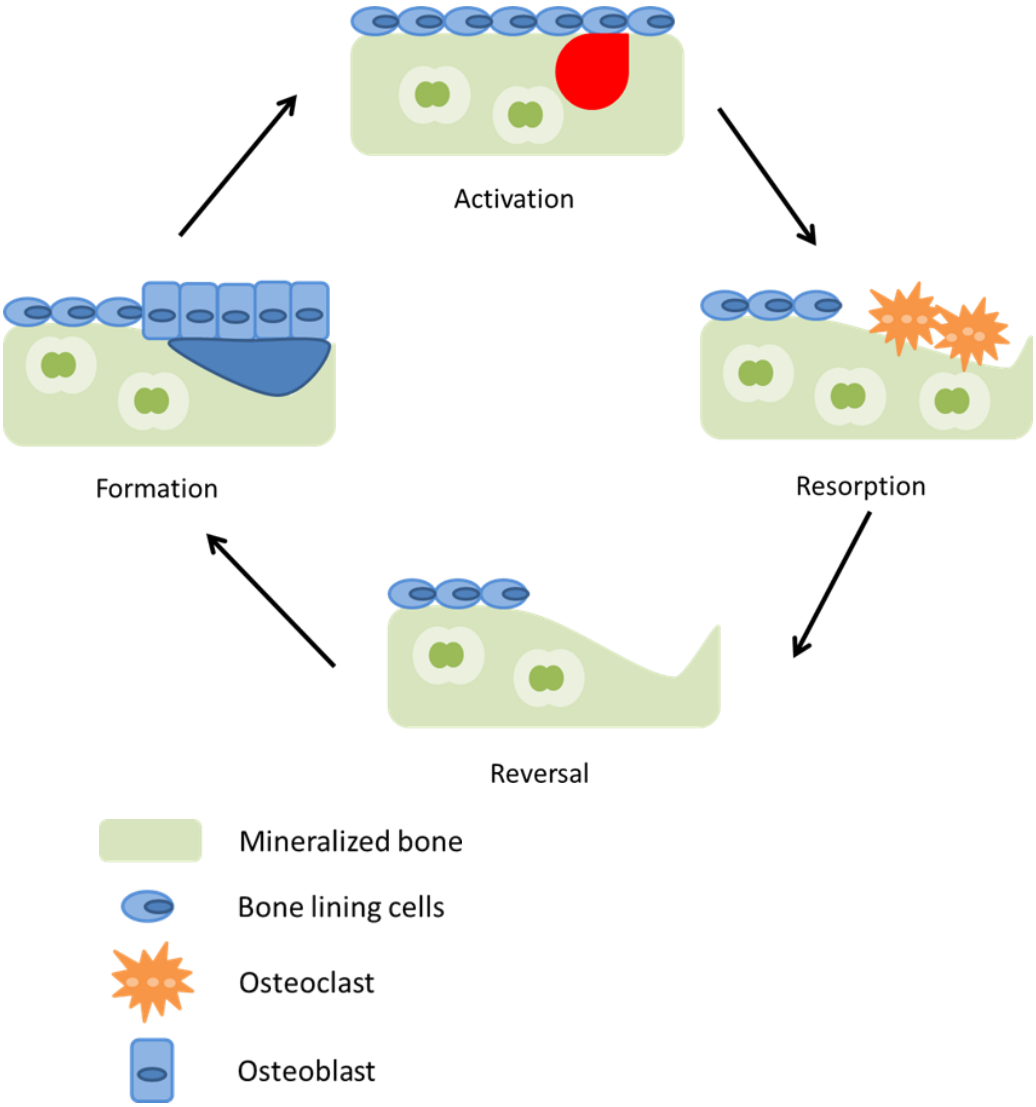

Figure 2.1 Phases of bone remodelling.

A cycle of bone remodelling process starts with an activation phase of osteoclast progenitors which later mature during resorption phase. Finally, osteoclasts die in reversal phase and new osteoblast progenitors mature on formation phase.

2.3 Osteoblast, osteoclast, and osteocytes

Osteoblasts are cuboidal cells that can be found at the intersection of newly synthesized bone. Osteoblasts are most active during the development of the embryonic skeleton; in contrast, adult osteoblasts are only activated when it becomes necessary to replace damaged bone. However, as the population of mature osteoblasts finally decreased, new osteoblasts will be differentiated from MSCs. (Long, 2012). The progenitor for osteoblasts is the MSCs. This is due to the multipotency ability of MSCs which they are able to regenerate into various types of cells including myoblast, osteoblasts, chondrocyte, and adipocyte lineages (Canalis, 2008). The differentiation of MSCs is said to be governed by so-called 'master transcriptional regulators' and for osteoblast lineages is *RUNX2*. The differentiation of osteoblasts begins with the activation of *RUNX2* which converts MSCs into preosteoblast. Then, preosteoblasts will undergo three stages of differentiation and each stage are characterised by specific molecular markers. The first stage of differentiation process continues with the proliferation of cells that expressing fibronectin, transforming growth factor- β (TGFβ) receptor 1, and osteopontin (*OPN*) during this stage. Stage 2 is characterised by the maturation of extracellular matrix and the expressions of *ALP* and collagen type 1 alpha 1 (*COL1A1*). Finally, stage 3 is when the organic scaffold is enriched with osteocalcin (*OCN*) that will promote the deposition of mineral substances such as calcium phosphate. During this stage, matrix mineralisation occurs and osteoblasts were observed to have a cuboidal shape (Long, 2012) (Figure 2.2).

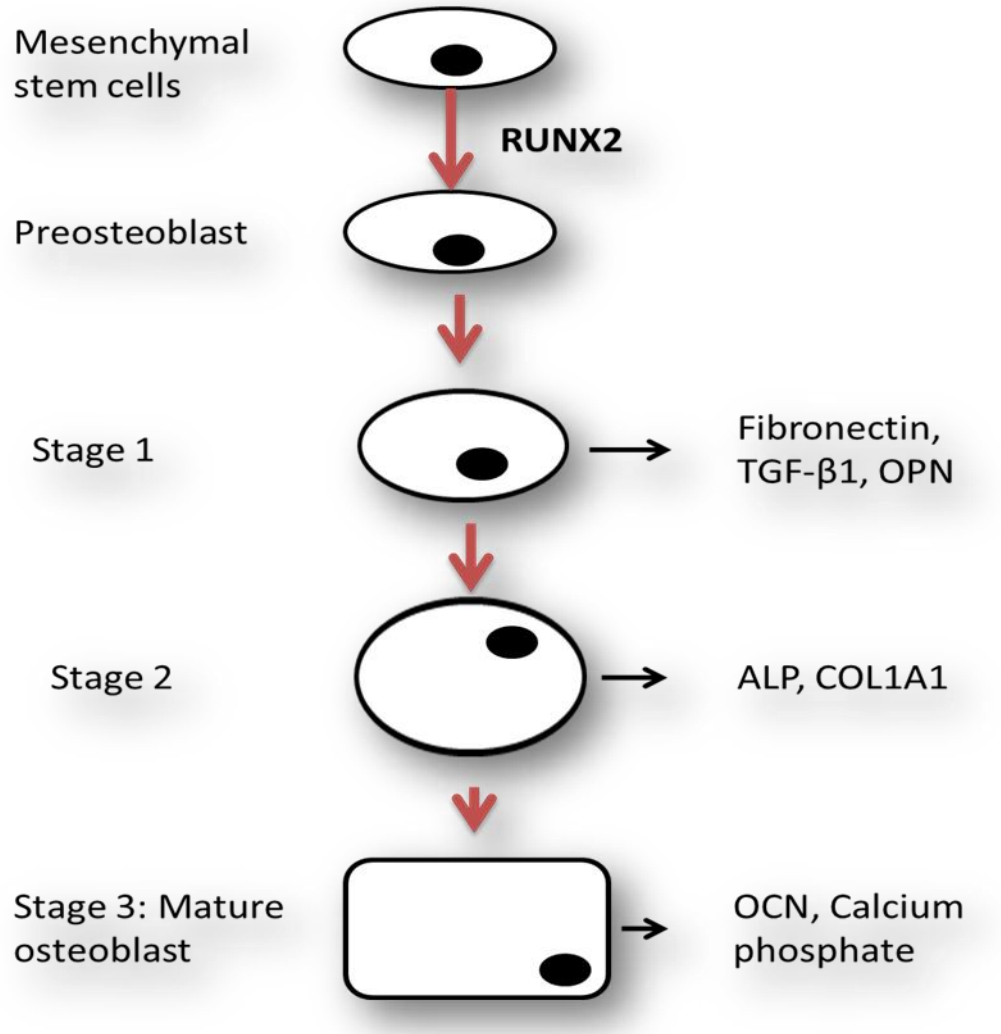

Figure 2.2 Osteoblastogenesis.

Osteoblasts are derived from multipotent mesenchymal stem cells that aregoverned by RUNX2 to become pre-osteoblasts which continued to differentiate into proliferative cells during the first stage. The differentiation continued to second stage where maturation of extracellular matrix occurs and the final stage which is characterized by matrix mineralisation cuboidal shape of mature osteoblasts.

Osteoclasts were originated from hematopoietic stem cells which were derived from cells in monocyte-macrophage lineage. It is characterised by the formation of osteoclast-like cells that express phenotypic characteristics of osteoclast and resorbed bone during the culture of peripheral blood monocyte under specific conditions (Matsuzaki et al., 1998). In the presence of a growth factor known as macrophage-colony stimulating factor (M-CSF) which induces the hematopoietic stem cell to give rise to macrophage colony-forming units (CFU-M), identified as precursor cells of macrophages and osteoclasts. Later, RANKL-RANK signalling will activate the precursor cells to differentiate further into mononucleated osteoclasts which subsequently fuse together to become multinucleated osteoclasts. Eventually, multinucleated osteoclasts will mature and resorb bone matrix by secreting acids (H+), proteases, and matrix metalloproteinase (MMP). Resorption happens when there is a tight junction between the basal membrane of osteoclasts and bone surface, forming a sealed compartment for osteoclastogenesis (Kim et al., 2020). Therefore, the earliest marker in osteoclast lineages is matrix metalloproteinase-9 (MMP-9) which then precode the expression of TRAP-positive in CFU-GM-derived cells after treatment with 1,25-dihydroxyvitamin D (Goldring et al., 1995). The overall process of osteoclastogenesis is illustrated in Figure 2.3.

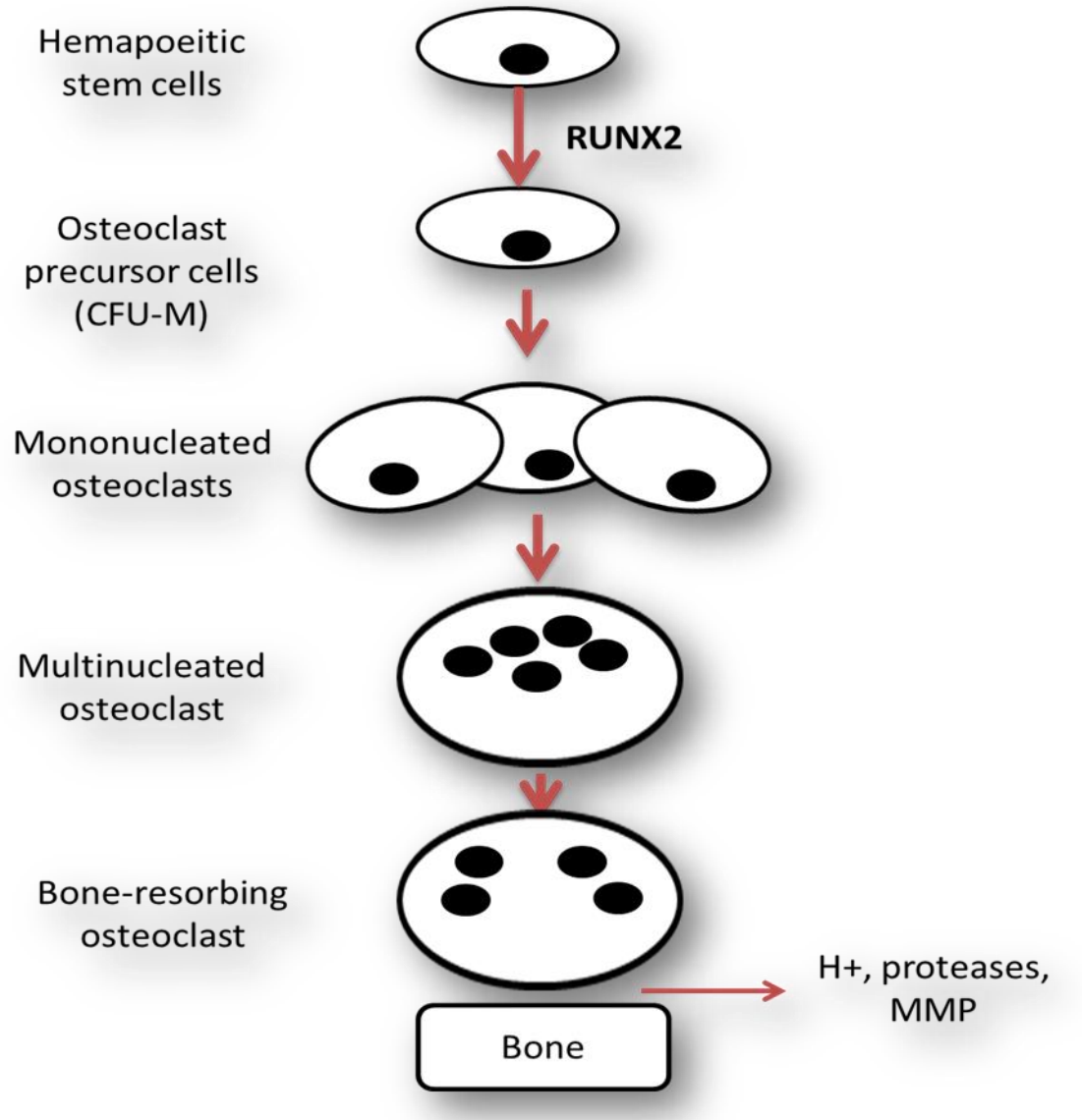

Figure 2.3 Osteoclastogenesis.

Osteoclasts are tissue-specific macrophages derived from hematopoietic stem cells which then form osteoclast precursor cells induced by M-CSF. Those precursor cells continued to differentiate into mononucleated osteoclasts which then fuse together to form multinucleated osteoclasts during the RANKL-RANK signalling. Fully matured osteoclast basal membrane will form a sealed compartment with bone surface and resorb bone matrix by secreting H+, proteases, and MMP.

Bone homeostasis involves osteoblast-osteoclast communications which is essential for bone remodelling. Interactions between membrane-bound molecules such as EFNB2-EPHB4, FAS-FASL, and NRP1-SEMA3A mediate a direct contact between osteoblasts and osteoclasts in order to regulate cell proliferation, differentiation, and survival. Osteoclasts secrete TGF-β and insulin-like growth factor 1 (IGF-1) from bone matrix to stimulate osteoblast-mediated bone formation. On the other hand, osteoblasts secrete M-CSF, RANKL, and WNT5A to promote osteoclast formation (Kim et al., 2020).

There are some known signalling pathways that have been shown to be involved in bone homeostasis process. The signalling pathway which has been discussed the most is the RANK/RANKL/OPG signalling pathway. It is an essential cellular signalling pathway for bone remodelling. RANK is a homotrimeric transmembrane protein of TNF receptor superfamily, meanwhile, RANKL is a membrane-bound on osteoblasts or secreted by activated T cells. Meanwhile, osteoprotegerin (OPG) can function as a soluble decoy receptor for RANKL which is secreted by many cell types including osteoblasts (Boyce and Xing, 2008). RANK is a signalling receptor for RANKL and the binding between those two will induce osteoclast differentiation, whereas OPG as a soluble decoy receptor for RANKL will act as a negative regulator of RANK signalling, thus inhibiting osteoclastogenesis (Walsh and Choi, 2014) (Figure 2.4).

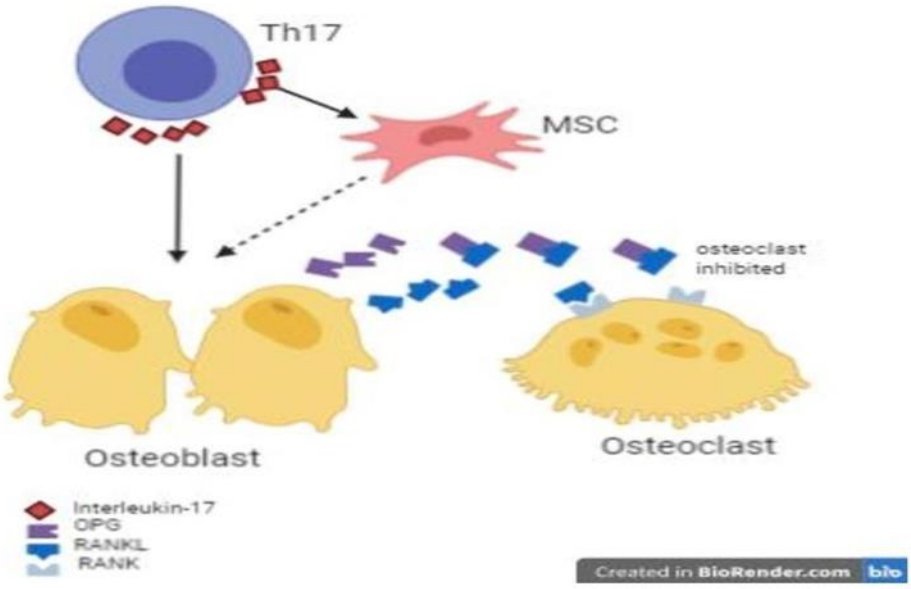

Figure 2.4 RANK/RANKL/OPG signalling pathway.

OPG and RANKL are two important markers that balanced the process of osteoblast and osteoclast in bone homeostasis. RANKL bind to RANK on osteoclast membranes and directs the osteoclastogenesis while OPG will act as decoy receptor to RANKL, regulating RANK signalling thus inhibiting the osteoclast differentiation.

2.3.1 Bone tissue engineering

Bone diseases are one of the most chronic diseases in elders over 50 years old and this could be a critical clinical challenge (Sozen et al., 2017). Although bone has its own ability to heal or regenerate, it still cannot be accomplished naturally for certain larger bone damages. Larger bone defects caused by traffic accidents, injuries, or bone tumor resection are serious problems in orthopaedic and could affect health and quality of life. Previously, the 'gold standard' treatment for bone defects was autologous bone grafting. However, bone grafting has many drawbacks and weaknesses causing it to be limited used in clinical settings. The field of bone tissue engineering (BTE) was long ago being investigated and developed. The main purpose for the introduction of BTE is to ideally eliminate the limitations of conventional solution or treatment for bone diseases such as limited availability, patients' immune rejection, and risk of infection at the operation site. However, the field of BTE was not yet fully studied to be successfully

assigned in clinical settings. There are indeed several important criteria of BTE including a biocompatible scaffold that mimics natural bone matrix, osteogenic cells as frame for bone tissue matrix, morphogenic signals that help to direct the cells, and sufficient vascularization supply (Amini et al., 2012). Cell-based therapy is the transplantation of human cells to replace or repair damaged tissues or cells and may be one of the successful therapy strategies in bone tissue engineering to circumvent conventional bone grafts. MSCs are multipotent adult stem cells and have been extensively studied for its therapeutic potential in BTE. Accordingly, there are several significant research notably on the osteogenic potential of MSCs obtained from different sources (Perez et al., 2018). However, there are critical procedures which need to be considered in utilizing cells for bone defect repair in clinical applications. Those include the quality and quantity of cells embracing the cell sources, cell isolation, and yield. Cell seeding efficiency, preculture conditions, and in vivo conditions should also be taken into consideration (Ma et al., 2014). Stem cells are the choice of many significant researches in bone tissue engineering due to its capability of self-renewal and ability to differentiate into specialised adult cell type (Biehl and Russell, 2009) Bone marrow-derived stem cells (BMSCs) are the most frequently studied and predominant source of adult MSCs due to their relatively easy harvesting, high proliferative capacity, and established regenerative potential. Plus, a study showed that BMSCs are more efficient at differentiating into osteoblasts compared to adipose-derived MSCs (D. S. Han et al., 2014). An effective alternative to autologous bone transplants has been demonstrated in a number of animal models of clinically severe bone abnormalities using cell-based therapy with allogenic BMSC grafts (Jones et al., 2016). Apart from that, one of the most popular being investigated recently is the dental pulp-derived stem cells (DPSCs). DPSCs have similar gene expressions as BMSCs.

They even have higher proliferative rate and produce higher yield of cells in harvested populations compared to BMSCs (Ma et al., 2014). A study also reported on DPSCs to have equal bone-forming capacity (Yamada et al., 2011). However, other studies reported only on the formation of connective tissue from DPSCs (W. Zhang et al., 2006), thus requiring in-depth investigation on the mechanism of bone-forming capacity by DPSCs. A better understanding on the properties of cells and bone disease will provide an understructure to design cell-based therapy in BTE.

2.4 Mesenchymal stem cells (MSCs)

MSCs are multipotent adult stem cells that can be isolated from different types of sources including bone marrow, adipose tissues, menstrual blood, umbilical cord, synovium, tooth and so forth (Zhao et al., 2016). The fact that MSCs exhibit high self-renewal capacity and have the ability to differentiate into various specialised cell types, MSCs have become an attractive tool for research and clinical setting. Generally, bone marrow becomes the main source of MSCs. However, only 0.01% of the cells in bone marrow are MSCs while it becomes a major source for hematopoietic stem cells (Brozovich et al., 2021). Furthermore, the challenging procedure and the necessity for anaesthesia when isolating MSCs from bone marrow contribute to the intricacy of the collection process, which, in turn, limits their practical application in clinical settings. Fortunately, nowadays MSCs can be derived from multiple tissues (Mushahary et al., 2018). Spindle-shaped cells resembling fibroblasts were identified in a 1976 study generated from bone marrow. Additionally, they displayed plastic adhering growth that multiplied across multiple passages in vitro (Y. Han et al., 2012; Mushahary et al., 2018). Moreover, MSCs are available for purchase or can be obtained as primary cells, and they can be characterised using universal markers found in MSCs derived from a

variety of sources. Some of the markers are strongly expressed cell adhesion markers, CD73, CD90, and CD105 while very weak or no expression of hematopoietic cell antigens such as CD34 and CD45 (Bharti et al., 2018; Mabuchi et al., 2021; Markmee et al., 2017). Additionally, the pluripotency of MSCs was approved through its differentiation ability into three cell lineages including adipocyte, osteocyte, and chondrocyte, confirmed by specific staining for each lineage (Pendleton et al., 2013). Besides that, MSCs have inherent immunomodulatory properties in both innate and adaptive immunity (N. Song et al., 2020). MSCs were said to be promising candidates for therapeutic strategies in some autoimmune diseases as studies have shown various immunomodulatory properties of MSCs. MSC suppress the proliferation, cytokine secretion, and cytotoxicity against HLA-class 1-expressing target, which some required cell-to-cell interactions or through paracrine activity (N. Song et al., 2020; Zhao et al., 2016). Besides that, MSC can modulate the functions of T cells and B cells, where it can suppress T lymphocyte proliferation and inhibit B lymphocyte proliferation via a cycle cell arrest occurring during the G0/G1 phase (Zhao et al., 2016). Interactions between MSCs and monocytes, macrophages, dendritic cells, T cells, B cells, and natural killer cells indicate different immunomodulatory features of MSCs. Nevertheless, unique interactions exist between monocytes, MSCs, and regulatory T cells, also known as Tregs. Monocytes and Tregs play a key role in immunomodulation through a complex interaction of MSC-secreted cytokines (Weiss and Dahlke, 2019).

2.4.1 Stem cells from human exfoliated deciduous teeth (SHED)

SHED can be obtained through a surgical procedure, collected from normal exfoliated human deciduous incisors from 6-8 years old children under strict aseptic conditions (Miura et al., 2003; Yasser et al., 2012; N. Zhang et al., 2016) (Figure 2.5). The dental pulp was separated from the pulp chamber, and then was digested in

collagenase type I (Yasser et al., 2012). Digested pulp will form single-cell suspension in a growth medium. The population of MSC isolated from the human exfoliated deciduous teeth have similar proliferative and differentiative properties as MSC isolated from other tissues, suggesting that human deciduous teeth may become a good source of MSC (Yasser et al., 2012). SHED grew as individual fibroblast-like cells and are spindle-shaped. Besides that, a study reported SHED to have stellate shape and also polygonal shape, indicating the diversity of proliferating pulp cells (Yasser et al., 2012). In addition, SHED possessed some characteristics of MSCs in which they are able to adhere to the bottom of the culture flask. A study observed that SHED on the 14th day grew up to 90% covering the culture flask. This shows that SHED are highly clonogenic and exhibit as a proliferative cell population (Yasser et al., 2012), which corresponded to the finding of a previous study describing that SHED exhibited a higher proliferation rate and a higher number of population doublings (Miura et al., 2003).

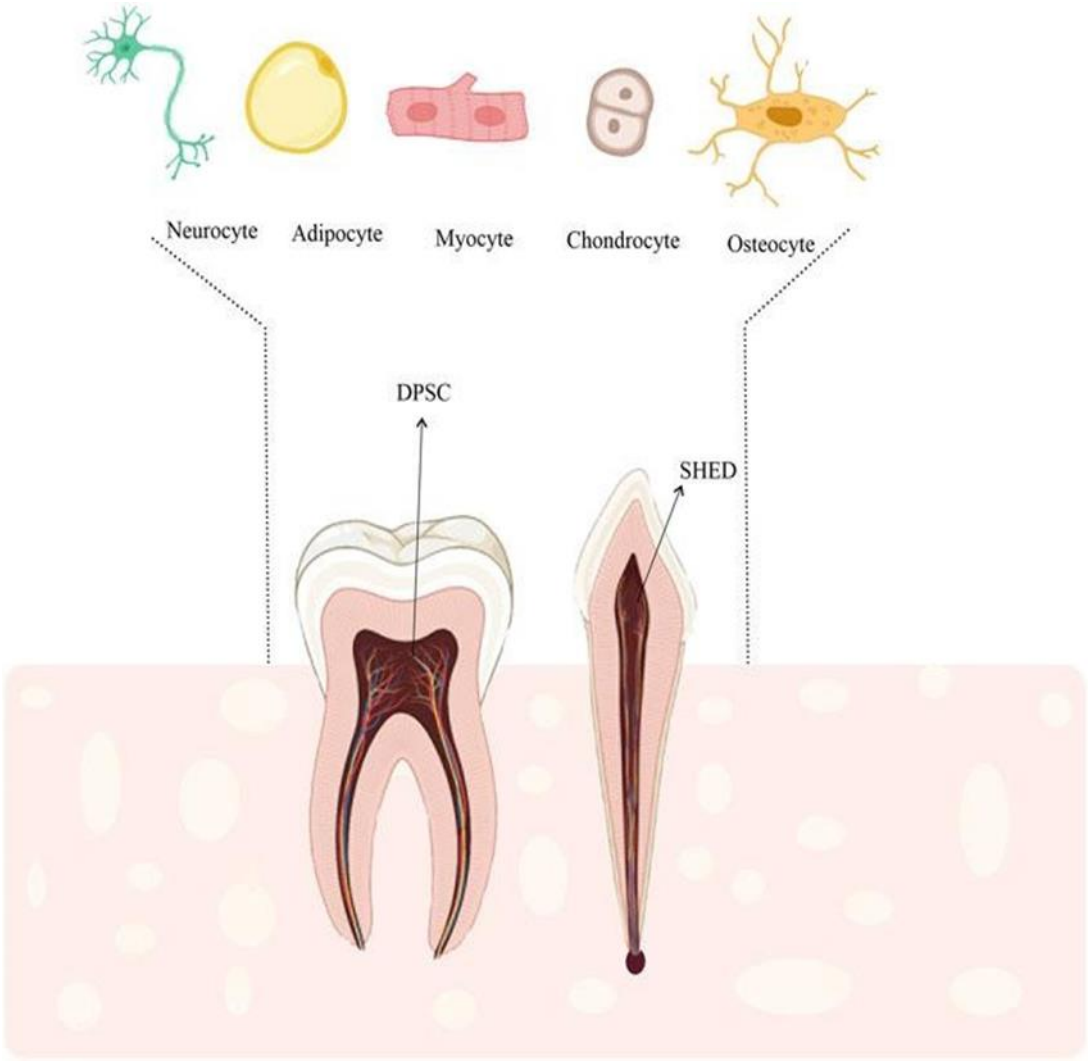

Figure 2.5 Dental pulp stem cells.

SHED is originated from deciduous teeth while DPSCs is originated from permanent teeth and both could differentiate into specified cells (Mahdavi-Jouibari et al., 2023).

On top of that, SHED also exhibits the characteristics of MSCs. They presented the classical surface markers of MSCs including cell adhesion markers CD73, CD90, and CD44. However, SHED is negative for hematopoietic cell marker such as CD34 (N. Zhang et al., 2016). Meanwhile, a previous study demonstrated that SHED exhibited early MSC markers such as STRO-1 and CD146 through immunohistochemistry staining. Most of the markers were positively stained around blood vessels, suggesting SHED developed from perivascular microenvironment (Miura et al., 2003).

Furthermore, SHED is able to differentiate into multiple cell lineages including bone, cartilage, fat, and skin under different conditions or induction medium such as osteogenic differentiation medium, chondrogenic differentiation medium, and adipogenic differentiation medium. SHED can differentiate into osteogenic cells under osteoinducing medium which is usually supplemented with L-ascorbate-2-phosphate, dexamethasone, and inorganic phosphate (Yasser et al., 2012; N. Zhang et al., 2016). Osteogenic differentiation of SHED shows the formation of a small number of calcified nodules that are positively red-stained with alizarin red s after two weeks of induction. The nodules are sometimes scattered among the cells. A larger number of calcified nodules were formed after 4 weeks of induction. Nodules formation show the accumulation of calcium suggesting SHED has undergone osteogenic differentiation (Yasser et al., 2012). The osteogenic differentiation of SHED can be further confirmed by western blot as well as RT-PCR, which can be indicated by higher expressions of osteogenic markers such as *ALP* and *RUNX2* (N. Zhang et al., 2016).

2.5 IL-17A in bone remodelling

2.5.1 Family members/receptors of IL-17A

Interleukin-17A (IL-17A) was first cloned from murine lymphoid cells and homologous to protein which was enhanced by open reading frame 13 (ORF13) gene in T Lymphotropic Herpesvirus Saimiri (Rouvier E, Luciani MF, Mattéi MG, Denizot F, 1993). As a cytokine with broad distribution receptors, the activity and responses may play an important role in tissue homeostasis as well as diseases progression. IL-17 has six members in the cytokine family: IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F (Davydova et al., 2023; Huangfu et al., 2023). Among the members, IL-17A is the most studied member of IL-17 cytokine family and primarily produced in activated T cells (Davydova et al., 2023; Huangfu et al., 2023). IL-17F is also produced in activated T cells while IL-17B, 1L-17C, IL-17D, and IL-17E can be found in a wide variety of tissues (Zenobia and Hajishengallis, 2015). IL-17B mRNA transcript was found to be expressed in a wide range of human adult tissues such as pancreas, small intestine, and stomach whereas IL-17C was constricted only as a rarely expressed sequence tag (EST) found in adult prostate and foetal kidney libraries (H. Li et al., 2000). However, a later study in 2011 showed that IL-17C was rapidly expressed on epithelial cells in response to bacterial and inflammatory stimuli (Ramirez-carrozzi et al., 2011). Meanwhile, IL-17D which is highly expressed in skeletal muscle, brain, adipose, heart, lung, and pancreas, exhibits similarities in their ability to stimulate the production of cytokines to indirectly modulate the immune response (Starnes et al., 2015). IL-17E-producing cells include dendritic cells, macrophages, T cells, eosinophil, basophil, mast cells, epithelial, and Paneth cells (X. Song and Qian, 2013). Moreover, IL-17E (IL-25) is produced by Th2 cells and mediates Th2 cell responses by inducing