IDENTIFICATION OF PREDOMINANT SIGNALING PATHWAY IN OSTEOGENIC GENE REGULATION OF DENTAL PULP STEM CELLS BY COLLAGEN TYPE 1 INDUCTION

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by

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LIST OF SYMBOLS AND ABBREVIATIONS

Akt	Protein kinase B
ALP	Alkaline phosphatase protein
ATF4	Activating transcription factor 4
bFGF	Basic fibroblast growth factors
BMP	Bone morphogenetic protein
BMSC	Bone marrow stem cells
BSP	Bonesialoprotein
CCM	Complete culture media
cDNA	Complementary DNA
Col 1	Collagen type 1
Col 1α1	Collagen type 1 alpha 1 chain
DFSC	Dental follicle precursor cells
Dlsx5	Distal-less homebox 5
DMP1	Dentin matrix protein 1
DPSC	Dental pulp stem cells
DT	Doubling time
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cells
FGF	Fibroblast growth factor
FHL2	Four and a half LIM domains protein 2
FoxOs	Foxhead box class O
GMSC	Gingiva-derived mesenchymal stem cells
hMSC	Human mesenchymal stem cells
IGF-1	Insulin-like growth factor 1
IL	Interleukin (inflammatory factors)
iPSC	Induced pluripotent stem cells
ISCT	International Society of Cellular Therapy ISCT
JNK	C-Jun N-terminal kinase
LY294002	Akt inhibitor
LY3200882	Smad inhibitor

MAPK	Mitogen activated protein kinase
MGP	Matrix Gla protein
MPK1	Mitogen-activated protein kinase 1 phosphatase
MSC	Mesenchymal stem cells
NF--*κB*	Nuclear factor kappa-light-chain-enhancer of activated B cells
OCN	Osteocalcin
OIM	Osteogenic inductive media
OPG	Osteoprotegerin
OPN	Osteopontin
Osx	Osterix
P13K	Phosphoinositide 3-kinase
PD	Population doublings
PD98059	ERK inhibitor
PDGF	Platelet derived growth factor
PDL	Population doubling level
PDLSC	Periodontal ligament stem cells
PGA	Poly-glycolic acid
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-bisphosphate
PLA	Poly-lactic acid
PMMA	Polymethyl methacrylate polymer
qRT-PCR	Quantitative Real Time polymerase chain reaction
RANK	Receptor activator of NF-*κb*
RANKL	Receptor activator of NF-*κb ligand*
RTK	Receptor tyrosin kinase
RT-PCR	Reverse transcriptase polymerase chain reaction
Runx2	Runt-related transcriptional factors 2
SCAP	Stem cells from apical papilla
SHED	Stem cells from human exfoliated deciduous teeth
TGF-β	Transforming growth factor beta
VEGF	Vascular endothelial growth factor
Wnt	Wingless

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PENGENALPASTIAN LALUAN ISYARAT UTAMA DALAM REGULASI GEN OSTEOGENIK BAGI SEL STEM PULPA GIGI OLEH KOLAGEN JENIS 1

ABSTRAK

Perubatan regeneratif berasaskan sel stem menawarkan strategi yang menyakinkan dalam perawatan kerosakan tulang dan pembinaan semula tulang kraniofasial. Interaksi harmoni antara sel stem, kerangka struktur dan faktor pertumbuhan adalah kunci kejayaan pembezaan osteogenik dan pembentukan tulang. Komponen utama matriks ekstraselular tulang, iaitu Kolagen jenis 1 (Col 1) telah dicadangkan secara meluas sebagai bahan yang sesuai untuk kejuruteraan tisu tulang, dengan bukti kukuh berkenaan keupayaan biokompatibiliti dan osteokonduktiviti. Walaubagaimanapun, laluan isyarat utama yang dikaitkan dengan percambahan dan pembezaan osteoblas yang disebabkan oleh Col 1 masih kurang diterokai. Kajian ini menyiasat kapasiti osteoinduktif Col 1 dalam mendorong pembezaan osteogenik sel stem pulpa gigi (DPSC). Sebanyak 10,000 sel/cm^2 sel dimasukkan ke dalam pelbagai kombinasi Col 1 + Matrigel™, tanpa kehadiran faktor-faktor pendorong luaran yang lain. Pewarnaan merah Alizarin dan analisis PCR dilakukan untuk memastikan pemendapan nodul tulang dan ekspresi gen-gen osteogenik (*OPN, OCN, Osx*). Analisis lanjut dilakukan dengan kehadiran perencat untuk menentukan laluan isyarat utama yang terlibat dalam induksi DPSC oleh Col 1 dalam pembentukan sel tulang. DPSC yang diinduksi dengan kerangka struktur pilihan iaitu Col 1 (2mg/ml), telah dirawat dengan perencat LY294002, LY3200882 dan PD98059 yang menyasarkan laluan isyarat AKT, Smad dan ERK. Ujian rencatan laluan isyarat dijalankan keatas

sel pada hari ke-7, 14 dan 21, diikuti dengan pewarnaan merah alizarin dan analisis kuantitatif RT-PCR. Col 1 (2mg/ml) menunjukkan keupayaan osteoinduktif secara spontan untuk mengubah DPSC menjadi sel tulang tanpa kehadiran sebarang perangsang osteogenik. Analisis ekspresi gen mendedahkan penglibatan semua laluan isyarat yang disiasat terutamana ERK dalam modulasi pembentukan tulang DPSC dengan induksi Col 1. Data yang dipelorehi daripada kajian ini, mengenai biologi sel stem dan komponen kerangka boleh digunakan untuk mereka bentuk rawatan berasaskan sel stem bagi mempercepatkan proses baik pulih serta penjanaan semula tulang.

IDENTIFICATION OF PREDOMINANT SIGNALING PATHWAY IN OSTEOGENIC GENE REGULATION OF DENTAL PULP STEM CELLS BY COLLAGEN TYPE 1 INDUCTION

ABSTRACT

Stem cells-based regenerative therapy offers a promising approach for the treatment of bone fractures and craniofacial bone reconstruction. A harmonious interplay between stem cells, scaffolds, and growth factors is the key to successful osteogenic differentiation and subsequent bone formation. The main component of the bone extracellular matrix, Collagen type 1 (Col 1) has been widely proposed as a suitable material for bone tissue engineering, with excellent evidence on biocompatibility and osteoconductivity. However, the key signaling pathway associated with Col type 1-induced osteoblast proliferation and differentiation remains underexplored. The present study investigated the osteoinductive capacity of Col 1 in inducing the osteogenic differentiation of dental pulp stem cells (DPSC). A total of 10,000 cells/cm2 cells were plated in various combination of Col I + Matrigel™, without the presence of other external inducing factors. Alizarin red staining and PCR analysis were performed to ascertain bone nodule deposition and osteogenic genes (*OPN, OCN, Osx*) expression. Further analysis was performed with inhibitors to assess the predominant signaling pathway involved in Col 1 induction of DPSC into forming committed bone cells. DPSC induced with selected scaffold- Col 1 at 2mg/ml were treated with pathway inhibitors LY294002, LY3200882, and PD98059 targeting AKT, Smad, and ERK signaling respectively. The pathway inhibition assay was performed on the cells at day 7, 14 and 21, followed by alizarin red staining and qRT-PCR analysis. Col 1 was described to induce spontaneous

osteoblast differentiation of DPSC in the absence of any osteogenic stimulant. Gene expression analysis revealed the involvement of all investigated pathways especially the ERK signaling in modulating DPSC osteogenesis by Col 1 induction. The data provided by this study, on the fundamental knowledge of stem cells biology and scaffold component may then be used to design an efficient stem cell-based treatment in accelerating the process of bone repair and regeneration.

CHAPTER 1

INTRODUCTION

1.1 Research background

Craniofacial deformities can be complex and require multi-specialty collaboration for successful treatments. Road traffic accidents have been identified as one of the main causes of craniofacial deformities in Malaysia and most of the time affected motorcyclists among the youths (Abosadegh et al., 2019; Chelvan et al., 2020; Mahalakshmi et al., 2023; Muhammad et al., 2012). Other than traffic accidents, craniofacial deformities can be caused by assaults, falls, congenital deformity, work injuries, sports injuries, and gunshot wounds. In severe circumstances, the affected individuals could suffer long-term problems such as facial disfigurement and sensory function loss as a result of nerve injury (Koons et al., 2020). A further worst possible result of craniofacial injuries is psychological problems that potentially disrupt the quality of life. The affected individuals may also face difficulties dealing with daily routines, as well as maintaining a healthy self-image dues to the affected physical appearance and function.

The current standard treatment for craniofacial deformities includes autographs and allografts bone surgery. Bone regenerative therapy has been among the best options to repair and regenerate defective bone areas, restoring their function and appearance. However, the standard bone graft surgery consists of various limitations, including donor site morbidity, limited harvest, risks of infection and lack of vascularisation (Simunovic & Finkenzeller, 2021; Winkler et al., 2018). Therefore, tissue engineering has provided potential methods for the restoration and regeneration of the craniofacial complex in order to overcome the limitations of

conventional bone graft surgery for the treatment of craniofacial abnormalities (Rai et al., 2015; Ward et al., 2010; W. Zhang & Yelick, 2018).

Numerous studies on the regeneration of bone by cell-based strategies have been conducted over the years, including mesenchymal stem cells and induced pluripotent stem cells (Borrelli et al., 2020). The use of autologous or self-derived stem cells may avert immunological rejection, meaning a risk-free application for the patient. Adult stem cells derived from bone marrow were the first and are still the most commonly employed for tissue engineering. The later discovery of multipotent dental stem cells has paved the way for more accessible supply of MSC (Gronthos et al., 2000). Dental stem cells are quite similar to most craniofacial bones, which are generated from the neural crest. The many types of dental stem cells are categorised based on their harvest site and tissue niche. In three-dimensional (3D) culture, the dental pulp stem cells (DPSC) have shown great osteogenic capacity to produce osteoblasts, implying a prospective application in bone tissue engineering (Chamieh et al., 2016; D'Aquino et al., 2008; Gronthos et al., 2002).

Scaffold serves as the foundation for stem cell-based bone tissue engineering. Scaffolds are 3D matrix formed out of biomaterials or synthetics that can promote cell adhesion, bone formation, and bone development while mimicking the properties and structure of bone extracellular matrix (ECM) (Ghassemi et al., 2018). Collagen-based scaffolds are widely used in bone tissue engineering because of their biocompatibility, ability to provide anchorage for cell differentiation, and ability to operate as a cell delivery system (Lin et al., 2019). Evidence suggests that collagen type I (ColI) can sufficiently stimulate osteogenesis. (Akhir & Teoh, 2020; C. H. Lee et al., 2001; Salasznyk et al., 2004).

Nevertheless, it remains unclear as to how reliable Col 1 can be used for the induction of osteogenic differentiation. On top of that, the underlying mechanism in the induction of osteogenic differentiation is still much to fathom. To assist the clinical application of bone regeneration, this study aims to investigate the role of Col I in the osteogenic induction of DPSC. The outcome of this study may provide substantial information on the mechanism of bone formation and pave the way for future application of targeted pathway as part of therapeutic strategies in accelerating bone regeneration.

1.2 Objective of study

1.2.1 General objective

To identify the predominant signaling pathway in differentiation of DPSC into osteoblasts by Col I induction.

1.2.2 Specific objectives

1. To characterise DPSC according to the minimal standard criteria for MSC and evaluate DPSC osteogenic potential.
2. To compare the osteoinductivity of DPSC with different concentration and combinations of Col 1 and Matrigel™.
3. To identify the predominant signaling pathway (P13K/Smad/ERK) activated in Col I induced osteogenesis of DPSC.

1.3 Research questions

- Does DPSC has the capacity to differentiate into osteoblast lineage by addition of Col 1 or Matrigel™?

- Are there any differences in the osteogenic capacity of DPSC grown in Col 1 or Matrigel™ by gene expression analysis?
- What is the most relevant pathway involved in Col 1- induced osteogenic differentiation of DPSC?

1.4 Hypothesis

- DPSC has the capacity to differentiate into osteoblast lineage by the addition of Col 1 or Matrigel™
- There is no significant difference in the osteogenic capacity of DPSC grown in Col 1 or Matrigel™ by gene expression analysis.
- The osteogenic differentiation capacity of DPSC cultured on Col 1 coated scaffold is mainly regulated via one predominant signaling pathway.

CHAPTER 2

LITERATURE REVIEW

2.1 Craniofacial repair and regeneration

The area of the head that houses the face structure and the brain is called as the craniofacial region. This craniofacial region's distinctive 3D design is significant for multiple sensory organs necessary for sight, smell, hearing, breathing, tasting, speech, and mastication, as well as for identification and recognition at the aesthetic level. Due to the complexity of craniofacial structure, treatment regarding craniofacial deformities typically necessitates advance procedures, ongoing monitoring, and substantial financial resources for proper healing (Winkler et al., 2018).

2.1.1 Type of injury to the craniofacial area

Craniofacial anomalies (including cleft lip and palate, craniosynostosis, and microsomia), trauma (such as nasal and mandibular fractures), burns, chronic illnesses like cancer, and surgical resection can all result in craniofacial bone defects. The effects of craniofacial defects can be divided into three categories either affecting the soft tissues, bones, or dental structures. Most of the craniofacial defects reported in Malaysia are due to traffic accidents and typically affect male, young adult motorcyclists (Abosadegh et al., 2019). The midfacial and mandible are major trauma sites, which in severe cases can alter the face structure (Abosadegh et al., 2019; Muhammad et al., 2012).

2.1.2 Current treatment modalities for bone repair

The general approach to treating craniofacial injuries is designed to promote both the optimal function and aesthetical features. To begin with, a mainstay like a prosthesis will be given to the injured area to reconstruct the affected bone structure.

However, this mainstay may prevent functional tissue repair and restricts development of the fractured bone. Bone graft surgery employing autografts and allografts is the prominent treatment option for craniofacial defects (Alonzo et al., 2021).

The most preferred method for repairing significant bone defects is autogenous bone grafting, given the benefits of reducing the chance of immune rejection and most likely to provide osteogenic potentials compared to other type of bone grafts (Pape et al., 2010; Sohn & Oh, 2019). Some sources of autogenous graft are hip bone (iliac crests), calvarium, mandible, shin bone (tibial) and rib graft. Calvaria bone grafts have been widely utilised in craniofacial reconstruction with minimal rates of complication (Movahed et al., 2017). Donor site morbidities, discomfort, and a finite number of harvests are the main downsides of autograft bone surgery.

Another means of bone grafting is through a donor, or allograft. Providing both a structural support framework and an osteoconductive scaffold, allograft serves as a template to help in bone repair. Additionally, taking allografts from cadavers may allow for a high volume of harvest. However, the risks of immunological rejection and infection remain a challenge. A high degree of processing is required to reduce graft's immunogenicity, which compromises the osteogenic and osteoinductive properties. Recent study was developing method for customisation of allografts bone with potential of lessen the pain after surgery and optimising surgical accuracy (Van Genechten et al., 2023).

Hence, the application of tissue engineering for bone regeneration has long been studied to overcome limitations of the current treatment modalities and provide better strategies. This includes using bone graft substitutes that involve the association of synthetic materials with biomaterials such as growth factors to enhance bone

healing processes. Advanced studies are using autogenous stem cells with scaffolds to introduce natural bone formation to restore both functionality and features of the fracture site.

2.2 Bone formation

In-depth understanding of the mechanism of bone formation is critical in attempting to develop viable bone tissue for craniofacial reconstruction. In general, the characteristics of craniofacial bone are similar to those of other skeletal bones. The first phase in the creation of skeletal elements is cellular condensation, which occurs when initially distributed mesenchymal cells condensate and form aggregation (Fröhlich et al., 2008; Hall & Miyake, 2000). Osteogenesis is the process by which pre-existing mesenchymal tissues convert into calcified bone tissue. Most craniofacial bones are formed through intramembranous ossification (Summerlee, 2002).

Additionally, the embryonic origin of craniofacial bones derived from the neural crest and some from the mesodermal lineage (Morikawa et al., 2016). Neural crest-derived mesenchymal cells have been shown to distinctly possess intrinsic osteogenic abilities as compared to the mesodermal lineage (W. Zhang & Yelick, 2018). During embryonic skeletal formation and fracture healing, bone tissues are formed as woven (immature) bone that will gradually be replaced by the lamellar (mature) bone. Woven bone forms rapidly but with random or disorganised collagen bundle, and low level of mineralisation (Nikita, 2017). Lamellar bones are highly organised and thus provide better mechanical properties. Lamellar bone structure can be found in most adults skeletal.

2.2.1 Bone structure

Bones can be classified depending on their shape and structural function. The craniofacial bones are made up of flat bones that cover the skull cranium and some of the facial bones, while the rest are irregular bones that contribute to the significant functional figures such as enclosing the eyeballs, nasal cavity, and supporting teeth in the upper and lower jaws.

Bones typically share a similar internal structure which consists of compact tissues and spongy tissues. The compact or cortical bone is the denser type of bone that provide strong support and protection. The spongy tissues or cancellous bone are much lighter and some of them house the red marrow. The bone tissue structure is considered as composites since it is mainly composed of collagen matrix and hydroxyapatite minerals (calcium, phosphorus, sodium, magnesium, and trace elements) (Hutmacher et al., 2007). The organic compound (collagenase protein) provides elasticity while the inorganic compound (minerals) offers strength.

Despite its rigid appearance, bone tissues are constantly under active maintenance process regulated by various metabolically active cells. The main bone cells are osteoprogenitor cells, osteoblast, osteocyte, osteoclast and bone lining cells (Nikita, 2017). Osteoblasts are important bone-forming cells. The bone matrix is formed when osteoblasts secrete a protein mixture called osteoid that serves as a template for mineralisation (Ansari, 2019; Nikita, 2017). The osteoid is mostly collagenase protein: collagen type I (90%), collagen type III and collagen type IV.

The osteoid later mineralised upon deposition of the hydroxyapatites. Osteoblast cells that are trapped within mineralised tissue will mature into osteocytes, the cells in charge of maintaining the bone microstructure. Tiny gaps within bone

matrix or lamellae homing the osteocytes are called lacunae (Junqueira & Carneiro, 2005). Osteoclasts are bone cells that originate from macrophage and white blood cells, functioning in the resorption of bone tissues. In adult skeleton, bone tissues are continuously being renewed by the balanced action of osteoclasts and osteoblast in a process called bone remodelling.

2.2.2 Type of bone formation

There are two modes of bone formation: intramembranous and endochondral ossification. Intramembranous ossification is a direct conversion of osteoblast cells into bone tissues, while endochondral ossification involves an intermediate phase of cartilage formation, which is later replaced by bone. Primarily, craniofacial bones are formed through intramembranous ossification which includes the development of flat bones of the face and most cranial bones.

2.2.2(a) Intramembranous ossification

Intramembranous ossification can be summarised in five stages; first is osteoblast differentiation, second is osteocyte differentiation, third is formation of trabecular and periosteum, fourth is formation of cortical bone and fifth is formation of red marrow (Breeland & Menezes, 2019).

Mesenchymal cells initially congregate and develop into osteoblasts. This process takes place in a cluster called the ossification centre during early osteoblasts. Following that, the osteoblasts secrete osteoid, which calcifies or hardens as mineral salts are deposited on it. At the same time, the peripheral mesenchymal cells continue to differentiate and secrete osteoid inwards toward the ossification centre. Osteoblasts cells that are entrapped within mineralised osteoid will mature into osteocytes. Osteocytes are considered inactive forms of osteoblasts, important in the regulation of

bone tissues microstructure, partaking in intercellular communication within mineralised matrix.

As osteoid continues to be deposited, it will be assembled in a random manner around the blood vessels. This process allows the formation of spongy bones or a trabecular matrix. Red marrow is formed within the spongy bones as crowded vessels condensed. Compact bones typically sandwiched the spongy middle matrix, and surrounding mesenchyme will develop into periosteum, fibrous sheath that covers bone surfaces. Figure 2.2 illustrate the schematic representation of intramembranous ossification process.

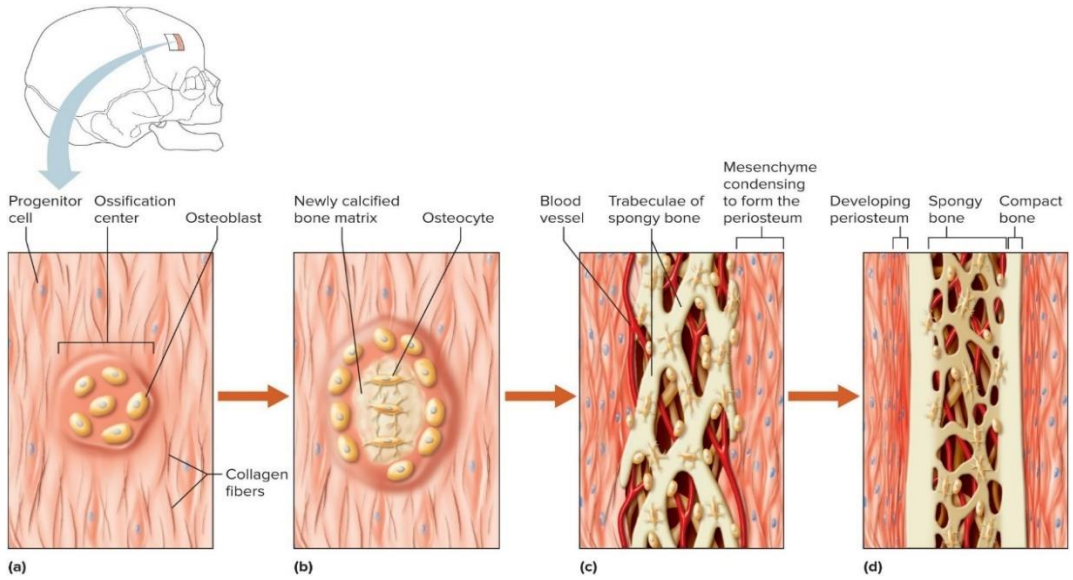

Figure 2.1 The process of intramembranous ossification. Image is adapted from Shier et al., 2018

2.2.2(b) Endochondral ossification

Endochondral ossification occurs during early fetal development and forms the long bones and bones at the base of the skull. Bone formation by the endochondral ossification mode requires a longer time to complete compared to intramembranous ossification. At first, mesenchymal cells differentiate into chondrocytes. The

chondrocytes will form hyaline cartilage, a semi-solid matrix consisting of hyaluronic acid, chondroitin sulfate, collagen fibres and water. The cartilage act as the template for bone formation. Blood vessels surrounding the cartilage outer membrane (perichondrium) will convey osteoblasts, depositing mineralised matrix in circles and forms the bone collar. Then, starting around centre of the cartilage, chondrocytes will undergo hypertrophic differentiation where they express collagen and mineralised matrix, before fated to apoptosis (Gruber et al., 2008). Nutrient deprivation at the centre of cartilage forces penetration of blood vessels, carrying along osteoblasts that later create the primary ossification centre. Ossification continues from the centre towards the edge of the bones, eventually replacing all the cartilage.

2.2.3 The stages of bone repair

Bone tissues are capable of self-healing which involves a unique repair mechanism distinctly from the regular scar-forming injury repair. There are two different bone healing action known as primary and secondary bone healing. In the primary bone healing, fractured segment is directly re-established when they are rigidly immobilised and be given adequate compression. The primary healing process is very slow and involve little or no inflammatory response at all (Maruyama et al., 2020). However, in most cases, bone healing follows the secondary action which involves overlapping phases of inflammatory action, reparation, and bone remodelling.

2.2.3(a) Inflammatory phase

In the occurrence of bone fracture, the blood vessels within can tear and leading to the death of some bone cells. Hematoma or blood clots at the damaged vessels causes swelling and pain. Immediate inflammatory response at the site of injury will then initiate the healing process. The immune system will start

accumulating inflammatory cells (such as neutrophils, macrophages, and platelets), and cytokines (such as Interleukin-1 (IL-1), IL-6, chemokine receptor 2 (CCR2) and tumour necrosis factor α (TNF α)) at the affected area (Maruyama et al., 2020).

Osteal macrophages are resident macrophage in bone tissues that contribute to bone homeostasis and the essential inflammatory cascade in bone healing (Maruyama et al., 2020). Mesenchymal progenitors and necessary growth factors such as platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factors (bFGF) are also directed to the affected area for angiogenesis and aid in inducing cells differentiation (Gruber et al., 2008). The inflammatory phase peaks during the first few days post-fracture and can be observed by the elevated level of inflammatory mediators and the expression of bone morphogenetic proteins (BMP) (Mountziaris & Mikos, 2008). The acute inflammatory response usually last for one week (Maruyama et al., 2020).

2.2.3(b) Reparative phase

About one-week after the injury, reparative phase will take place. Usually, woven bones are produced simultaneously through intramembranous and endochondral ossification during bone tissue repair (Junqueira & Carneiro, 2005). At the periosteum site of fractured bone, mesenchymal progenitors will differentiate into osteoblasts regulating direct formation of the woven bones. New vessels rise to replace the damaged blood vessels in the process of angiogenesis and vasculogenesis.

At the endosteum where oxygen is starved and there are least mechanical stabilities, soft callus are formed to fill in the gaps within fracture (Gruber et al., 2008). In few weeks, the soft callus hardens to form bony callus, and later fully be

replaced with woven bones (Maruyama et al., 2020). The bone formation at reparative phase is very much alike to those during initial bone development.

2.2.3(c) Remodelling phase

The last stage of bone repair involves adaptive modelling and remodelling processes, which occur around 6 weeks after the injury. Pro-inflammatory factors such as interleukin (IL)-1, IL-6, IL-11 are highly expressed, and osteoclast formation are promoted (Maruyama et al., 2020; Mountziaris & Mikos, 2008). Osteoblasts and osteoclasts cells regulate the renewing and resorptive actions, which then allowed lamellar bone to replace the initial woven bone.

Even if the bone has restored the structural and mechanical properties, cells metabolism will keep driving the remodelling process until the bone fully returns to the original shape. It might take years until the remodelling phase is completed. The homeostasis of skeletal and serum calcium levels relies heavily on the balanced action of bone resorption and new bone formation (Hienz et al., 2015). Bone remodelling occurs in daily basis and is critical in maintaining the healthy bone structure throughout human life.

2.2.4 Transcriptional control of bone formation

There are numerous variables and signalling networks that direct osteoblast differentiation and activity at various molecular levels. The two main transcriptional factors involve in osteoblast lineage commitment are Runt-related transcriptional factors 2 *(Runx2)* and osterix *(Osx)*. *Runx2* is considered as master regulator of osteogenesis, crucial in early osteoblast differentiation (Huang et al., 2007). The expression of *Runx2* is the highest at early osteoblasts and lowest in mature osteoblasts (Chan et al., 2021). Whilst *Runx2* regulate early osteoblast differentiation,

Osx plays significant role in osteoblast maturation since high expression of *Osx* can be observed in late or mature osteoblasts. *Osx* can also be expressed independent of *Runx2* via activation of *Dlx5* by BMP2 (Chan et al., 2021). Other transcription factor that can regulate osteoblast differentiation are Foxhead box class O (*FoxOs*), activating transcription factor 4 (*ATF4*), and dentin matrix protein 1 (*DMP1*) (Amarasekara et al., 2021; Chan et al., 2021; Huang et al., 2007).

The development of osteoblast differentiation into bone tissues can be classified into three stages. Firstly, pre-osteoblast cells actively proliferate following the osteoblasts lineage commitment. At this stage, ECM proteins such as fibronectin and collagen is synthesised for anchorage (Amarasekara et al., 2021; Huang et al., 2007). The osteoblasts also ligate existing matrix via β1 integrins to form monolayer linked by cadherins (Huang et al., 2007). Cells proliferation is later downregulated, followed by the synthesis of matrix Gla protein (MGP), collagen type 1 αlpha 1 chain (Col 1 α1) and alkaline phosphatase (ALP) by mature osteoblasts (Amarasekara et al., 2021; S. W. Tsai et al., 2010).

The final stage is matrix mineralisation. At the mineralisation stage, various markers are expressed including osteocalcin (OCN), osteopontin (OPN) and bonesialoprotein (BSP). The OPN protein has several calcium binding domains (Riccio et al., 2010). The *OCN* gene has been described as the most unique to osteoblasts., which play the roles of regulating calcium metabolism and minerals deposition on ECM (Amarasekara et al., 2021; Ducy & Karsenty, 1995; W.Huang et al., 2007). Figure 2.2 shows a schematic representation of osteoblast differentiation stages.

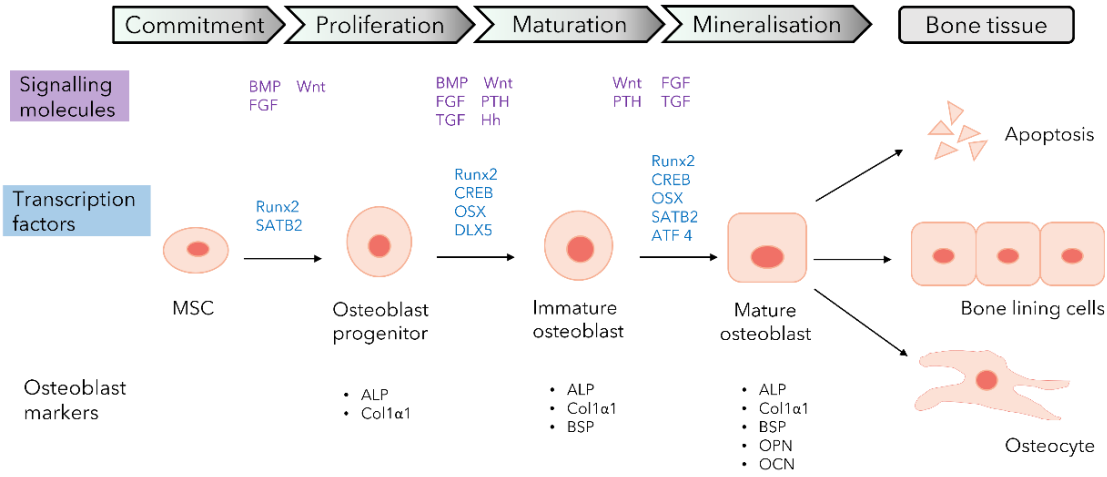

Figure 2.2 Schematic representation of MSC commitments towards osteoblast differentiation. Image adapted from Amarasekara et al., 2021.

During bone remodelling process, bone resorptions occur simultaneously along the bone formation. The recruitment of osteoclast towards the bone resorption sites are regulated by the osteocytes. The osteocytes induce expression of receptor activator of NF-κB ligand *(RANKL)* by the local osteoblast cells (Boyce & Xing, 2008). The regulation of bone resorption or bone homeostasis is modulated by the RANKL/RANK/OPG signaling system (Tobeiha et al., 2020). RANKL is the receptor that binds with RANK, while osteoprotegerin (OPG) is the inhibitor that acts on RANKL and prevents binding of RANK/RANKL.

2.2.5 Key signaling pathways in bone formation

Signaling pathways in a cell refers to a complex series of chemical reactions that are responsible for carrying out specific cell functions such as, cell division, cell differentiation or cell death. These signaling pathways also play crucial role in the body's response to specific environments, such as the presence of specific chemicals or hormones. There are three main stages in a typical cellular signaling pathways: reception, transduction, and response.

In the reception stage, a specific signal molecule or ligand binds to a receptor that either located on the cell membrane or inside the cell. Type of receptors include G-protein coupled receptors, receptor tyrosine kinase or intracellular receptors. Second, the transduction stage converts the signal into a form that can bring about a specific cellular response. The signal is often relayed and amplified through a sequence of changes in a series of different molecules along the pathway. The series of changes are often referred to as a signal transduction cascade. Lastly, the transduced signal finally triggers a specific cellular response such as expression of certain genes.

The osteoblast lineage is determined by complex interplay of multiple signaling networks. Figure 2.3 depicted some of the relevant pathways activated during bone formation such as the wingless (Wnt), Notch, transforming growth factor-β (TGF-β), BMP, FGF/FGF receptor, Hedgehog, ERK and phosphoinositide 3-kinase (P13K/AKT) pathway (Amarasekara et al., 2021) .

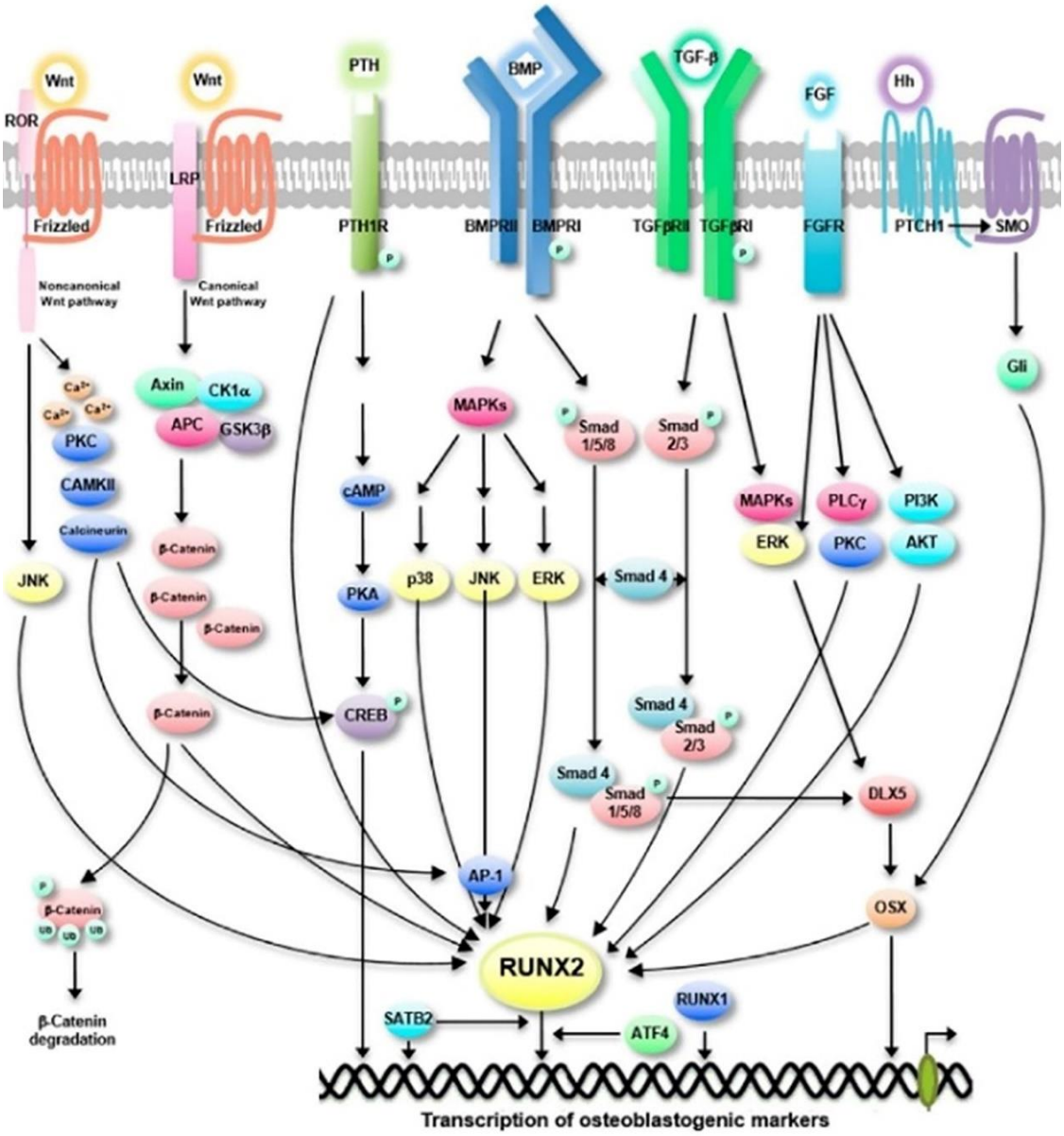

Figure 2.3 Key signaling pathways in osteoblast commitment. Image reproduced from Amarasekara et al., 2021.

2.2.5(a) P13K/Akt pathway

The P13K lipid kinase is an intracellular signal transduction pathway involves in many cellular functions such as proliferation, survival and migration. P13K pathway is stimulated upon ligand activation by extracellular signals and mediated through serine and threonine phosphorylation. The key proteins in P13K/AKT pathway are phosphatidylinositol 3-kinase (P13K) and AKT/protein kinase B, including the receptor tyrosin kinase (RTK), phosphatidylinositol-4,5-bisphosphate

(PIP2), and phosphatidylinositol-3,4,5-bisphosphate (PIP3). Studies suggested that P13K is a critical survival network for osteoblasts differentiation (Nasir et al., 2023).

AKT pathway is associated with BMP and Runx2 in the regulation of osteoblasts differentiation (Mukherjee et al., 2010). The osteogenic differentiation of hMSC was described to be stimulated upon AKT kinase activation upstream the BMP-2 signaling, and AKT kinase activation downstream the Mammalian target of rapamycin (mTOR) signaling (Viale-Bouroncle et al., 2015). Furthermore, inhibition of AKT pathway has been shown to suppressed both BMP/Smad pathway and osteoblast differentiation in dental stem cells (Ghosh-Choudhury et al., 2002; Viale-Bouroncle et al., 2015). Apart from that, downstream regulation of AKT is postulated to play an important role in endochondral ossification (Guntur & Rosen, 2011).

2.2.5(b) BMP/TGF-β pathway

The cytokines BMP and TGF-β belong to the TGF-β superfamily. The Smad molecule which can bind to the serine/threonine kinase receptors of the TGF-β superfamily, marks the signaling cascade of BMP and TGF-β pathway. The phosphorylated Smad is called receptor-regulated Smad (R-Smad), consists of Smad 1,2,3,5 and 8. The BMP extracellular signals activate Smad 1 and 5, while TGF-β extracellular signals activate Smad 2 and 3 (Hayrapetyan et al., 2015). R-smads can forms complexes with common Smad (Smad 4) that accumulates in nucleus and modulate target gene expression (Teixeira et al., 2020).

Of note, the Smad protein is responsible for regulation of transcription factors including Runx2, which is necessary for differentiation of osteoblast. The canonical TGF-β activity are mostly associated with TGFβ receptor 1 or also known as activin receptor-like kinase 5 (ALK5) (K. Zhang et al., 2019). ALK 5 activation increases the

expression of phosphorylated Smad 2/3 which induce cells differentiation towards osteoblast lineage (Matsunobu et al., 2009; Nasir et al., 2023). The BMP/Smad signaling pathway activation is also known to led to the transcription of vital proteins in osteoblasts differentiation and development, which are Col1α1, alkaline phosphatase and osteocalcin (Plotkin & Bruzzaniti, 2019). In recent study, *in vitro* osteogenic induction of stem cells on collagen/hydroxyapatite scaffold demonstrated upregulation of BMP2 and Smad 3 gene expression (Mazzoni et al., 2021).

2.2.5(c) ERK pathway

The extracellular signal-regulated kinase (ERK) is one of the classified MAPK pathways among the other two (P38 and JNK). Mitogens such as growth factors IGFs and FGFs are dominant activators of the ERK pathway (Chau et al., 2009). The MAPK pathway are also activated by ECM (Franceschi & Ge, 2017). The *in vivo* roles of MAPK pathways, in particular P38 and ERK signalling, in the formation of the skeleton are well known (Franceschi & Ge, 2017; Ge et al., 2007). The ERK and P38 kinases stimulated phosphorylation and transcriptional activity of osteoblast markers including the Dlx5, Runx2 and Osx (Franceschi & Ge, 2017; Ge et al., 2007; Khodabandehloo et al., 2020).

Other than that, Smad-induced Runx2 acetylation and stabilisation are promoted by the ERK-activated pathway, indicating the role of ERK signaling (from BMP/TGF-β) in enhancing stability and transcriptional activity of Runx2 (Hayrapetyan et al., 2015). The ERK 1/2 and focal adhesion kinase (FAK) in MAPK signaling, along with P13K was reported to activate integrin subunit alpha 5 (ITGA) which driven osteogenic differentiation of hMSC by inducing IGF2 and IGFBP2 expression (Khodabandehloo et al., 2020; Noda et al., 2019). ITGA5 was associated with the proliferation of DPSC (Cui et al., 2014). Previous study also showed that Col

1 matrix activates α2β1 integrin which are hypothesised to stimulate osteoblast differentiation *in vitro* via MAPK's FAK activation (Ge et al., 2007).

2.3 Tissue engineering

The application of tissue engineering in bone reconstruction is called-for an improved success rate in bone graft surgery. The three components of bone tissue engineering include stem cells as the basic element, a suitable scaffold for the mechanical structure, and also biomolecules (e.g., growth factors) that can synergistically stimulate bone tissue regeneration and enhance the healing process. The knowledge on cellular actions that lead to bone regeneration is vital for the application of tissue engineering strategies, noting each type of bone injury may differs between patients. The current concern in regeneration of bone tissues is adopting minimal invasive treatment and ensuring vascularisation following the integration of scaffolds.

2.3.1 Stem cells

2.3.1(a) Classification of stem cells

The gem of tissue regeneration is the population of cells that possess the ability to proliferate and differentiate into various lineages, known as stem cells. Due to its plasticity, most cells-based studies focused on the potential of stem cells for medical application. Stem cells can be classified into three main types: embryonic stem cells (ESC), adult tissue/somatic stem cells and induced pluripotent stem cells (iPSC). Apart from the origins, stem cells can also be classified based on their potency. Figure. 2.4 illustrated the origins of various types of stem cells.

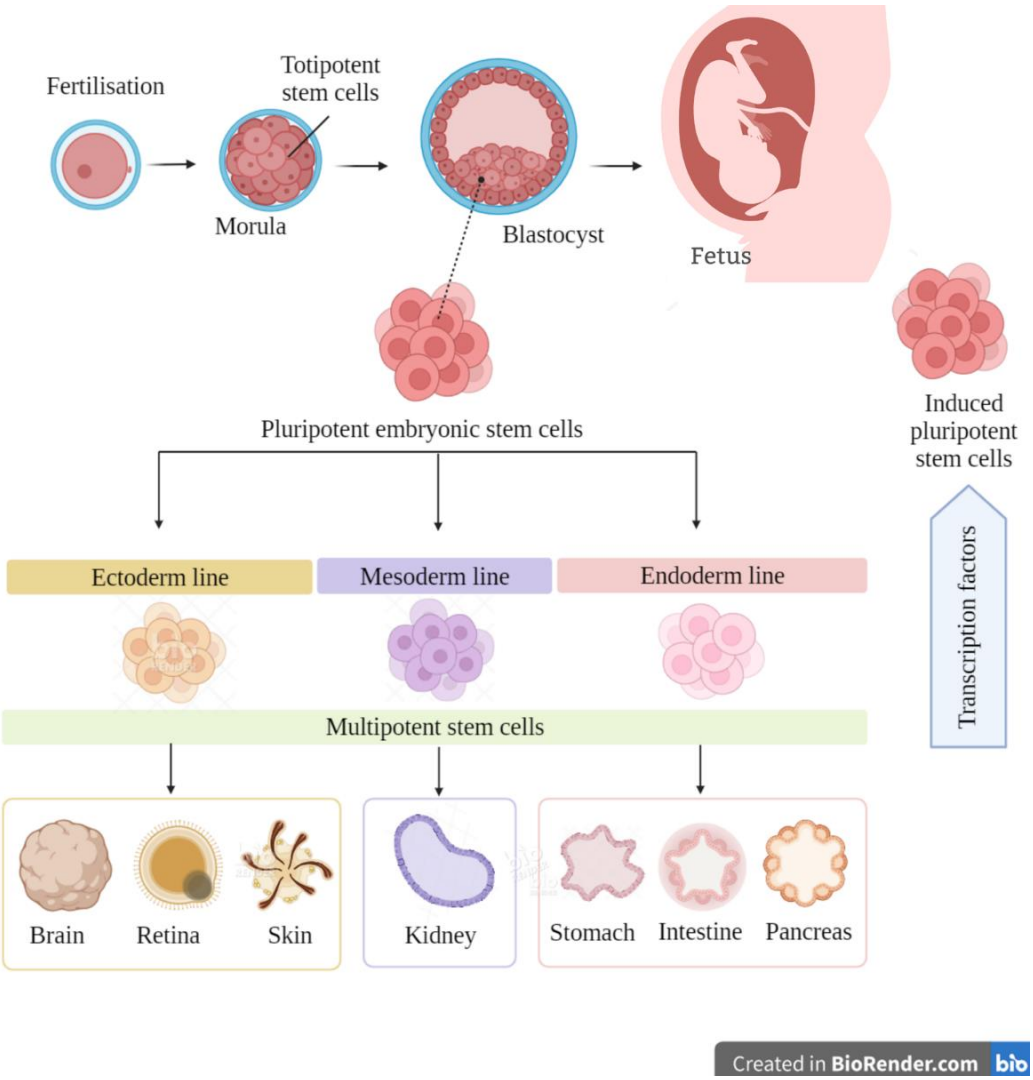

Figure 2.4 Origin of various types of stem cells.

The highest level of potency is totipotency. Totipotent cells are present at early fertilisation such as zygote and morula. As cells continue dividing, the inner cell mass inside the blastocyst will develop into an embryo became pluripotent cell type. The ESCs do not have the ability to differentiate into placenta or cells that form the amniotic sac, instead they can give rise to different types of cells within the three germ layers of the human body (Fortier, 2005). Next, multipotent stem cells generally refer to the adult stem cells present in specific tissues or organs which can give rise to

limited type of cells in a particular germ layer. The iPSCs are cells derived from adult somatic cells that is reprogrammed to generate pluripotency.

2.3.1(b) MSC potential for tissue regeneration

Mesenchymal stem cells (MSC) is the multipotent adult stem cells harvested from various tissues in the human body. MSCs are ideal for bone tissue regeneration given its ability to differentiate into various type of tissue within the mesenchymal lineage including bone, fat, cartilage as well as neuron cells (Dominici et al., 2006). Common sources of MSC for tissue engineering are the bone marrow stem cell (BMSC)- the pioneer described stem cells population, and stem cells from adipose tissues which are preferred for its abundance. Other discovered sources of MSC include the umbilical cord, amniotic fluid, skeletal muscle, liver tissue and periodontal tissue. The immune privilege, effective *in vitro* expansion and associated with low ethical concerns has allowed MSC to be of importance in the study and application for tissue regenerative therapy (Ayala-Cuellar et al., 2019).

2.3.1(c) Dental stem cells

The discovery of adult stem cells from the oral cavity in recent decades has encourage their implications for regenerative dentistry. Several sources stem cells from the oral cavity include dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSC), gingiva-derived mesenchymal stem cells (GMSC), and dental follicle precursor cells (DFSC), as shown in Figure 2.5. Dental stem cells are believed to be originated from the cranial neural crest, like most of the craniofacial bones (Bakopoulou & About, 2016).

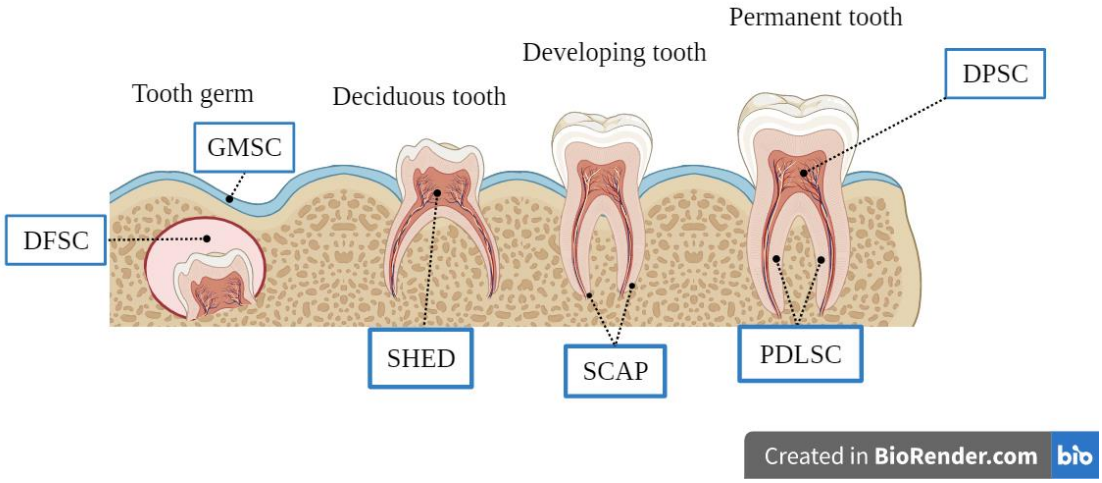

Figure 2.5 Different populations of dental stem cells. Image adapted from Li et al., (2022).

2.3.1(c)(i) Dental pulp stem cells (DPSC)

Dental pulp stem cells (DPSC) are harvested from permanent teeth, usually as impacted third molars or supernumerary teeth which commonly been disposed as medical waste (Gronthos et al., 2000; Ledesma-Martínez et al., 2016). DPSC has been characterised for plasticity hallmark of stem cells and capable to differentiate into osteoblasts, chondrocytes, odontoblasts, adipocytes, and myocytes (Gronthos et al., 2000; Pilbauerova et al., 2019). *In vitro* culture of DPSC revealed high capacity of clonogenicity, proliferative capacity and ability to form calcified colonies, suggesting its potential application in bone regeneration (Alipour et al., 2021; D'Aquino et al., 2008). DPSC also showed higher proliferation capacity compared to BMSC (Tamaki et al., 2013; X. Yang et al., 2018).

DPSC and SHED are dental derived stem cells that have excellent osteogenic differentiation potential (Kotova et al., 2021; Lau et al., 2022; Sabbagh et al., 2020). Furthermore, transplantation of collagen scaffold seeded with DPSC demonstrated regeneration of compact bone tissue *in vivo* (Chamieh et al., 2016; Giuliani et al.,

2013). The non-invasive harvesting procedure, high proliferative capacity, differentiation potential and established interaction with biomaterials has made DPSC a favorable subject for the application in bone tissue engineering (Leyendecker Junior et al., 2018).

2.3.1(c)(ii) Stem cells from human exfoliated deciduous teeth (SHED)

Stem cells from human exfoliated deciduous teeth (SHED) can be harvested from the dental pulp of primary teeth. SHED has been described to be multipotent, highly proliferative and clonogenic (Ledesma-Martínez et al., 2016). SHED also shows the ability to differentiate into odontoblasts, adipocytes, osteoblasts, and neural cells (Miura et al., 2003). The significant difference is that SHED exhibits higher embryonic markers while DPSC exhibits higher neurogenic markers (Shi et al., 2020). Studies show that SHED can generate functional odontoblast both *in vitro* and *in vivo* (Bakopoulou & About, 2016).

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

Stem cells from apical papilla (SCAP) is another type of dental stem cell recently discovered which is associated with dentin root development and thus notable of plasticity and potency (Sonoyama et al., 2008). Studies have shown that SCAP holds great potential of dentin regeneration given its initial function as root tissues (Nada & El Backly, 2018). SCAP are also comparable with DPSC in term of osteogenic and odontogenic differentiation, which appear to be superior to BM MSC (G. T. J. Huang et al., 2010; Nada & El Backly, 2018; Sonoyama et al., 2008).