PROTEOMICS APPROACHES IN IDENTIFICATION OF KEY SIGNALLING PATHWAYS ASSOCIATED WITH COLLAGEN TYPE 1-INDUCED OSTEOGENIC DIFFERENTIATION OF DENTAL STEM CELLS

NUR JULIA NABILA BINTI NASIR

UNIVERSITI SAINS MALAYSIA

2024

PROTEOMICS APPROACHES IN IDENTIFICATION OF KEY SIGNALLING PATHWAYS ASSOCIATED WITH COLLAGEN TYPE 1-INDUCED OSTEOGENIC DIFFERENTIATION OF DENTAL STEM CELLS

by

NUR JULIA NABILA BINTI NASIR

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

July 2024

ACKNOWLEDGEMENT

Alhamdulillah praise be to Allah, this project has finally come to an end after months of experimenting although there was a lot of challenges and hiccups along the journey. I would like to express my deepest gratitude to my supportive supervisor, Dr. Norhayati Yusop, who contributed substantially to the content of this thesis. I am very thankful for her endless guidance, patience, supervision throughout this journey. I am also extremely grateful to my co-supervisor, Dr. Norsyahida and Dr. Khairul Bariah for assisting me in various experimental tasks, managed to monitor the progress of my project, and provide suggestions on the problems.

I would like to express my appreciation to School of Dental Sciences for the support provided through student research grant. I am also thankful to the staff of Craniofacial Science Laboratory as well as Institute for Research in Molecular Medicine (INFORMM) for providing the equipment and materials needed and their helps throughout this project. I would like to acknowledge the contributions of my peers from both School of Dental Sciences and INFORMM, Siti, Athirah, Ain, Miza and Meha, who patiently have guided me in the experiments, insights, and expertise to enhance the overall quality of this research. A heartfelt gratitude to my parents for their constant encouragement, understanding, and patience. Their unwavering belief in my abilities has been a driving force behind the completion of this thesis. Finally, I extend my gratitude to the Ministry of Higher Education (FRGS/1/2020/SKK06/USM/03/3) for their research funding, which has enabled me to pursue and complete this research.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SYMBOLS

α	alpha
β	beta
γ	gamma
ζ	zeta
°C	celcius
%	percent
kDa	Kilo dalton

LIST OF ABBREVIATIONS

AKT	Ak strain transforming
ALP	Alkaline phosphatase
AMPK	Adenosine monophosphate-activated protein kinase
ANXA2	Annexin A2
ATF4	Activating transcription factor 4
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BLC	Bone lining cell
BMP	Bone morphogenetic protein
BM-MSC	Bone marrow mesenchymal stem cells
BMSC	Bone marrow stem cells
BMU	Bone multicellular units
BSA	Bovine serum albumin
BSP	Bone sialoprotein
BTE	Bone tissue engineering
CCM	Complete culture media
CD	Cluster of differentiation
CFL1	Cofilin 1
CO_2	Carbon dioxide
Col-1	Collagen type 1
COL-1A1	Collagen type 1 alpha 1 chain
CS	Calcium silicate
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DFSC	Dental follicle stem cells
DKK1	Dickkopf-1
DLX5	Distal-less homeobox 5
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPSC	Dental pulp stem cells
DTT	Dithiothreitol

ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ENO1	Enolase 1
ERK	Extracellular signal-regulated kinase
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FDA	Food and drug administration
FGF	Fibroblast growth factor
FM	Freezing media
FLNA	Filamin A
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GF	Growth factor
GO	Gene ontology
GSK	Glycogen synthase kinase
HA	Hydroxyapatite
HCl	Hydrochloric acid
HRP	Horseradish peroxide
HSC	Hematopoietic stem cell
HSPA8	Heat shock protein family A member 8
IGF-1	Insulin like growth factor 1
IL-8	Interleukin-8
iPSC	Induced pluripotent stem cell
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lithium borate buffer
LC	Liquid chromatography
LGALS1	Galectin 1
LY294002	2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one
MALDI-TOF/TOF	Matrix assisted laser desorption/ionization time-of-flight/time-of-flight
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MEK1	Mitogen-activated protein kinase
MEM	Minimum essential media
MF	Molecular function

MS	Mass spectrometry
MSC	Mesenchymal stem cell
MSX-1	Msh homeobox 1
MSX-2	Msh homeobox 2
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NFATC1	Nuclear factor of activated T-cells, cytoplasmic 1
NH4OH	Ammonium hydroxide
OCN	Osteocalcin
OIM	Osteogenic induction media
OPN	Osteopontin
OSX	Osterix
PBS	Phosphate buffered saline
PCL	poly(ε-caprolactone)
PCR	Polymerase chain reaction
PD98059	2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one
PDA	Polydopamined
PDGF	Platelet-derived growth factor
PDL	Population doubling level
PDLA	Poly(D-lactic acid)
PDLC	Periodontal ligament stem cell
PES	Polyethersulfone
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PLGA	Poly(lactide-co-glycolide)
PLLA	Poly(L-lactic acid)
PTEN	Phosphatase and TENsin homolog
PVDF	Polyvinylidene fluoride
RANK	Receptor activator of nuclear factor kappa beta
RANKL	Receptor activator of nuclear factor kappa beta ligand
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
RT	Room temperature

RTK	Receptor tyrosine kinase
RUNX2	Runt-related transcription factor 2
SCAP	Stem cells from apical papilla
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHED	Stem cells from exfoliated deciduous teeth
SOST	Sclerostin
SP7	Osterix transcription factor
SPP1	Secreted Phosphoprotein 1
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
TBS	Tris buffer saline
TBST	Tris buffer saline-Tween
β-TCP	beta-tricalcium phosphate
TE	Tissue engineering
TGF-β	Transforming growth factor beta
THBS1	Thrombospondin 1
TPI1	Triosephosphate isomerase 1
TGX	Tris-Glycine eXtended
VEGF	Vascular endothelial growth factor
VCL	Vinculin
VIM	Vimentin

LIST OF APPENDICES

PENDEKATAN PROTEOMIK DALAM MENENTUKAN LALUAN ISYARAT UTAMA YANG BERKAITAN DENGAN PEMBEZAAN OSTEOGENIK DALAM SEL STEM GIGI MENGGUNAKAN KOLAGEN JENIS 1

ABSTRAK

Kolagen jenis 1 (*Collagen type 1*; Col-1) ialah bahan perancah yang berpotensi dalam penjanaan semula tulang. Walau bagaimanapun, laluan isyarat utama yang terlibat apabila sel stem pulpa gigi (*dental pulp stem cell*; DPSC) dibiakkan pada Col-1 masih kurang difahami. Kajian ini menganalisis laluan isyarat utama dan profil proteomik untuk menentukan proses yang terlibat di sebalik osteogenesis oleh Col-1. Sel-sel telah dikumpulkan kepada kumpulan yang berbeza iaitu medium kultur lengkap (*complete culture medium*;CCM; kawalan negatif), medium induksi osteogenik (*osteogenic induction medium*; OIM; kawalan positif) dan Col-1 tanpa dan dengan tiga perencat laluan berbeza: LY294002 (perencat PI3K/AKT), LY23200882 (perencat TGF-β/Smad) and PD98059 (perencat MAPK/ERK). Analisis Western blot selama 7, 14, dan 21 hari serta profil proteomik LC-MS/MS pada hari ke-21 mendedahkan bahawa laluan isyarat PI3K/AKT adalah penting dalam proses pembezaan osteogenik DPSC dalam kedua-dua kumpulan OIM dan Col-1. Laluan PI3K/AKT adalah yang paling dominan sepanjang 21 hari pembezaan osteogenik menggunakan Col-1, manakala laluan MAPK/ERK dan TGF-β/Smad, masing-masing lebih relevan pada peringkat awal dan akhir. Interaksi anatara molekul isyarat menunjukkan kebergantungan dua arah atau sehala, di mana pengaktifan AKT boleh dipengaruhi oleh Smad, tetapi tidak sebaliknya. Begitu juga, pengaktifan Smad boleh dipengaruhi oleh ERK1/2, tetapi tidak sebaliknya. Profil proteomik osteogenesis yang

diinduksi oleh Col-1 menunjukkan sebilangan besar protein berkaitan terlibat dalam proses penghasilan gula, karbon, asid amino dan lekatan fokus.

.

PROTEOMICS APPROACHES IN IDENTIFICATION OF KEY SIGNALLING PATHWAY ASSOCIATED WITH COL 1-INDUCED OSTEOGENIC DIFFERENTIATION OF DENTAL STEM CELLS

ABSTRACT

Collagen type 1 (Col-1) is a promising scaffolding material in bone regeneration approach. However, the predominant signalling pathway involved when dental pulp stem cells (DPSC) cultured on the scaffold are still poorly understood. This study analysed the stemness of the DPSC prior to the studies of relevant signalling pathways involved and the proteomic profiles to determine the mechanism underlying the Col-1 induced osteogenesis. Characterisation of DPSC were analysed via its morphology, MSC surface markers, population doubling level as well as differentiation capacity. For the signalling pathways analysis, cells were grouped into complete culture medium (CCM; negative control), osteogenic induction medium (OIM; positive control) and Col-1 without and with three different pathway inhibitors: LY294002 (PI3K/AKT inhibitor), LY23200882 (TGF-β/Smad inhibitor) and PD98059 (MAPK/ERK inhibitor). Western blot analysis over 7, 14, and 21 days and LC-MS/MS proteomic profiling on day 21 revealed that the PI3K/AKT signalling pathway is crucial for the osteogenic differentiation of DPSC on both OIM and Col-1 group. PI3K/AKT pathway was predominant throughout the 21 days of Col-1 induced osteogenesis, while MAPK/ERK and TGF-β/Smad was more relevant at earlier and later stage, respectively. Crosstalk between signalling molecules showed bidirectional or unidirectional dependent to each other, where AKT activation can be influenced by Smad, but not vice versa. Likewise, Smad activation can be influenced by ERK1/2, but not the other way around. Proteomic profiling of Col-1 induced osteogenesis

showed majority of the proteins were associated with glycolysis, carbon metabolism, biosynthesis of amino acid and focal adhesion.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Road traffic accidents (RTA) are among the prevalent aetiology of craniofacial injury present in an emergency department in Malaysia. Data provided by the Malaysian Ministry of Transport indicates a gradual increase of 27.9% in the occurrence of RTA over a ten-year period from 2013 to 2023 (Malaysia Ministry of Transport). Studies over the years revealed the pattern of injuries occur in mandibular area which causes facial disfigurement (Noorali et al., 2023; Nordin et al., 2015; Ramli et al., 2008, 2011). Although craniofacial bone injuries are rarely life-threatening, the injuries may cause a variety of long-term effects physiologically, functionally, and aesthetically (Nordin et al., 2015).

Craniofacial bone structures serve fundamental functions in the body by protecting the brains and organs responsible for speech, smelling, mastication, breathing and hearing. In addition, the craniofacial bone structures provide the aesthetic purposes aside from the support for adjacent soft tissues and dental structures attachment (Petrovic et al., 2012). Craniofacial bone reconstruction assists in recovering the functions and aesthetic structure of the defective bone areas as it provides the aid for adjacent soft tissues and embedment for dental structure, hence, leading the structural stability as well as the appearance of the face (Petrovic et al., 2012).

Tissue engineering has a significant possibility in aiding bone regeneration process including repairment of large craniofacial defects. However, challenges persist in providing an optimum condition to create a scaffold that is adequate to the needs of controlled bone regeneration (Tollemar et al., 2016). A suitable combination of cell

types, scaffold properties, and growth factor supplementation is being considered to optimise the progress in achieving proper restoration of craniofacial defects.

Stem cell-based tissue engineering provides an alternative for tissue regeneration, which also includes bone tissue, due to stem cells' properties in proliferation, differentiation, and flexibility in developing into various specialised cells (D'Aquino et al., 2008). Mesenchymal stem cells (MSC) have been extensively used in bone tissue engineering for its ability to differentiate into osteoblasts and endothelial cells, to support haematopoiesis. Mesenchymal stem cells specifically isolated from dental pulp tissue is a promising source for cell-based therapy due to the non-invasive purification procedures, long-term proliferative properties, and multilineage differentiation capability (Awais et al., 2020; Yasui et al., 2016).

However, limitation does exist in the application of stem cell for a therapeutic context including the need to identify accessible sites within the human body to obtain sufficient stem cell samples for *in-vitro* expansion, risk of invasive procedures, and ethical concerns (Hoang et al., 2022). In the recent years, studies in dental pulp stem cells (DPSC) proved to be efficient in overcoming the limitations in the therapeutic use of stem cells. Isolation and expansion of DPSC involve non-invasive protocol, exhibit high efficacy in stimulating tissue regeneration, and exhibit promising interactivity with biomaterials for tissue engineering applications (Rodas-Junco & Villicaña, 2017). A noticeable feature of DPSC is the capability of regenerating a dentin-pulp-like complex that consists of a mineralised matrix with tubules lined with odontoblasts as well as fibrous tissue containing blood vessels in a position like the dentin-pulp complex in normal human teeth (Staniowski et al., 2021).

Scaffold serves as a three-dimensional (3D) matrix that encourages the attachment and proliferation of osteoinductive cells on its surface, as well as allowing for guided bone regeneration (Ghassemi et al., 2018). Scaffolds can be classified into two classes, which are the synthetic polymers, and natural extracellular biomaterials such as proteins, polysaccharides and polynucleotides. Collagen is a natural polymeric scaffold and being is the most abundant protein component of the extracellular matrix (ECM) (Rico-Llanos et al., 2021). Collagen type 1 (Col-1) used in bone scaffolds acting as a physical support for cells to attach to and develop along, by influencing the cell behaviour and outcome through receptor-mediated interactions (Rico-Llanos et al., 2021). Col-1 provides a support structure similar to an extracellular matrix, to stimulate stem cells to attach and proliferate on the scaffold surface upon implantation (Ghassemi et al., 2018; Reddy et al., 2021). However, the basic understanding of the signalling pathway activation when stem cells are exposed to Col-1 during osteogenesis is lacking.

The capability to comprehend the predominant signalling pathway in osteogenic differentiation induced by an optimised scaffold will offer a better opportunity to promote bone tissue regeneration. Nevertheless, most of the studies on dental stem cells only focus on its potential in tissue engineering, without further understanding of the effect of scaffold and the activation of the key signalling pathway involved. Moreover, the stimulation of the signalling pathway in cell is commonly not sufficient to assess their significant importance because protein modifications of many types can affect the functioning of the protein in the cell.

Therefore, the present study was performed to investigate the predominant signalling pathway induced by Col-1, to gain a clearer insight into primary activated

pathway in osteogenic differentiation at the protein level. Further proteomics analysis may assist in providing more information on protein-protein interaction, downstream targets, and inhibitory networks, as well as the kinetics of osteogenic activation. The findings gathered from this study may then be used in manipulating internal or external factors to stimulate the outcome of Col-1-induced, cell-based bone regeneration by targeting the key pathway in osteogenesis.

1.2 Problem statement and rationale of the study

DPSC is a promising source of stem cells for cell-based therapy in bone repair and regeneration. Although many treatment strategies in bone repair have successfully demonstrated the efficacy of using Col-1 in inducing bone formation, the exact cellular mechanisms involved at protein level remains partially understood. The data gathered from the present study will provide additional insights into the osteogenic induction of DPSC by Col-1, focusing on the relevant signalling pathways and mechanism involved in bone regeneration. It will also explore into its potential in craniofacial bone repair. This will facilitate the manipulation of the activated signalling to stimulate healing process in clinical context. Furthermore, the key findings of this study will enable researchers to conduct further experiments on exploring the potential of dental stem cells in association to the signalling pathway, chemoattractant and mechanisms involved during osteogenic differentiation.

1.3 Objectives of study

General objective

To elucidate the predominant signalling pathway in osteogenic differentiation of dental pulp stem cells (DPSC) by collagen type 1 induction.

Specific objectives

1. To investigate the MSC characteristics and osteogenic properties of DPSC.
2. To assess the key signalling pathway involved in Col-1 induced osteogenesis of DPSC by Western blot analysis.
3. To evaluate protein profile of Col-1 induced osteogenesis of DPSCs via proteomics analysis.

1.4 Hypothesis

1. Col-1 able to induce osteogenic differentiation of dental pulp stem cells without osteogenic induction medium.
2. The osteogenic differentiation capacity of DPSC cultured on Col-1 coated scaffold is regulated via a single predominant signalling pathway.

CHAPTER 2

LITERATURE REVIEW

2.1 Bone biology

Bone provides a variety of essential functions in the human musculoskeletal system by protecting vital organs, preserving the bodily structure, and facilitating body movement. Moreover, bones also possess a distinct role in bone repair and regeneration, enabling them to return to their fully functioning, pre-injury form. The process of bone formation involves both cellular and molecular components such as bone cells, and bone-forming transcription factors (Bahney et al., 2019).

Bone cells consist of four distinct types which are: osteoblasts, osteocytes, osteoclasts, and bone lining cells, as shown in Figure 2.1. The different types of bone cells play a vital role throughout the process of bone formation and remodelling.

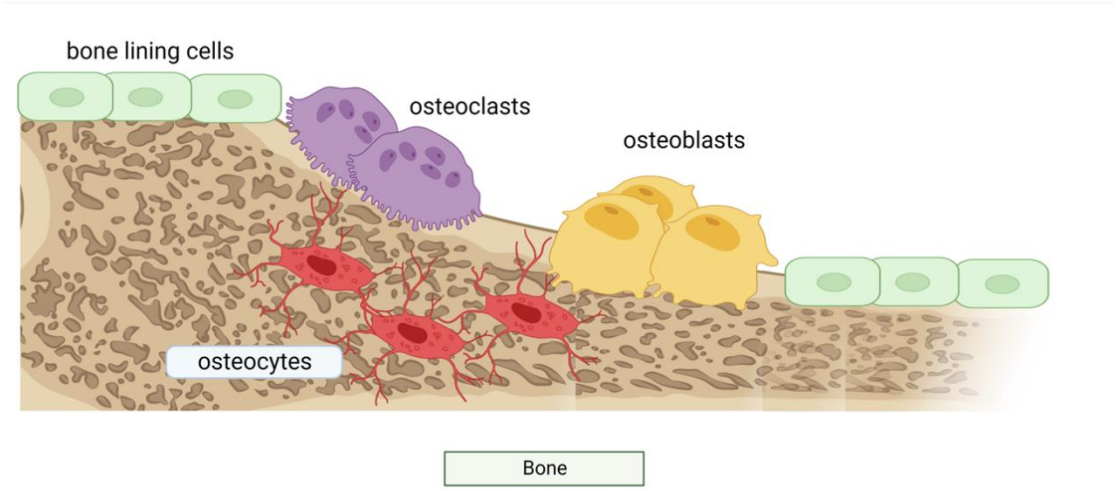

Figure 2.1 The different type of cells in bone formation and remodelling

Osteoblasts are bone-forming cells that are derived from immature mesenchymal stem cells (MSC) or osteoprogenitor cells (Bassi et al., 2011). In bone remodelling, osteoblast precursors are employed from the bone marrow by cytokines and growth factors, such as, transforming growth factor-β (TGF-β) and insulin-like growth factor 1 (IGF-1) that are released during the resorption phase (Dirckx et al., 2019). After several round of proliferation, the cell then matures into a cuboidal, bone-synthesising osteoblast, located in the most active site of bone formation. Hence, induction of osteogenesis in MSC involved the activation of various signalling molecules and transcription factors. Essentially, osteoblast differentiation of MSC is regulated by several transcription factors such as distal-less homeobox 5 (*DLX5*), Runt-related transcription factor 2 (*RUNX2*), and osterix (*OSX*) (Augello & Bari, 2010; Capulli et al., 2014).

The second type of cells is osteocytes, a former osteoblast trapped in lacunae of the mineralised matrix which make up around 90-95 percent of the majority of bone cells (Bonewald, 2010). Osteocytes involve in responding to mechanical strain or injury and circulating signals for bone formation or resorption to the bone surface to adjust the microenvironment. This is due to osteocytes' ability to synthesise proteins such as sclerostin that are able to regulate osteoblast and osteoclast differentiation (Jilka et al., 2008).

Meanwhile, osteoclasts originated from haematopoietic stem cells (HSC) located in bone marrow cavities, from the macrophage lineage or monocytes (McNamara, 2011). Osteoclasts can be characterised based on the development of the resorption lacunae on a mineralised surface (Bonewald, 2010). The primary role of osteoclast in bone regeneration is to resorb old or damaged bone to allow osteoblasts

to be recruited to the affected area to replace bone removed by osteoclasts (Iaquinta et al., 2019).

Bone-lining cells (BLC) is a population of inactive osteoblasts covering bone surfaces that are flat in shape. Since BLC are post-mitotic osteoblast lineage cell and possess stem cell-like genetic markers, it can be a good alternative to osteoblasts and proliferating pre-osteoblasts instead of bone marrow mesenchymal stem cells (BM-MSC) (Matic et al., 2016). BLC are most likely involved in bone remodelling, by coupling of bone resorption to bone formation, removal of the non-mineralised collagen fibrils, and deposition of smooth layer of collagen over the surface (Everts et al., 2002). However, the exact role of BLC in bone regeneration is poorly understood.

2.2 Craniofacial bone structure

The craniofacial region consists of the cranium, face, and structures of the ventral neck that are developed from the embryonic pharynx. Craniofacial complexes can be categorised into three regions which are neurocranium that encloses the brain, face that includes maxillary and mandibular structure, and oral apparatus (Chu et al., 2013). The complexity of craniofacial includes basic components such as blood vessels and nerves, as well as various distinct tissues such as craniofacial bones, cartilages, muscles, and ligaments, as shown in Figure 2.2. The region also consists of specialised organs which contribute to bodily functions, such as speech and chewing (W. Zhang & Yelick, 2018).

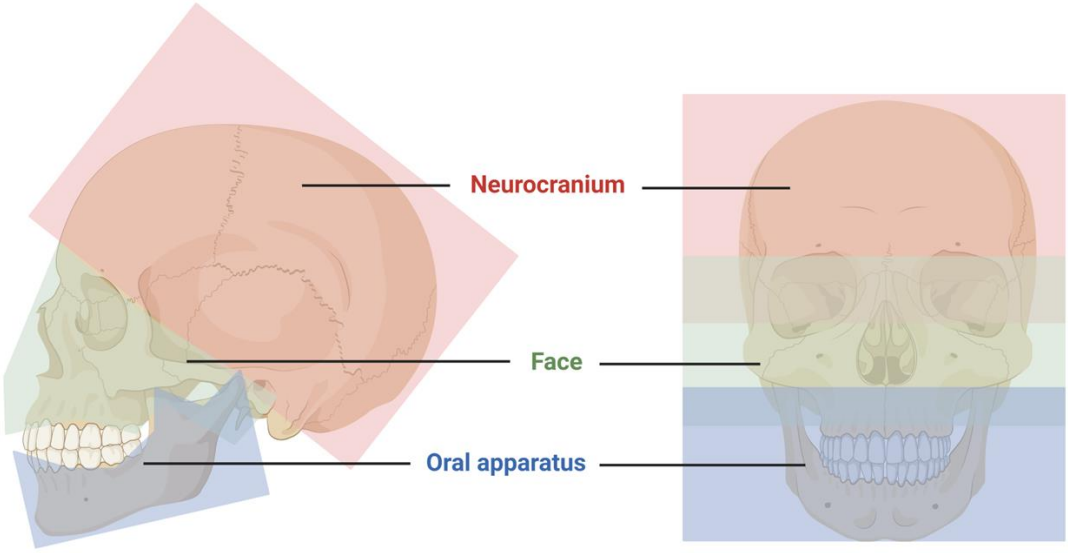

Figure 2.2 Anatomy of craniofacial region.

Craniofacial bone development involves the differentiation of mesenchymal progenitor or neural crest cells into osteoblasts by intramembranous ossification during embryonic development (Runyan & Gabrick, 2017). Intramembranous ossification is an essential process of osteogenesis of the mandible, clavicle, and majority of the skull bone that directly transforms the mesenchymal tissue into a bone (Shah et al., 2021).

2.2.1 Craniofacial deformities and the repair mechanisms

Bone injuries will undergo healing through two distinct mechanisms: the direct and indirect healing. Regardless of the type of bone involved, a general bone healing process consists of three overlapping stages, which are the inflammatory phase, reparative phase and the bone remodelling phase. Inflammation is a crucial part in bone healing. The inflammatory phase sets in immediately upon injury and triggers the formation of haematoma surrounding the fracture which releases a growth factor that stimulates bone formation (Lieberman et al., 2002). In direct bone healing, the

reparative phase involves intramembranous ossification meanwhile, indirect bone healing involves endochondral ossification (Sheen & Garla, 2022). The bone remodelling phase in general is the formation of lamellar bone that gives rigidity and stability to the bone (Elhawary et al., 2021).

Direct or primary bone healing involves intramembranous ossification that occurs only under solid attachment with minor bone fragments movement (M. Maruyama et al., 2020). The flat bones of the face, and most of the cranial bones are formed via intramembranous ossification. In intramembranous ossification, mesenchymal condensations differentiate directly to osteoblasts (Lee et al., 2023). The primary healing mechanism takes place via contact healing which involves direct remodelling of lamellar bone, the Haversian canals (osteon), and blood vessels (Marsell & Einhorn, 2011). The canals and surrounding lamellae are called a Haversian system or osteon, which are responsible for osteoblasts transportation to the defective area by blood vessels (J. N. Kim et al., 2015). Bridging osteons then developed into lamellar bone which results in fracture healing without periosteal callus formation, and fully depending on the osteoblasts and osteoclasts activity to connect adjacent bone fragments (Claeset al., 2012; Ghiasi et al., 2017; Marsell & Einhorn, 2011). Therefore, cranial bone utilises intramembranous ossification to form compact bone with closely packed osteon which is important to protect vital organs like brain (Szpalski et al., 2010).

Indirect bone healing involves the development of haematoma into granulation tissue for cartilage formation via chondrogenic differentiation by MSC within the local region with the aid of various growth factors. The primary soft callus proceeds to mineralise and resorbed to form a hard bony callus. The final phase, bone remodelling

takes place to reconstruct the hard callus into a lamellar bone structure via resorption and deposition by osteoclasts and osteoblasts, respectively (Bigham-Sadegh & Oryan, 2014; Marsell & Einhorn, 2011). Figure 2.3 exhibits differences between direct and indirect bone healing with the stages involved in both processes.

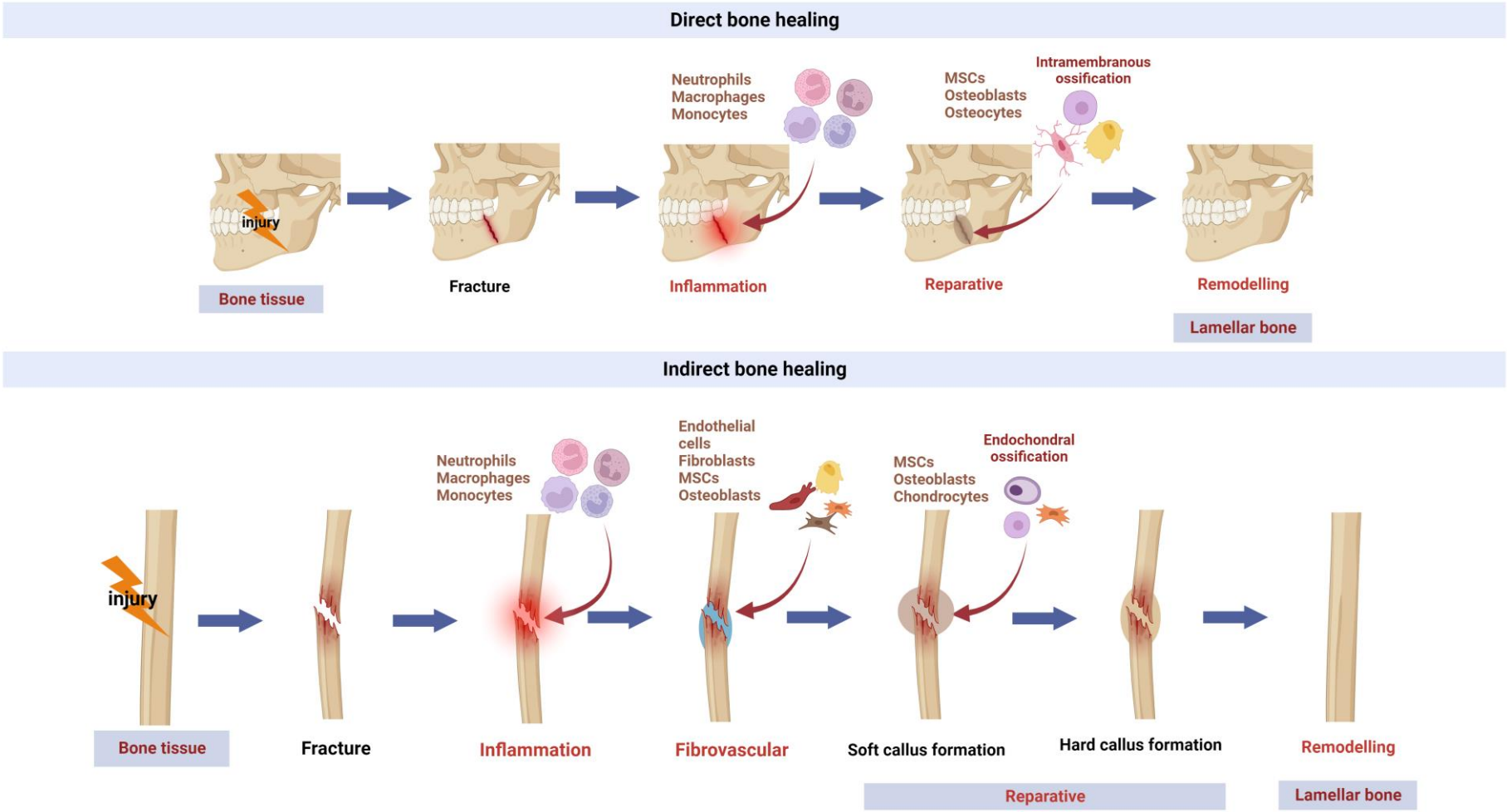

Figure 2.3 The different stages of bone healing mechanism

2.3 Tissue engineering for craniofacial bone repair

Regenerative therapy was primarily introduced to address the limitation in donor availability and grafts rejection by the immune response (Mhanna & Hasan, 2016). Current regeneration strategies in tissue engineering field can be achieved through techniques such incorporating stem cells, scaffold fabrication to improve the administration of biomolecules and 3D bioprinting of tissues and organs (Dzobo et al., 2018; Lynch et al., 2021) . Bone tissue engineering aims to regenerate new bone tissues by optimising the three major components which are biomaterials, cells, and growth factors in the defective areas which are then remodelled by the individual's own cells (Koons et al., 2020; Mhanna & Hasan, 2016). Interaction of these components under an ideal environment, along with adequate nutrients and growth conditions allow the cells to grow and differentiate into the desired tissue.

Craniofacial bones and long bones exhibit distinct challenges and considerations in bone tissue engineering due to the difference in anatomical locations, functions, and structural properties. Craniofacial regeneration can be more challenging as it demands both functional and aesthetic recovery (Zhang et al., 2020). Craniofacial bone regeneration often requires stem cells originated from cranial sutures, dental tissues, or nearby adipose tissue (Chamieh et al., 2016; Maruyama et al., 2016; Oliver et al., 2020). In this case, the source of mesenchymal stem cells also plays an important role in craniofacial bone repair and remodelling mechanisms due to the germ layers origin during embryogenesis (Aghali, 2021; W. Zhang & Yelick, 2018). Although craniofacial bone tissue engineering may focus more on restoring the form and function surrounding nerve tissues and blood vessels, it is also important to include the regeneration on the structural support and movement aid, especially in mandibular

area (Aghali, 2021; Novais et al., 2021). Therefore, most of MSC sources that have been proposed as potential cell sources in craniofacial bone regeneration have also showed the potential in long bone regeneration (Chamieh et al., 2016; Lorusso et al., 2020).

2.3.1 Comparison of cell-based scaffold and the conventional bone grafts

Tissue engineering and traditional bone grafts differ in terms of source of bone tissue, scaffold, mechanism of bone formation, availability, and donor site morbidity. Tissue engineering incorporates the use of stem cells such as bone marrow-derived cells to form new bone tissues within a structured scaffold, whereas bone grafts require the transplantation of bone tissue directly from the donor site to the defective areas of the receiving patient (Colnot, 2011). Scaffold being one of the key components in tissue engineering to mimic the natural extracellular matrix (ECM) to provide the structural support for cell growth and tissue regeneration while bone graft relies on the bone tissue itself as the matrix (Nicolas et al., 2020).

The mechanism of bone formation differs for bone graft and other type of tissue engineering, in terms of the application of biomaterial used in scaffolds, stem cells, and growth factors involved. Bone grafts, on the other hand, involve the natural processes of inflammation, remodelling, and incorporation into the host body. In contrast to bone grafts, tissue engineering offers the accessibility to expandable source of bone tissue as stem cells, that can be grown *in vitro* and the tissue harvesting process can reduce the risk of morbidity and complications (immunogenic rejection, disease transmission)at the harvest site (Betz, 2002; Rogers & Greene, 2012). While bone grafts actively contribute to natural bone repair mechanisms by providing osteogenic cells, tissue engineering utilises the potential of scaffold properties. Tissue engineering aims to conform to specific requirements, including promoting osteoblast activity,

providing mechanical support, facilitating necessary cellular processes such as attachment, migration, and nutrient transfer (Oliva & Chen, 2022; Pina et al., 2019). These regulated scaffolds enable the effective regeneration of functional bone tissue.

2.4 Stem cells and bone tissue engineering

Stem cells can be classified into three types depending on the source, which are: embryonic stem cells (ESC), adult stem cells and induced pluripotent stem cells (iPSC). ESCs are categorised as pluripotent stem cells and can be obtained from the inner cell mass of pre-implantation embryos (Zakrzewski et al., 2019). *In vitro* and *in vivo* differentiation of ESC, however, are exposed to the risk of allogeneic immune rejection by recipients and possibility of hESC being exposed to immunogenic and pathogenic animal component during proliferation stages (Khan et al., 2018).

Meanwhile, adult stem cell populations are defined as multipotent cells residing in tissues of adult organisms that help to regulate the local microenvironments (Łos et al., 2019). To date, the most common source of adult stem cells used in cell-based therapy is bone marrow, followed by adipose tissue (Berebichez-Fridman & Montero-Olvera, 2018; Freitas et al., 2020). Next, iPSCs that are genetically modified and derived from adult somatic stem cells demonstrate similar characteristics to ESC to mitigate the ethical concern with ESC (Kato et al., 2022). However, reprogramming of iPSC involves the incorporation of retrovirus and lentivirus that imposes the risk of tumour formation (Singh et al., 2015).

2.4.1 Characteristics of mesenchymal stem cells

Mesenchymal stem cells (MSC) are among the widely studied adult stem cells for cell-based bone regenerative therapies. The International Society for Cellular Therapy has outlined the minimal characteristics of MSC as follows; plastic adherence

growth, positively expressed MSC markers, and confirmation of different lineage potential (Dominici et al., 2006). Due to MSC distinct property of multipotency, a proper control of cellular differentiation is crucial to ensure the stem cell differentiate into desired cell lineage (Pittenger et al., 2019).

The stem cell lineage determination can be influenced by either internal or external factors. Internal factors that regulate cell differentiation consists of involvement of signalling molecules (Jothimani et al., 2020), activation of lineage-specific transcription factors (Frith & Genever, 2008), and epigenetic modification (Rojas et al., 2015; Sepulveda et al., 2017). Meanwhile, the external factors involve the combination of chemical and mechanical cues, growth factors, activation of the downstream signalling pathway (Cheng et al., 2019; Guo et al., 2020).

2.4.2 Type of dental stem cells

Similar to general characteristics of MSC, dental stem cells exhibit the exact characteristics and hence qualify as an alternate source of MSC. Figure 2.4 exhibits the different type of dental stem cells.

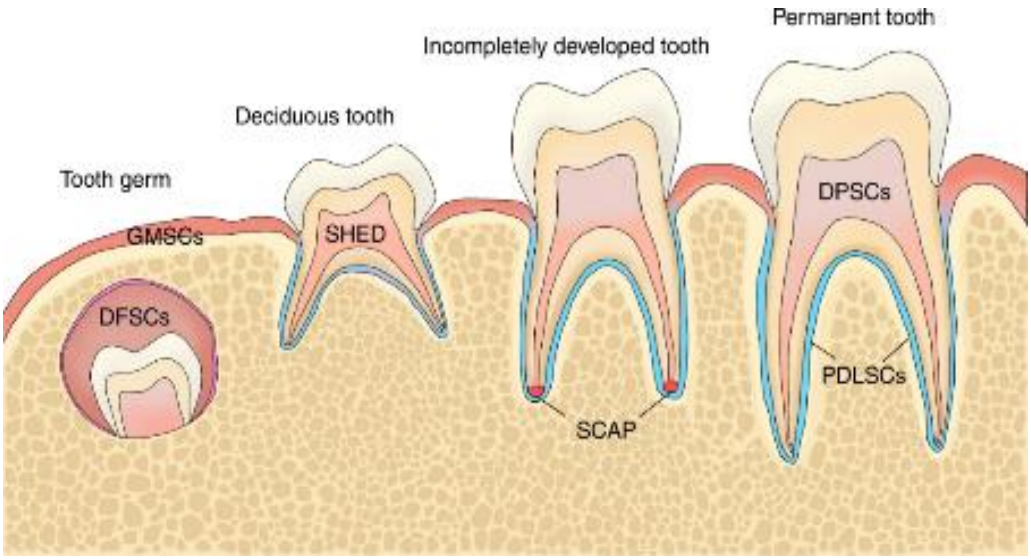

Figure 2.4 Type of dental stem cells from different source (Li et al., 2022).

Dental stem cells population can be isolated from various dental origins, such as dental pulp stem cells (DPSC), stem cells from exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), dental follicle stem cells (DFSC), and periodontal ligament stem cell (PDLC) (Hollý et al., 2021). Isolation of dental stem cells rely on non-invasive protocol, and sustainable approach which aim to make use of available tissues that are usually regarded as clinical waste.

2.4.2(a) Dental pulp stem cells (DPSC)

In tissue engineering context, DPSC remains as one of the promising sources of MSC as it possesses great proliferation and multilineage differentiation capability, as well as feasibility of cell purification. DPSC can be obtained from impacted third molars whereby the pulp was extracted from the crown and root, followed by enzymatic digestion process in collagenase/dispase and physical straining of the digested tissue (Gronthos et al., 2000). Furthermore, DPSC possesses better potential in terms of proliferative and clonegenic as well as odontogenic capability when compared to bone marrow stem cells (BMSC) (Tamaki et al., 2013a).

Isolated DPSC expressed MSC surface markers such as CD29, CD44, CD59, CD73, CD90, CD105, and CD146 (Jeon et al., 2015). While DPSC and MSC share identical transcription factors, the expression levels of these transcripts vary based on the source of the cells and external factors such as isolation methods, passage stage, and experimental conditions (Borciani et al., 2020). DPSC also express osteoblast-like cells bone markers, such as alkaline phosphatase, collagen type-1 (Col-1), bone sialoprotein (BSP), osteocalcin (OCN), osteopontin, transforming growth factor β (TGF- β), and bone morphogenetic proteins (BMPs). DPSC can undergo osteogenic differentiation when cultured under osteoinductive conditions (H. Liu et al., 2006).

Hence, DPSC are widely used in surgical regeneration studies, as the cells were stimulated from neural crest and assists in development of craniofacial structure region.

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

SHED is another type of dental derived stem cells which can be obtained from exfoliated primary teeth that are considered as readily accessible postnatal human tissues (Rosa, 2013; Rosa et al., 2011). Stem-cell characteristics of SHED can be applied to regenerate damaged tooth structures and enhance bone regeneration due to their extensive proliferation and multipotential differentiation properties (Miura, et al., 2003). Besides, SHED also possesses expression of neuronal and glial cell markers that may be associated with neural crest-cell origin of dental pulp where neural crest cells are known as the vital component in embryonic development, inducing various type of cells such as smooth muscle, craniofacial cartilage, and bone (Chai et al., 2000; Labonne & Bronner-Fraser, 1999).

2.4.2(c) Periodontal ligament stem cells (PDLSC) and other dental stem cells

PDLC exhibits characteristics of MSC, found located in the perivascular space of the periodontium (Zhu & Liang, 2015). The periodontal tissues arise from migrated neural crest cells during tooth development, however, when extracted from mature periodontal ligaments, exhibit stem cell properties like MSC instead of neural crest cells (Chai et al. 2000). In addition, dental follicle stem cells or DFSCs are obtained from dental follicle tissue of the tooth germ, derived from neural crest cells which descended from the ectoderm (Zhang et al., 2019). Similar to other dental stem cells, DFSCs also exhibit the ability to differentiate to multiple lineages including osteogenic

differentiation. Stem cells from the apical papilla (SCAP) that can be found in the apical papilla of immature permanent teeth depicting another source of dental MSC that have the capability to proliferate at a high rate, self-renewal ability, as well as low immunogenicity (Sonoyama et al., 2006).

2.5 Growth factors

Growth factors are natural substances produced by specific tissue that bind to receptors on the cell membrane of the target cell. The binding of the growth factor ligand to the extracellular domain receptor results in the activation of protein kinase in the intracellular domain, which leads to a cascade reaction that activates gene transcription into mRNA (Solheim, 1998). In addition, the capability of the growth factor to transmit signals to a specific cell is determined by several factors such as the target cell number, type of receptors and the intracellular signalling cascade upon the binding to the receptor Lee et al., 2011).

Growth factors are often utilised to stimulate various cellular development including cell proliferation, migration, differentiation, leading to tissue regeneration (Ren et al., 2020). The mechanism of action comprises signal transmission initiated along with growth factor secretion by producer cell, leading to growth factor to instruct cell behaviour by binding to specific transmembrane receptors on the target cells (Lee et al., 2011). Combination of growth factor with scaffolds allows loose association with structural material, hence, enabling the desired stimulatory molecules to be released in a better way (Tollemar et al., 2016).

Several growth factors involved in bone formation are bone morphogenetic proteins (BMPs), insulin-like growth factors I and II (IGF-1 and IGF-II), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), along with

fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF) (De Witte et al., 2018). Also, recombinant BMP-2, BMP-7, and PDGF are among the growth factors approved by Food and Drug Administration (FDA) for bone regeneration (Gillman & Jayasuriya, 2021).

2.6 Scaffold in bone tissue engineering

Scaffolds remain one of the vital components in bone tissue engineering where it gives temporary mechanical integrity at the damaged site until the tissue is repaired (Amini et al., 2012). The mechanical strength of scaffold greatly affects the mechanotransduction of the adherent bone cells which contribute to improved osteoinductive ability for bone repair process (Amini et al., 2012; Pioletti, 2010). Besides, scaffold in BTE also important in inducing the incorporation of bone in-growth into the porous scaffold and integrating with the local tissue, providing transportation means for bioactive molecules to stimulate the healing process, and enhancing the osteogenic capability of the cells via molecular signalling (Filippi et al., 2020). To achieve functional and effective scaffolding, the scaffolds need to fulfil several important criteria which will be further elaborated in the next section.

2.6.1 Ideal scaffold design for BTE

Other than the mechanical properties, several other important characteristics also need to be considered to achieve an ideal scaffold design, such as biocompatibility for cell adherence, biodegradability for scaffold to degrade efficiently for proper bone remodelling process, porosity where pore size need to be optimised for proper cell migration and tissue in-growth, osteoinductivity which enable the signalling molecules to stimulate the cells proliferation and differentiation, and osteoconductivity that allow the scaffold to induce the bone cells growth on its surface (Filippi et al., 2020; Polo-

Corrales et al., 2014; Sarker et al., 2016; Tariverdian et al., 2019). However, designing an ideal scaffold is a challenging process as one needs to consider the possible varying effects of mechanical stimuli on cells at different maturity levels along with the microenvironment issues.

In BTE, scaffolds for bone tissue engineering consists of four category which are polymeric, ceramic, composite, and metallic scaffolds (Ghassemi et al., 2018). Polymeric scaffolds can be further categorised into natural and synthetic polymers. Commonly used synthetic polymers consist of aliphatic polyesters such as poly(ε-caprolactone) (PCL), polylactide (PDLA,PLLA), and poly(lactide-co-glycolide) (PLGA) (Chocholata et al., 2019). Meanwhile, natural polymers can be divided into proteins (collagen, silk, fibrin gels) or polysaccharides (alginate, chitin/chitosan, starch, and hyaluronic acid derivatives) (Filippi et al., 2020). The current study will be focusing on the application of natural polymers, specifically collagen.

2.6.2 Overview of collagen type 1 (Col-1)

Collagen is the most abundant protein which represents the fibrous part of the extracellular matrix in all multi-cellular animals (Alberts et al., 2002). To date, there are up to 28 types of collagen discovered (Shenoy et al., 2022). Collagen type I, II, III, IV, and V are the most common type being the most abundant in the human body (Correlo et al., 2011). Collagen type 1 (Col-1) is the most prevalent type of protein that constituted more than 90% of the organic mass of bone (Fan et al., 2023; Rico-Llanos et al., 2021). Therefore, Col-1 has been used as the scaffold material in bone tissue engineering due to its ability to mimic the ECM of natural bone to promote the cell adhesion, proliferation, and differentiation (Rico-Llanos et al., 2021).

In cell-based therapy and scientific research, Col-1 is utilised as coating substrate to infuse the stem cells into it, stimulating the osteoblast differentiation and collagen production (Amirrah et al., 2022; Mochizuki et al., 2020). Figure 2.5 shows the chemical structure of collagen and the individual unit of collagen, tropocollagen. The following sections further elaborate the structure, source and post translational modifications of Col-1 used in the current study.

The procollagen is transported and excreted outside of the cells and undergoes peptide cleavage at both ends to form tropocollagen (Climov et al., 2016). In addition, Col-1 undergoes molecule packing that produces hexagonal and quasi-hexagonal shape conformation, forming an elongated fibril (Naomi et al., 2021). The molecular packing arrangements of Col-1 to form an elongated fibril is essential for the strength and stability of the collagen fibril (Orgel et al., 2001).

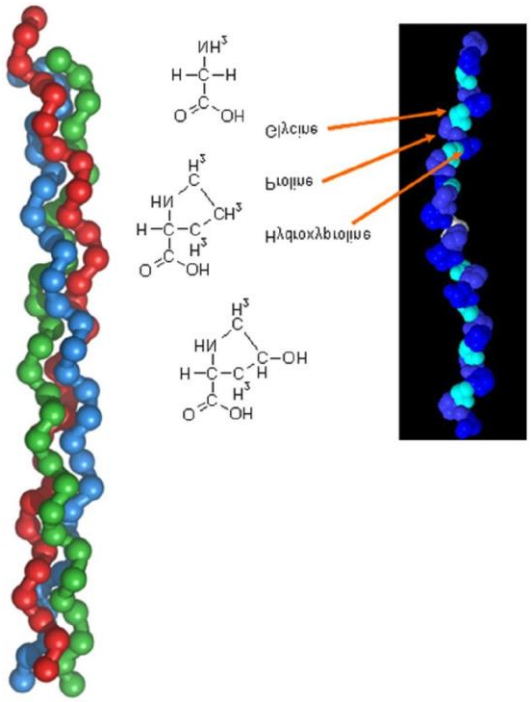

Figure 2.5 Chemical structure of collagen and the basic unit of collagen, tropocollagen. Accumulation of tropocollagen molecules form collagen fibrils which will further aggregate and cross-link to form larger collagen fibres. (Rau & Kajzar, 2014).

Col-1 can be produced from different species such as mammalians, amphibians, fish, marines, birds, and human recombinant collagen (Naomi et al., 2021). In normal tissue, Col-1 is found in bone, skin, tendons, and connective tissue and part of the dental component (D'souza et al., 2020). Tendon is a favourable source of collagen due to the higher concentration of Col-1 compared to other tissue sources. Moreover, tendons are easy to isolate, and the supply of tendon-rich anatomical structures is readily available, especially from the surgical remnants of animal experimentation (Rittié, 2017). For example, rat-tail tendon, bovine tendon, porcine tendon, or equine tendon (Techatanawat et al., 2011).

2.6.3 Application of Collagen type 1 in osteogenic induction

Collagen type 1 is predominantly synthesised by osteoblasts which plays a central role in both bone formation and remodelling. The mechanism of action of Col-1 as a scaffold involves various processes such as cell adhesion, cell migration, osteogenic differentiation, and extracellular matrix deposition. Col-1 facilitates the initial cell attachment by providing specific binding sites (integrin-binding motif) on its molecule, which interact with the cell surface receptors, aiding in cell adhesion (Rico-Llanos et al., 2021; Somaiah et al., 2015). In addition, the porous nature of Col-1 provides favourable conditions for cells to penetrate the scaffold matrix, aiding the migration of the cells throughout the scaffold (Chocholata et al., 2019; Y. Li et al., 2021). Larger pore size also enables the blood vessels to grow and support bone formation (Moncayo-Donoso et al., 2021).

Furthermore, osteogenic differentiation by Col-1 scaffold is mediated by the involvement of growth factor, cell signalling as well as the interaction with the scaffold itself. For instance, cells cultured on collagen scaffold exhibited an upregulation of

p38/MAPK pathway along with the enhanced migration and adhesion (Hiew & Teoh, 2022). Similarly, another study on mineralised collagen scaffold induced osteogenic differentiation via BMP receptor and improved the matrix mineralization of hMSC (Zhou et al., 2017).

To date, most of application of collagen scaffold is combined with other additional materials such as hydroxyapatite and polycaprolactone to enhance the mechanical properties (Calabrese et al., 2016; Pupo et al., 2021; Sadeghzadeh et al., 2022). Therefore, the present study aims to conduct osteogenic induction solely by using Col-1, and further investigate the direct interactions between Col-1 and pro-osteoblastic cells for new bone formation.

2.7 Signalling pathways in bone tissue repair

It is crucial to understand the regulatory pathway in cellular metabolism which can be further used as an alternative strategy in formulating bone tissue regeneration. Wnt, BMP/Smad, Hedgehog and Notch signalling are among well-known signalling pathways involved in osteogenic differentiation (Ahmadi et al., 2022; Thomas & Jaganathan, 2022). Besides, several other studies also demonstrated that PI3K/AKT, TGF-β/Smad and MAPK/ERK pathways involvement in osteogenic differentiation of cells (Dong et al., 2020; Kim et al., 2022; Li et al., 2021; Ye et al., 2019; Yu et al., 2017; Zhang et al., 2020; Zhang et al., 2019; Zhang et al., 2019; Zheng et al., 2022). Hence, PI3K/AKT, TGF-β/Smad and MAPK/ERK are targeted in the current study to induce osteogenic differentiation of DPSC by Col-1 scaffold.

2.7.1 Important signalling molecules in bone tissue repair

Determination of osteoblast lineage from MSC precursor is heavily influenced by the locally produced molecules such as transcription factors, growth factors and