# ELUCIDATION OF INTERLEUKIN-17A IN REGULATING OSTEOGENIC DIFFERENTIATION OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

**by**

**ALPHY ALPHONSA SEBASTIAN**

**Thesis submitted in fulfilment of the requirements**

**for the degree of**

**Doctor of Philosophy**

**January 2018**

## ACKNOWLEDGEMENT

I am extremely grateful to many who have supported and guided me all through this endeavor. First and foremost, my heartfelt thanks to God Almighty who has chosen me for this PhD program and bestowed me with blessings to ensure a smooth journey all through. I treasure my experience of doing research work at Universiti Sains Malaysia which has helped me imbibe multitude of skills and ideas. I have learnt throughout the journey that determination, continuous moral support and blessings can lead us to any extent.

My special thanks to my supervisor, Dr. Nurul Asma Abdullah who has been both my guide, role model and friend. I stand with a thankful heart for her proper guidance, innovative ideas, moral support, amiable behavior and being an icon of inspiration. My sincere thanks to my co- supervisor Assoc. Prof. Dr. Thirumulu Ponnuraj Kannan who has always been the perfect person to look up to. Their expertise, experience, guidance and advice in the research arena highly motivated me in overcoming hurdles and further strengthened my research orientation. Their supervision has helped me not only in achieving academic success but also in broadening my outlook towards the world. My sincere appreciation to Prof. Dr. Adam Husein for his encouragement, support, advices and wise suggestions throughout the study.

I would like to thank my friends and colleagues Dr. Hasan Subhi Azeez, Siti Nurshazwani, Johnathan Malagobadan, Shaminea Sritharan, Raihaniah Binti Abd Rahman for their guidance and moral support. I am also grateful to Mr. Jamaruddin Mat Asan for his valuable efforts.

My special thanks to my dear husband Nibu Raj Abraham who was with me in all my ups and downs throughout my research, who understood the importance of my work, and have remained my pillar of support. My heartfelt thanks to my beloved uncle, Dr. Cyriac Kottayarikil, my loving father, late mother, late father-in-law, mother-in-law, my brothers and sisters for motivating me during the last 3 years, through their constant support, care, love and prayers due to which I have made it through all the obstacles to reach this point in life.

My sincere thanks also goes to all laboratory technologists, Ms. Asiah Abu Bakar, Ms. Siti Fadilah Abdullah, Mr. Marzuki Yusof, Mr. Mohd Yusof Soon Abdullah, Ms. Eda Sharip, Ms. Khadijah Mohd Ali, Ms. Nora Aziz, Ms. Khairiena, and Mr. Mohamad Hairie Sahabudin of the Craniofacial Laboratory, who guided and helped me in my laboratory work.

My deepest appreciation credited to the Global fellowship from Universiti Sains Malaysia. This research project was financially supported by Fundamental Research Grant Scheme (FRGS) from Ministry of Higher Education, Malaysia (203/PPSG/6171172).

## TABLE OF CONTENTS

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

| | |
|---|---|
| ALP | Alkaline Phosphatase |
| ANOVA | Analysis of Variance |
| APS | Ammonium Persulphate |
| A-MEM | Alpha- Minimum Essential Medium |
| BMMSC | Bone marrow mesenchymal stem cells |
| BMP | Bone Morphogenic Protein |
| BMU | Basic multicellular unit |
| cDNA | Complimentary DNA |
| CFU-F | Colony Forming Unit Fibroblasts |
| c-Jun | Jun Proto-Oncogene |
| CO2 | Carbon Dioxide |
| COL1A1 | Collagen Type 1 Alpha 1 |
| DEPC | Diethyl Pyrocarbonate |
| DPSC | Dental Pulp Stem Cells |
| ECL | Enhanced Chemiluminescent |
| ECM | Extra Cellular Matrix |
| EDTA | Ethylene diamine tetra acetic acid |
| EGF | Epidermal growth factor |
| ELISA | Enzyme-linked immunosorbant assay |
| ERK | Extra cellular signal-regulated kinase |
| et al | et alii- and others |
| FBS | Fetal Bovine Serum |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| hMSC | Human mesenchymal stem cells |

| | |
|---|---|
| HRP | Horse Radish Peroxide |
| IL | Interleukin |
| JNK | c-Jun NH2-terminal kinases |
| kD | Kilo dalton |
| MAP2K | Mitogen-activated protein kinase kinase |
| MAP3K | Mitogen-activated protein kinase kinase kinase |
| MAP4K | Mitogen-activated protein kinase kinase kinase kinase |
| MAPK | Mitogen-activated protein kinase |
| MAPKAPK | Mitogen-activated protein kinase-activated protein kinase |
| MKNK1 | MAP kinase interacting serine/threonine kinase 1 |
| miRNA | micro RNA |
| ml | Milliliter |
| MRI | Measurement of mean relative intensity |
| mRNA | Messenger ribonucleic acid |
| MSC | Mesenchymal stem cell |
| ng | Nanogram |
| OCN | Osteocalcin |
| OD | Optical density |
| OM | Osteogenic media |
| OPG | Osteoprotegerin |
| OPN | Osteopontin |
| PBS | Phosphate Buffered saline |
| PCR | Polymerase chain reaction |
| PVDF | Polyvinylidene fluoride |
| qRT-PCR | Quantitative real time polymerase chain reaction |

| | |
|---|---|
| RANK | Receptor activator of nuclear factor kappa-B |
| RANKL | Receptor activator of nuclear factor kappa-B ligand |
| rBMP | Recombinant bone morphogenic protein |
| rIL-17A | Recombinant interleukin 17A |
| RIPA | Radio Immunopreceipitation assay |
| rpm | Revolutions per minute |
| RT | Reverse Transcriptase |
| Runx2 | Runt-related transcription factor 2 |
| SDS | Sodium dodecyl sulphate |
| SHED | Stem cells derived from exfoliated deciduous teeth |
| TEMED | Tetramethylethylenediamine |
| Th 17 | T helper 17 |
| α | Alpha |
| β | Beta |
| γ | Gamma |
| µg | Microgram |
| µl | Microliter |

# PENJELASAN INTERLEUKIN-17A DALAM PENGAWALATURAN PEMBEZAAN OSTEOGENIK SEL STEM DARIPADA GIGI SUSU MANUSIA YANG TERLUPAS

## ABSTRAK

Sehingga kini, data yang menunjukkan penglibatan IL-17A di dalam pembentukan semula tulang amat terhad. Pengaktif reseptor ligand NF-κB (RANKL) telah menunjukkan peningkatan dalam penyerapan tulang manakala osteoprotegerin (OPG) pula menyokong osteogenesis. Oleh itu, tujuan kajian ini dilakukan adalah untuk mengenal pasti potensi peranan IL-17A dalam mengawal selia proliferasi, pembezaan osteogenik dan sistem OPG / RANKL sel-sel stem dari gigi susu manusia yang terlupas (SHED). SHED telah dikultur di dalam dua keadaan yang berbeza; di dalam media alfa minimum essential (α-MEM) dan di dalam α- MEM yang ditambah dengan reagen osteogenik (OM). Kedua-dua kumpulan ini dirawat dengan kepekatan rIL-17A yang berbeza seperti 5, 10, 25, 50 dan 100ng/ml. Sel yang dirawat kemudian dianalisa untuk aktiviti proliferasi menggunakan asai MTS dan aktiviti pembezaan menggunakan asai alkalin fosfatase, aktiviti mineralisasi menggunakan Alizarin merah dan Von Kossa dan ekspresi penanda tulang dan sel stem serta apoptosis menggunakan analisis 'flow cytometry'. Potensi pembezaan osteogenik seterusnya dinilai dengan mengukur penanda osteogenik yang terpilih seperti ALP,COL1A1, RUNX2, OCN, OPN, OPG, and RANKL menggunakan 'real time PCR' dan 'Western blot'. Penglibatan laluan pengisyaratan MAPK dan miRNA-145 dalam mekanisme pembezaan osteogenik oleh SHED yang dirawat dengan rIL-17A turut dinilai dalam kajian ini. Rawatan rIL-17A ke atas SHED dan SHED+OM menyebabkan peningkatan proliferasi dan aktiviti ALP yang dipengaruhi oleh dos yang digunakan. Samajuga, pewarnaan Alizarin merah dan Von Kossa

menunjukkan peningkatan aktiviti mineralisasi oleh rawatan rIL-17A di dalam kedua-dua sel SHED dan SHED+OM Menariknya, sel-sel yang dirawat dengan rIL-17A menunjukkan penurunan penanda sel stem (c-Myc dan Nanog) secara beransur-ansur di dalam kedua-dua sel sementara peningkatan ekspresi penanda osteogenik (ALP dan COL1A1) telah dibuktikan seiring dengan masa. SHED yang dirawat dengan rIL-17A juga berkemampuan untuk membantutkan apoptosis dalam kedua-dua kumpulan. Tambahan lagi ekspresi ALP, COL1A1, RUNX2, OCN, OPN dan OPG telah dikawalnaik dengan signifikannya di dalam SHED yang dirawat dengan rIL-17A dalam kedua-dua kumpulan. Walau bagaimanapun, ekspresi RANKL telah dikawalturun. Yang menariknya lagi, nisbah OPG/RANKL telah meningkat dengan signifikan dalam kumpulan yang dirawat dengan rIL-17A. Kami juga telah menunjukkan laluan pengisyaratan MAPK yang diaktifkan oleh IL-17A melalui kawalnaik yang signifikan semua pengaktif huluan dan sasaran hiliran ERK, P38 dan laluan JNK. Sebagai tambahan, SHED yang dirawat dengan rIL17A turut menunjukkan peningkatan dalam ekspresi miR-145. Buat pertama kalinya, penemuan ini menunjukkan rIL-17A menggalakkan proliferasi dan meningkatkan osteogenesis dengan menggalakkan pembezaan osteogenik dengan mengubah laluan isyarat OPG/RANKL dan meningkatkan aktiviti mineralisasi. Kajian ini juga menunjukkan kemungkinan peranan laluan pengisyaratan MAPK dan miR-145 dalam pembezaan osteogenik oleh SHED yang diaruh oleh IL-17A. Penemuan ini sekaligus mencadangkan peranan penting IL-17A dalam mekanisme pembentukan semula tulang.

# ELUCIDATION OF INTERLEUKIN-17A IN REGULATING OSTEOGENIC DIFFERENTIATION OF STEM CELLS FROM HUMAN EXFOLIATED DECIDUOUS TEETH

## ABSTRACT

To date, limited studies describe the involvement of interleukin-17A(IL-17A) in bone remodelling. Receptor activator of NF-κB ligand (RANKL) has been shown to augment bone resorption whereas osteoprotegerin (OPG) favours osteogenesis. Hence, the aim of the present study was to determine the potential role of IL-17A in osteogenic differentiation and regulation of OPG/RANKL system of the stem cells extracted from human exfoliated deciduous teeth (SHED). SHED were cultured in 2 different conditions; one in complete alpha minimum essential medium (α-MEM) and the other in complete α-MEM medium supplemented with osteogenic medium (OM). Both groups were treated with various concentrations of rIL-17A i.e. 5, 10, 25, 50 and 100ng/ml respectively. Treated cells were then analyzed for proliferative activity by MTS assay, differentiation by alkaline phosphatase assay, mineralization activity by Alizarin red and Von Kossa stainings, expressions of bone and stem cell markers and apoptosis by flow cytometry analysis. Osteogenic differentiation potential of SHED was further evaluated by measuring the expressions of selected osteogenic markers i.e. ALP, COL1A1, RUNX2, OCN, OPN, OPG and RANKL by quantitative PCR and Western blot. The involvement of MAP kinase (MAPK) signalling pathway and miRNA-145 in the osteogenic differentiation mechanism of rIL-17A-treated SHED were also evaluated in the study. Treatment of rIL-17A on SHED and SHED + OM demonstrated increased proliferation and ALP activities in a dose-dependent manner. Similarly, stainings by Alizarin red and Von

Kossa indicated increased mineralization activity by rIL-17A-treated SHED and SHED + OM. Interestingly, treatments of cells with rIL-17A showed gradual declining of stem cells markers (c-Myc and Nanog) in both groups while increased expression of osteogenic markers (ALP and COL1A1) was evident as the days progressed. SHED-treated with rIL-17A also inhibited apoptosis in both groups. Moreover, the expressions of ALP, COL1A1, RUNX2, OCN, OPN and OPG were significantly up-regulated in rIL-17A-treated SHED in both groups. However, the RANKL expression was downregulated. Interestingly, the OPG/RANKL ratio was significantly enhanced in rIL-17A-treated groups. We also demonstrated that IL-17A activated MAPK signalling pathway by significant upregulation of all upstream activators and downstream targets of ERK, p38 and JNK pathway. In addition, rIL-17A-SHED also showed increased expression of miR-145. These findings demonstrated for the first time that IL-17A promotes proliferation, enhances osteogenesis by promoting osteogenic differentiation through the alteration of OPG/RANKL signalling pathway and increases mineralization activity. We also demonstrated possible roles of MAPK signalling pathway and miR-145 in osteogenic differerentiation of SHED induced by IL-17A. These findings suggest the pivotal role played by IL-17A in bone remodelling mechanism.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Background of the study

Bone serves as the basic foundation of the body and performs various functions such as providing fortification for internal organs, supporting muscles, producing red and white blood cells apart from acting as a warehouse for minerals (Kanczler *et al.*, 2008). Therefore, this construct is designed such that it constantly undergoes "remodelling" mechanism to sustain its original strength and structural integrity. It is regulated by two opposing forces, namely osteoblast activity (bone formation) and osteoclast activity (bone resorption) (Mulari *et al*., 2004). Osteoblasts influence the initiation and mineralization of bone at early stage of development followed by bone remodelling. Osteoblasts also modulate osteoclast differentiation and resorption (Neve *et al*., 2011). Osteoclasts are large, multinucleated cells that are specifically adapted to remove mineralized bone matrix.

Receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG) are the two regulatory factors in the metabolism of bone architecture (Alkady *et al*., 2011). The discovery of the RANKL/RANK (receptor activator of nuclear factor kappa-β)/OPG system and its role in the regulation of bone resorption helped in identifying factors that regulate cell function. In normal bone remodelling and diverse pathologic conditions, RANKL/RANK signalling controls the formation of multinucleated osteoclasts from their precursors as well as their activation and survival (Kawashima *et al*., 2007). On the other hand, the OPG protects the skeleton from excessive bone resorption by binding to RANKL and inhibiting it from binding to its receptor, RANK (Saidak and Marie, 2012). Thus, RANKL/OPG system

appears to be vital for controlling the bone remodelling process and is an important determinant of bone mass and skeletal integrity (Whyte *et al.*, 2013).

Stem cells are undifferentiated cells which have the potential to differentiate into specialized cells. Although, bone marrow-derived mesenchymal stem cells (BMMSCs) which have good osteogenic differentiation potential are a source of seed cells, procuring BMMSCs involve excessive trauma and high expense (Li *et al*., 2011). Dental stem cells are an easily available source of stem cells which have the capability for self-renewal and the capacity to differentiate into multiple lineages (Sedgley and Botero, 2012). Dental stem cells can be isolated from several soft tissues of the tooth located at different parts of the mouth. These include stem cells from pulp tissue which were isolated from permanent tooth (Gronthos *et al.*, 2000) and stem cells isolated from human exfoliated deciduous teeth (SHED) (Miura *et al.*, 2003). Dental pulp stem cells (DPSCs) have the capability to differentiate into osteoblasts and form bone-like tissues, and further are devoid of the various disadvantages associated with BMMSCs, thus making it the most suitable option for bone regeneration.

SHED stand leaps ahead of both DPSCs and BMMSCs, in their faster rate of proliferation, higher cell population doublings, easy access, absence of tissue destruction at donor site, lesser pain, multi-lineage differentiation and capacity for self-renewal (Jo *et al*., 2007). SHED also demonstrated osteoinductive capacity in vivo, which was absent in DPSCs. SHED also readily form bone/dentin in single colony-derived SHED clones when injected to immunodeficient mice (Yamada *et al.*, 2010). Exfoliated deciduous tooth is identical in some aspects to an umbilical

cord containing stem cells, thus offering an alternate but unique stem-cell resource for various potential clinical applications.

A feature common to both SHED and BMMSCs was their osteogenic differentiation capacity (Yamaza *et al.*, 2010). A previous study demonstrated that SHED transplanted *in vivo,* became an ideal source for osteoblasts and mineralized tissues to form bone (Laino *et al.*, 2006). The repairing capability of SHED was displayed in a study by Seo *et al*. (2008) wherein the calvarial defects in mice were replaced with substantial bone formation.

The interleukin-17 (IL-17) cytokine family is a group of proinflammatory cytokines consisting six members: IL-17A to IL-17F (Kolls and Linden, 2004; Kawaguchi *et al.*, 2004). Cluster of differentiation (CD) $4^+$ memory T cells, $CD8^+$ memory T cells, neutrophils, and eosinophils are the major cellular sources of IL-17 cytokines (Ferretti *et al.*, 2003). Added to this, Th17 cells have also been recently identified as a novel subset of $CD4^+$ effector T cells that produce IL-17A and IL-17F (Langrish *et al.*, 2005).

IL-17 group of cytokines perform various functions. IL-17A and IL-17F manage tissue inflammation by triggering the release of other proinflammatory cytokines and inducing neutrophil chemotaxis (Kolls and Linden, 2004). Moreover, IL-17 has been associated with different human ailments such as systemic lupus erythematosus, asthma, rheumatoid arthritis (RA), and allograft rejection (Langrish *et al.*, 2005). The high levels of IL-17 serves as an indicator of the disease in RA patients (Kawashima *et al.*, 2007).

A study by Huang *et al.* (2009) revealed the role of IL-17A in the bone microenvironment. Apart from stimulating the proliferation of human BMMSCs (hBMMSCs), IL-17 also induced the expression of macrophage colony-stimulating factor (M-CSF) and RANKL on human mesenchymal stem cells (hMSCs), thereby stimulating osteoclastogenesis, both *in vitro* co-culture and *in vivo* implant experiments. According to Ma *et al*. (2015), IL-17 hindered the mineralized deposition induced by MSC-hBMMSC and MSC-SHED. A study by Osta *et al*. (2014) proved that the complex interaction between IL-17A and tumor necrosis factor (TNF) - α can trigger osteogenic differentiation of hMSCs. Even though studies have upheld the role of IL-17 in the regulation of the hMSC function towards the maintenance of bone homeostasis, the other functional roles of IL-17A on bone regulation, including RANKL/RANK/OPG system, still remains to be thoroughly explored.

**1.2 Statement of the problem**

Numerous studies have confirmed the favourable biological functions of IL-17A and its ability to induce hard tissue formation. The use of IL-17A in osteogenic differentiation of SHED and its clinical application has not been discussed elsewhere. In addition, the overall mechanism of RANKL/RANK/OPG in osteogenic differentiation of SHED induced by IL-17A has not been studied so far. The aim of the present study is to understand the role of IL-17A in modulating the RANKL/RANK/OPG system of SHED cultured in two different conditions. This study will lead to a better understanding of the roles of IL-17A in the mechanism of bone remodelling, and perhaps will pave the way for future development of a more

conducive environment for bone tissue engineering as well as provide related information regarding pathogenesis of bone diseases.

### 1.3 Justification of study

Bone remodelling is a continuous process of bone resorption and formation for the purpose of maintaining normal bone mass. Remodelling is a complex, tightly regulated process carried out by two key cell types: osteoclasts and osteoblasts. An imbalance in the bone remodelling process, favouring either osteoclast or osteoblast activity, leads to a number of clinical disease conditions eg: osteoporosis and RA. However, the exact mechanisms responsible for normal bone remodelling are still largely unclear.

The role of IL-17A in MSCs has been studied. The study states that IL-17A stimulates the proliferation and differentiation of hMSCs and enhances bone remodelling by regulating massive bone turn over. But the role of IL-17A in bone remodelling of SHED have not been studied so far.

In addition, bone regenerative medicine is currently a treatment modality that is continuously developed. SHED were reported to have similarity in their osteogenic differentiation capacity with BMMSCs, thereby indicating its potential of becoming a future bone regenerative agent. Therefore, understanding the cellular and molecular mechanisms involving SHED in bone remodelling will possibly provide important insights for therapeutic and tissue engineering developments.

## 1.4 Research objectives

### 1.4.1 General objective:

To elucidate the role of IL-17A in osteogenic differentiation of SHED cultured in two culture conditions.

### 1.4.2 Specific objectives:

1. To determine the effect of IL-17A on proliferation, differentiation and mineralization activities of SHED.
2. To investigate the effect of IL-17A on cell death and apoptosis activities of SHED.
3. To quantify the effect of IL-17A on osteogenic gene expression levels of SHED by quantitative PCR.
4. To verify the effect of IL-17A on the mitogen - activated protein kinase (MAPK) signalling pathway in SHED using the RT² Profiler™ PCR Array.
5. To quantify the effect of IL-17A on osteogenic proteins expression of SHED by Western blotting.
6. To quantify the effect of IL-17A on miR-145 expression level of SHED by quantitative PCR.

## 1.5 Research questions

1. What is the effect of IL-17A on differentiation activity of SHED?
2. What is the effect of IL-17A on mineralization activity of SHED?
3. What are the effects of IL-17A on cell death and apoptosis activities of SHED?
4. What are the roles of IL-17A on osteogenic markers expression of SHED?
5. Does IL-17A modulate RANKL-RANK-OPG in SHED?

6. Does IL-17A promote bone remodelling mechanism in SHED?

### 1.6 Research hypothesis

IL-17A promotes osteogenic differentiation by modulating RANK/RANKL/OPG system of SHED cultured in two culture conditions.

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1 Bone

Bones are the building blocks of the skeletal framework, which further acts as the basic structure on which the human body is constructed. Apart from providing structural integrity and thus regulating bodily movement, bone acts as a storage house for minerals and fats like triglycerides. It plays a key role in mineral hemostasis (calcium and phosphorus). The internal organs find a protective barrier in the skeletal framework. Bone also participates in the endocrine regulation of energy metabolism. Production of blood cells (haematopoiesis) is done by bone marrow, which is an integral part of the bones (Peel, 2009). Thus, bones are a boon to humans in multiple ways.

The major activities associated with bones, apart from longitudinal and radial growth, are modelling and remodelling. The primary mechanism of bone growth, both longitudinal and radial, which mostly occurs during childhood and adolescence (Clarke, 2008), is followed by mineralization resulting in new bone formation. Bone modelling occurs in response to physiological or mechanical forces, like damages and aging, leading to overall changes in the shape and structure of bones (Peel, 2009; Crockett *et al.*, 2011). Bone is constructed in such a manner that it is constantly subjected to "remodelling" mechanism. This is definitely to preserve its structural integrity and strength. Osteoblasts and osteoclasts, the two contrasting forces, regulate remodelling in the bone microenvironment (Mulari *et al.*, 2004). Osteoblasts apart from being key regulators in osteoclast differentiation and resorption, also play

a prominent role in the initiation, early stage bone mineralization and bone remodelling (Neve *et al.*, 2011).

### 2.2 Bone remodelling

Remodelling is a major bone regulatory mechanism, which occurs mostly during adulthood. The bone homeostasis is maintained by two processes namely bone formation and resorption. In detail, bone remodelling involves basically the breaking down of the old bones followed by deposition of the inorganic matrix and further apposition of the proteinaceous matrix (Peel, 2009). This continuous mechanism, mainly focused on cortical and trabecular sites, controls cellular activity through the life-span of the bone (Ralston, 2005; Kazama *et al.*, 2010; Crockett *et al.*, 2011; Sims and Martin, 2014). Bone remodelling revolves around two fundamental cells, namely, osteoblasts which are induced by mesenchymal cells and osteoclasts, induced by hemopoietic stem cells (HSCs). They are closely interconnected by regulatory proteins which help in the formation of new bone and the resorption of old bone (Rucci, 2008; Shahnazari *et al.*, 2013).

According to Bellido *et al.* (2014), bone remodelling is the balance of resorption and formation. In the remodelling mechanism, the coupling process of osteoblasts and osteoclasts occurs at definite sites, called basic multicellular units (BMU). The BMU in cortical and trabecular bone differs morphologically, although the mechanism is the same. The larger surface area of the trabecular bone helps in prompt remodelling than the cortical bone (Hadjidakis and Androulakis, 2006). The precursors of remodelling are supplied and distributed by bone marrow and bloodstream (Sims and Martin, 2014). Osteoprogenitor synthesizes osteoblasts

which further makes up the lining cells, of which some are stored as osteocytes (Downey and Siegel, 2006). The active osteoclast is characterized by its ruffled border with deep plasma membrane folds where the active bone resorption takes place (Andrades *et al.*, 2013). The different phases in bone remodelling are as shown in Figure 2.1.

### 2.2.1 Osteoblasts: formation and function

The cuboidal osteoblasts, originating from osteoprogenitor MSCs, are the key players in bone formation (Caetano-Lopes *et al.*, 2007). The process of osteoblastogenesis from mesenchymal progenitors to a mature bone forming cell involves 3 stages. First stage is commenced by the upregulation of chondrocyte master transcription factor sex-determining region Y (SRY)-box 9 (SOX9) in the mesenchymal progenitors (Akiyama *et al*., 2005). Thereafter, the osteoblast master transcription factor runt-related transcription factor 2 (RUNX2) transforms the proliferating precursor cells to the osteoblast lineage resulting in the formation of osteochondroprogenitors. Subsequently SOX9 is downregulated and thereby leads to the formation of RUNX2-positive osteoprogenitors (Robling *et al*., 2006). At this stage, Osterix (OSX), a second osteoblast master transcription factor mediates the expression of collagen type 1 (COL1) and alkaline phosphatase (ALP) (Nishio *et al*., 2006). This leads to the formation of preosteoblasts also known as immature osteoblasts. Additionally, the transcription factor activating transcription factor -4 (ATF-4) also plays a key role in osteoblast differentiation. Hence, besides the transcription factors, certain genes are essential for the activation of pre-osteoblast proliferation (eg: c-Fos, c-Jun and c-Myc) and cell cycle progression (eg: histones and cyclines) are expressed along with the genes of the growth factors such as

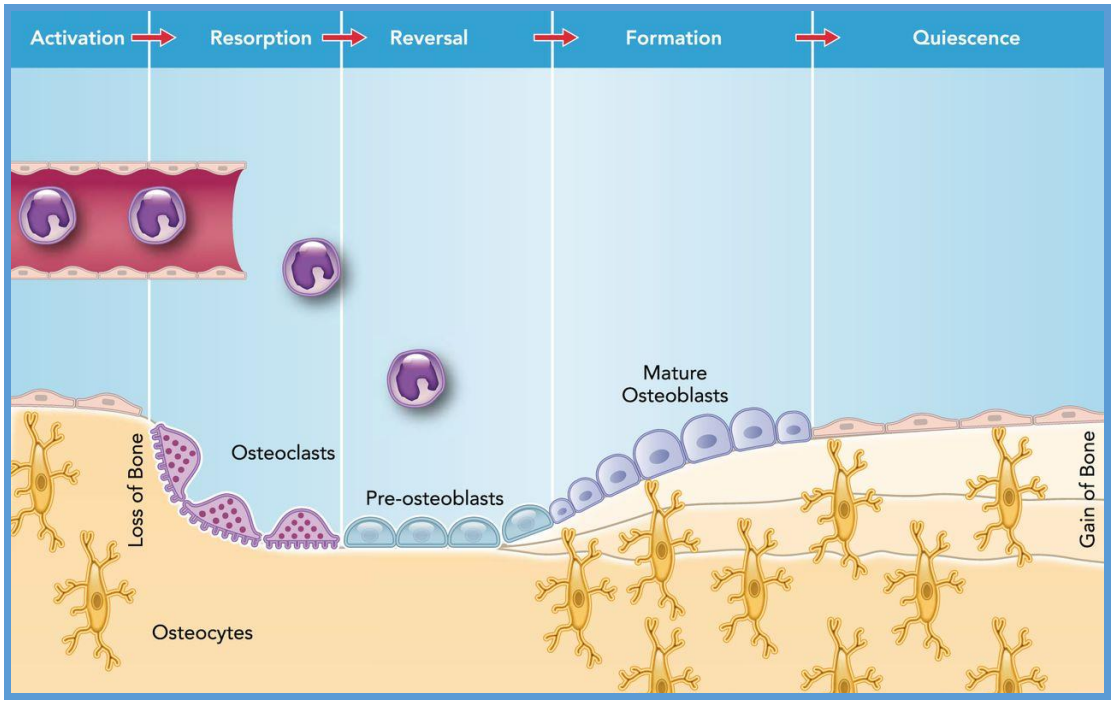


**Figure 2.1:** Phases of bone remodelling (adapted from Siddiqui and Partridge, 2016).

fibroblast growth factor (FGF), insulin-like growth factor 1 (IGF-1), transforming growth factor (TGF), bone morphogenetic protein (BMP), cell adhesion proteins and COL1 (Feldman, 2013). The second stage starts with the maturation and organization of the bone extracellular matrix (ECM) following the proliferatory phase. This stage is characterized by the continuation of collagen synthesis and cross-link maturation. Gene responsible for this phase is ALP (Feldman, 2013). Under alkaline conditions, the crucial role of ALP in bone calcification is characterized by hydrolysis of ester bonds in organic phosphate compounds. This suggests that high ALP activity corresponds to ECM formation before moving to the mineralization phase (Zhu *et al*., 2010; Costa-Pinto *et al*., 2011).

The third stage is characterized by the downregulation of the genes that aid in proliferation as well as upregulation of genes involved in mineralization phase. During mineralization phase, maximal expression of genes encoding the non-collagenous proteins such as osteocalcin (OCN), osteopontin (OPN) and bone sialoprotein (BSP) occurs (Feldman, 2013). In the final phase, collagenases are elevated and 50-70% of osteoblasts undergo apoptotic activity (Clarke, 2008; Feldman, 2013).

Osteoblasts regulate the development, maintenance and repair of bone thereby regulating the bone remodelling process (Tanaka *et al.*, 2005). The other functions performed by osteoblasts are osteoclast differentiation and expansion of HSCs. The bone formation is characterized by the synthesis of ECM proteins such as COL1, OCN, OPN and BSP on which osteoid is deposited (Neve *et al.*, 2011; Kini and Nandeesh, 2012). Osteoblasts generate increasing levels of bone specific ALP

mainly on the outer membrane, which catalyzes mineralization (Golub and Boesze-Battaglia, 2007). Osteoblasts secrete these proteins into the blood which act as signals for bone formation (Harimoto *et al.*, 2012).

Apart from regulating the flow of calcium to and from the bone, the osteoblast cells which form the ECM of bone, act as a warehouse for a variety of growth factors. Also, to be noted is the structural integrity and strength provided by these cells to the bone (Long, 2012). They also signal peculiar proteins which induces the osteoclasts (Calabrese *et al.*, 2012). Moreover, the non-collagenous proteins and COL1 secreted by the osteoblasts control bone mineralization (Andrades *et al.*, 2013). The major functions of osteoblast are pictorially depicted in Figure 2.2.

### 2.2.2 Osteoclasts: formation and function

Osteoclasts are large multi-nucleated cells formed by the fusion of the precursors of the monocyte macrophage lineage originated from hematopoietic cells (Crockett *et al*., 2011). RANKL plays a pivotal role in osteoclast formation whereas M-CSF is essential for the proliferation, survival and differentiation of osteoclast precursors (Boyce and Xing, 2008). Medical science suggests that most of the bone ailments in humans are characterised by a process known as bone resorption. An inflammation always transmogrifies resorption of bone usually through three important processes. Primarily, the pro-inflammatory cytokines modulate the osteoclast function by means of RANK and its functional ligand (RANKL) also known as TNF related activation induced cytokine (TRANCE).

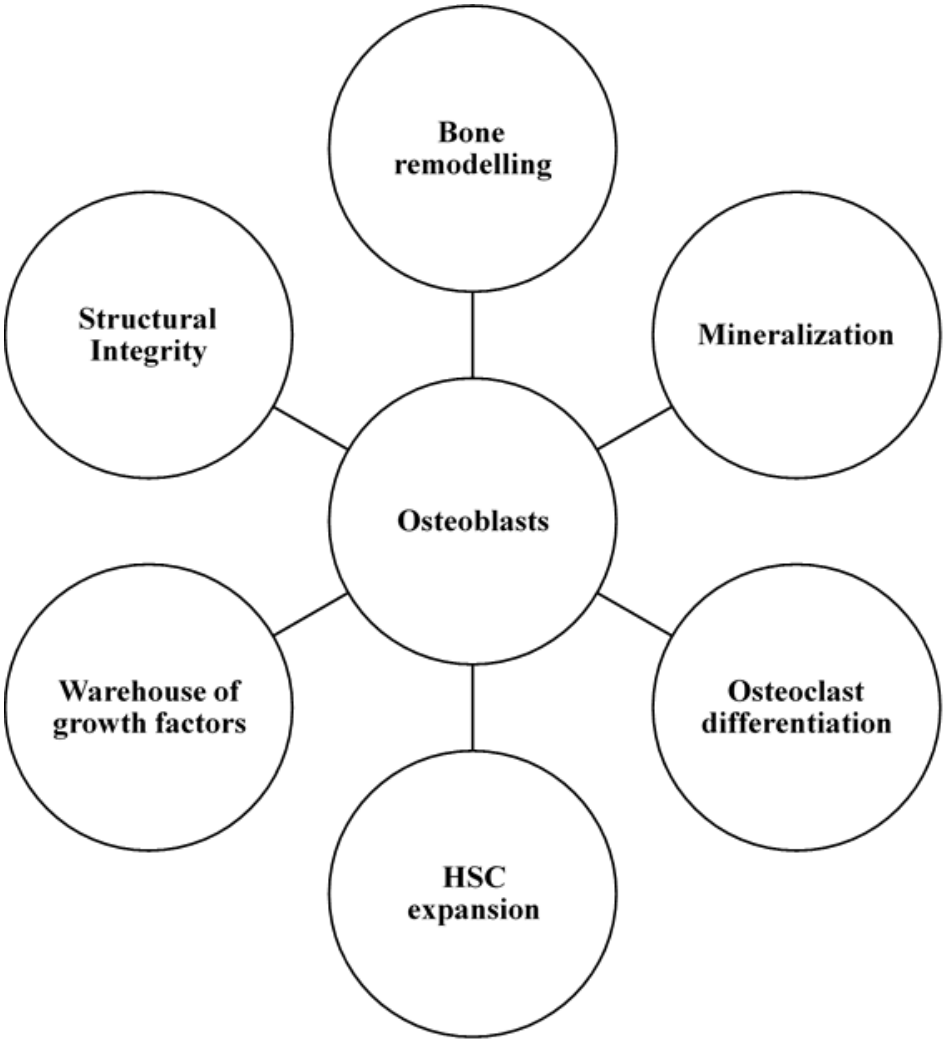


**Figure 2.2**: Functions of osteoblasts. Abbreviation: HSC; Haemopoetic Stem Cells (Adapted from Calabrese *et al*., 2012; Harimoto *et al*., 2012; Long, 2012).

Secondly, it happens through the crucial role of M-CSF and, thirdly, through transcription factor, PU.1. M-CSF passes its osteoclastogenic signals to the cell through the stimulating factor receptor, c-fms. They further stimulate the expression of RANK in monocyte-macrophage precursor cells. The transcription factor PU.1 actually regulates the development of osteoclasts by controlling the expression of c-fms (Raggat and Patridige, 2010). The major pathways of osteoclast differentiation are pictorially depicted in Figure 2.3.

In one of these pathways, the RANKL/RANK signalling cascade induces osteoclastogenesis, which is commenced by the activation of TNF receptor-associated factor 6 (TRAF6), described by the pioneers Armstrong *et al*. (2002). The two most common downstream mediators in this pathway are nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κB) and activated protein 1(AP-1). Several co-stimulatory molecules are present in the other pathway such as osteoclast associated receptor (OSCAR), paired immunoglobulin-like receptor A (PIR-A), signal-regulatory protein β (SIRPβ) and triggering receptor expressed on myeloid cells (TREM2) which are essential for osteoclast activation. They initiate calcium ions (Ca2+) signalling cascade through immunoreceptor tyrosine-based activation motifs (ITAM) phosphorylation. The Ca2+ signalling cascade along with the action of transcription factors such as NF-κB and c-fos result in the auto amplification of nuclear factor of activated T cells cytoplasmic1 (NFATc1), which is the master regulator of osteoclastogenesis (Koga *et al*., 2004; Aliprantis *et al*., 2008).

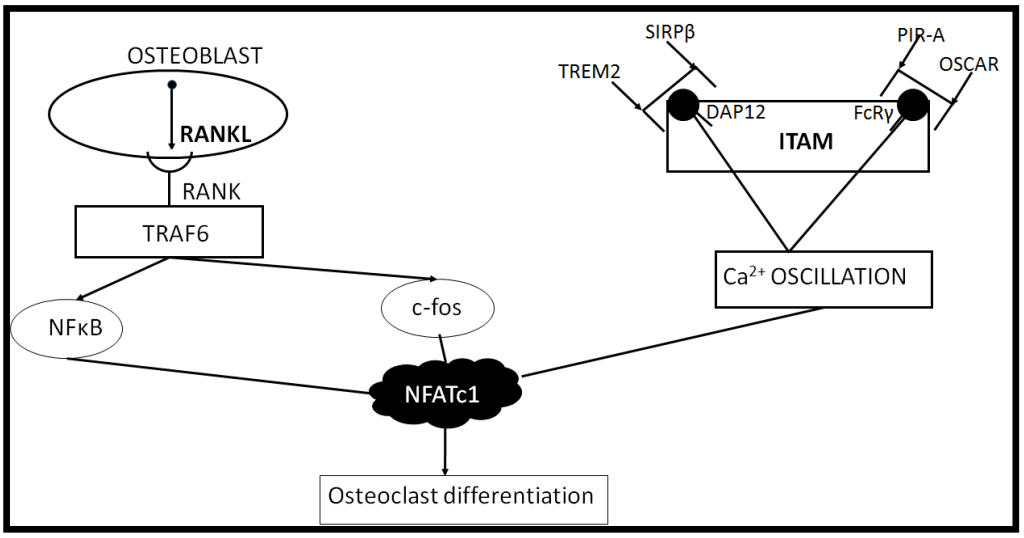


**Figure 2.3**: Major pathways of osteoclast differentiation. Footnotes: RANKL, Receptor activator of nuclear factor-κB ligand; RANK, receptor activator of nuclear factor kappa-β; TRAF6, TNF receptor-associated factor 6; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; NFATc1, nuclear factor of activated T cells cytoplasmic1; TREM2, triggering receptor expressed on myeloid cells; SIRPβ, signal-regulatory protein β; PIR-A, paired Ig-like receptor A, OSCAR, osteoclast associated receptor; ITAM, immunoreceptor tyrosine-based activation motif (Adapted from Yamashita *et al*., 2012)

Activated osteoclasts generate an acidic microenvironment in the active bone resorption site via secretion of hydrogen ions that dissolve the bone minerals. Additionally, several proteases and hydrolases degrade the organic matrix resulting in the destruction of its main constituent, COL1 (Novack and Teitelbaum, 2008).

### 2.2.3 Osteocytes: formation and function

Osteocytes constitute 95% of all bone cells. These cells are terminally differentiated osteoblasts formed when osteoblasts get implanted in the matrix. Prompt spurts of bone calcium across filipodial gap junctions accelerate communication between osteoblasts on the bone surface and osteocytes inside the bone (Dallas and Bonewald, 2010; Plotkin, 2011). During its implantation in the bone matrix, osteoblasts totally change their morphology into a stellar shape with 50 or more thin extensions known as osteocyte processes. During this transformation the osteoblast cells lose over 70% of cell organelles and cytoplasm (Rochefort *et al*., 2010). The resulting osteocyte network provides microporosity in the mineralised bone. Osteocyte bodies are contained within spaces referred to as lacunae, whereas their connected processes are contained within channels (termed canaliculi) – together they make up the lacunar–canalicular network.

Osteocytes surveil mechanical stress (i.e. pressures or cracks) in the bone and further generate prostaglandins. Thus, they are mechanosensory cells belonging to the osteoblastic family that play a pivotal role in the regulation and adaptation of bone (Calabrese *et al.*, 2012; Link, 2013). They initiate osteoclasts into dissolving the bone (Xia *et al.*, 2010). But it is now evident that they also sense metabolic signals.

(Manolagas and Parfitt, 2010). The osteocytes positively regulate osteoblasts through the production of messengers, such as nitric oxide and prostaglandin E2, and they also negatively regulate osteoblasts through secretion of sclerostin (Rochefort *et al*., 2010).

### 2.2.4 RANK/RANKL/OPG signalling

A close study of bone metabolism reveals the involvement of OPG and RANKL in balancing the whole metabolism (Alkady *et al.*, 2011). The discovery of RANKL/RANK - OPG system can be considered as a quantum leap in the understanding of bone biology. It was first identified in the 1990s. In 1997, Simonet *et al.* discovered OPG as an inhibitor of osteoclastogenesis. It was followed by the discovery of RANKL in 1998 as an inducer of osteoclastogenesis. The interruption in this system is responsible for a disrupted bone homeostasis. The newly found cytokine RANKL is the corner stone in the osteoclast generation and its activity. RANKL, RANK and OPG are pivotal regulators of bone metabolism belonging to TNF receptor and ligand families. RANKL, a type 2 ligand protein of TNF ligand family is an essential activator for osteoclast differentiation. This factor exists as a homotrimeric protein and is expressed as a membrane - bound protein on the surface of osteoblasts, osteocytes, marrow stromal cells, activated T cells, and B-cells. M-CSF, along with most osteotropic factors such as IL-1, IL-11, prostaglandin E2 and 1,25-(OH)2D3, induces osteoclast formation by binding to marrow stromal cells, which in turn express increased levels of soluble or membrane forms of RANKL (Herman *et al*., 2008).

RANK is a heterotrimeric transmembrane protein member of the TNF receptor superfamily that appears to be expressed in osteoclast precursors, mature osteoclasts, dendritic cells, mammary glands and some cancer cells, like breast and prostate cancers. RANKL binds to its receptor RANK, expressed by hematopoietic cells and thereby initiates osteoclast development. This action is indispensable for osteoclast differentiation, function and survival (Kawashima *et al.*, 2007; Papadopouli *et al*., 2008; Trouvin and Goeb, 2010).

OPG which is a secreted member of TNF receptor family, is produced similar to RANKL molecule, by osteoblasts and marrow stromal cells. Its main action is to inhibit osteoclast formation and activity. OPG, the name itself signifies protector of bone. It is the first molecule of the three different key players that can control osteoclast function. Also known as osteoclastogenesis inhibitor factor, it is a soluble protein secreted by osteoblasts, 64% of which is produced by B-cells. OPG production is controlled by cytokines, growth factors, hormones and Wingless protein (Wnt)/beta-catenin. OPG acts as a soluble inhibitor of osteoclast maturation and osteoclast activation *in vitro* and *in vivo*. In the RANKL-RANK-OPG mechanism, OPG binds with RANKL hindering further interaction of RANKL with RANK. This process inhibits the differentiation of preosteoclasts into mature osteoclasts (Figure 2.4) (Saidak and Marie, 2012). Thus, RANKL and OPG appear to be the major determinants in the control of the bone remodelling process (Whyte, 2013).

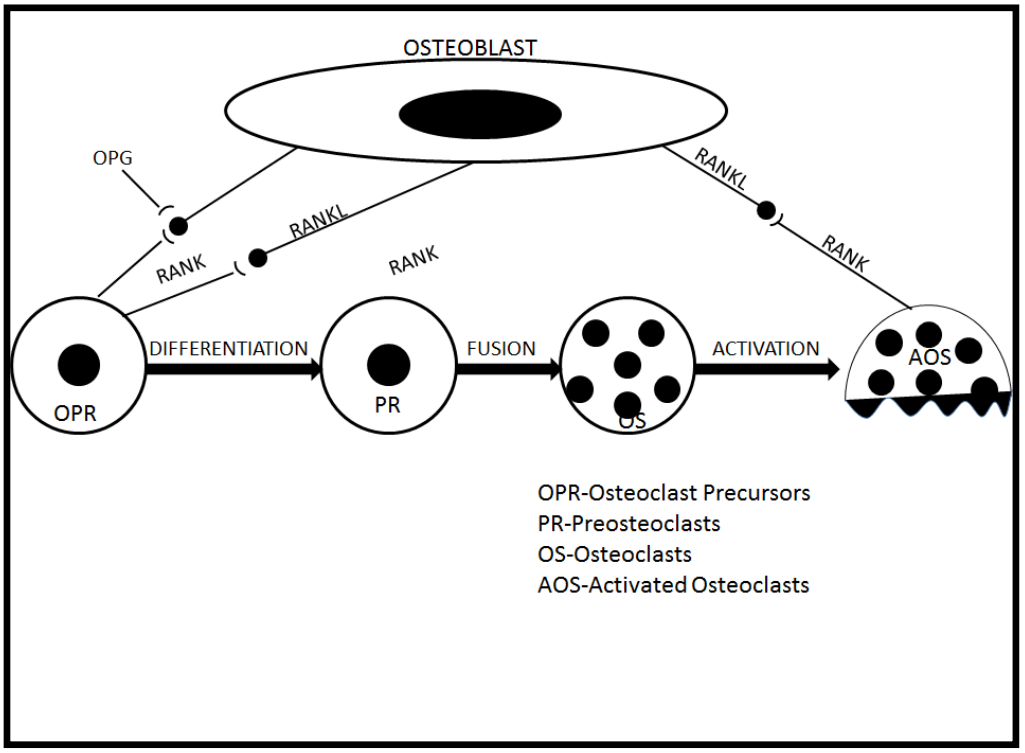


**Figure 2.4:** RANKL-RANK-OPG mechanism (Adapted from Lee, 2010)

OPG-RANKL system acts as a platform to delve into the intricacies of bone remodelling mechanism (Boyce and Xing, 2008). In metabolic bone diseases, variations in the OPG-RANKL system has resulted in severe complications (Hofbauer *et al.*, 2004). Studies have indicated that excessive production of RANKL being inhibited by the high levels of OPG have accelerated bone metastasis *in vivo* (Haynes *et al.*, 2003). OPG/RANKL ratio is a major determinant regarding the performance of adipocyte-secreted factors towards bone formation (Kuhn *et al.*, 2012). Another study have dealt with the effectiveness of RANK/RANKL/OPG system towards measuring the oxidative and anti-oxidative properties of metals in bone activity (Brzoska and Rogalska, 2013).

It has been stated that deviations in the RANK/RANKL/OPG network can result in bone diseases such as RA, cancerous bone metastases (osteolytic metastases) and osteoporosis (Terpos *et al.*, 2005; Wada *et al.*, 2006). Studies have also dealt with the effectiveness of OPG/RANK/RANKL marker levels as an indicator of the presence of bone metastases in breast cancer (Ibrahim *et al.*, 2011). It can be inferred that OPG/RANKL/RANK modulation holds a pivotal role towards understanding bone regulation and inhibiting bone diseases (Boyce and Xing, 2008).

### 2.3 Necessity of bone substitute

Bone substitutes are essential for healing traumatic bone injury through orthopaedic, craniofacial, and dental reconstructions. Bone is the second most commonly transplanted tissue (Shegarfi and Reikeras, 2009)). It is estimated that greater than 200 million people are affected with osteoporosis (WHO, 2015). A report published on National Osteoporosis Foundation website accessed on August 2, 2016 stated the

following facts regarding osteoporosis patients in US. Osteoporosis was the major cause for the approximately 1.5 million fractures occurring annually in the US. Majority of the 10 million Americans suffering from osteoporosis were at immediate risk of hip, spine (vertebra) or wrist (distal radius) fractures. Further, osteoporosis related fractures are presumed to follow an increasing pattern for the upcoming 60 years. In a survey conducted in 27 countries of the European Union (EU27), 22 million women and 5.5 million men were suffering from osteoporosis. Among these patients, 610,000 suffered from hip fractures, 520,000 from vertebral fractures, 560,000 from forearm fractures, and 1,800,000 from other fractures like fractures of the pelvis, rib, humerus, tibia, fibula, clavicle, scapula, sternum, and other femoral fractures, totalling 3.5 million new fractures (Hernlund *et al*., 2013). For this ailment having a low cure rate of 10-15%, one of the most effective treatment modalities is bone graft substitutes (Larsson and Procter, 2011)). Annual global estimates of patients undergoing this treatment modality are approximately 2.2 million (International Osteoporosis Foundation, 2009).

Major treatment modalities for bone tissue loss are autografts, allografts, implantation of substitute materials and more recently, application of recombinant BMPs. Certain limitations are associated with these procedures. Autograft, a mostly redundant procedure, was hindered by the difficulty in procuring healthy tissue, high chance of donor site morbidity, and pain associated with the harvest (Chou *et al*., 2007). Allografts are limited by the decreased biological and mechanical properties after the procedure, high possibility of disease transmission and hostile immunological host response (Reikeras *et al*., 2008), leading to a high rate of failure (Reichert *et al*., 2011). Synthetic materials which account for approximately 10% of

bone substitutes suffer from various limitations, namely, absence of osteogenic or osteoinductive component, toxicity, wear, biodegradability, acute inflammations in the host and chances of rejection due to insufficient biomechanical compatibility (Bueno and Glowacki, 2009).

Due to these drawbacks associated with bone substitutes, there exists an exigent need for substitutes which are osteoinductive, osteogenic, osteoconductive, angioinductive, permit growth of blood vessels, degrade within a certain time period and possess biomechanical stability.

### 2.4 Tissue engineering

Tissue engineering comes as a possible alternative by developing biological substitutes which can restore, preserve or ameliorate tissue function (Holzapfel *et al*., 2012; Jakob *et al*., 2012). The multitude of approaches to bone tissue engineering involve either or all of the following vital components: harvested cells, scaffolding matrix and regulatory/inductive/stimulatory signals to direct osteoblastic differentiation (Bose *et al*., 2012; Frey *et al*., 2012; Yun *et al*; 2012).

#### 2.4.1 Cells for bone tissue engineering

The ideal cell for cell-based tissue engineering should be effortless to acquire and expand, non-immunogenic, genetically modifiable, ethically non-controversial and possesses the capacity to differentiate to the osteogenic lineage. Majority of the cells which are presently studied for bone tissue engineering are multipotent MSCs (Bianco *et al*., 2008). According to Hutton and Grayson, (2014), MSCs are a category of bone marrow cells which possess the capacity to differentiate to bone.

These were also termed as colony-forming unit-fibroblast (CFU-F) because of their ability to form colonies when plated at low density (Bianco *et al*., 2013). In 1991, Caplan had coined the term, MSCs and highlighted their capability to differentiate to all cells of the mesodermal lineage (Caplan, 1994).

Various sources of MSCs have been discovered for the clinical applications over the years (Annibali *et al*., 2014) such as umbilical cord blood (Erices *et al*., 2000), dental pulp (Gronthos *et al*., 2000), SHED (Miura *et al*., 2003), synovial membrane (De Bari *et al*., 2001), adipose tissue (Zuk *et al*., 2002), placenta (In't Anker *et al*., 2004), Wharton's jelly (Wang *et al*., 2004), scalp tissue (Shih *et al*., 2005), umbilical cord perivascular cells (Sarugaser *et al*., 2005), skeletal muscle perivascular cells (Crisan *et al*, 2008), amniotic fluid (Nadri and Soleimani, 2007), and breastmilk (Patki *et al*., 2010).

**2.4.1(a) Bone marrow stromal cells (BMSCs)**

The most commonly used MSCs in bone tissue engineering are BMSCs. Even though BMSCs have cured different bone defects (Neman *et al*., 2012; Geng *et al*., 2013), the extraction of bone marrow is difficult and the yield of CFU-F is low, approximately 1:10,000 at birth diminishing to 1:250,000 in adult marrow (Caplan, 2009). Moreover, the osteogenic capacity of the cells is inversely proportional to the age of the donor (Mueller and Glowacki, 2001). Although BMMSCs were used as the main cell source for bone tissue engineering (Phinney and Prockop, 2007) and showed favourable results when used instead of autogenous bone grafting in clinical trials (Yamada *et al*., 2004; Yamada *et al.*, 2008). The BMMSCs are not used frequently nowadays because of its disadvantages such as invasive harvest of cells,