DIFFERENTIATION AND PROLIFERATION ACTIVITIES OF HUMAN FOETAL OSTEOBLAST CELL LINE (hFOB 1.19) TREATED WITH A POLYPHENOL, TANNIC ACID ALONE OR IN COMBINATION WITH PAMIDRONATE

By

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Dissertation submitted in partial fulfillment of the requirement for the Degree of Master of Science (Biomedicine)

December (2018)

CERTIFICATE

This is to certify that the dissertation entitled “Differentiation and proliferation activities of human foetal osteoblast cell line (hFOB 1.19) treated with a polyphenol, tannic acid alone or in combination with pamidronate” is the bona fide record of research work done by Ms Nor Munira binti Hashim during the period from March 2018 to January 2019 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfillment for the degree of Master of Science (Biomedicine).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	percentage
<	less than
=	equal to
>	more than
°C	degree celcius
µg/ml	microgram per millilitre
$AgNO_3$	silver nitrate solution
ALP	alkaline phosphatase
ANOVA	analysis of variance
ARS	Alizarin Red S
ATCC	American Type Cell Culture
BMD	bone mineral density
BMPs	bone morphogenetic proteins
BMUs	basic multicellular units
BPs	bisphosphonates
BRC	bone remodelling compartment
BSP	bone sialoprotein
Ca^{2+} ions	calcium ions
CaLG	calcium lactate-gluconate
CaSR	calcium-sensing receptor
cDNA	complementary DNA
CI	Combination Index
Col1A1	alpha-1 type I collagen

CT	condensed tannin
dH2O	distilled water
Dlx-5	distal-less homeobox 5
DMEM D12	Dulbecco’s modified eagle F12 medium
DMP	dentin matrix protein
DMP-1	dentin matrix-protein-1
DMSO	dimethyl sulphoxide
DXA	dual X-ray absorptiometry
EC_{50}	half maximal effective concentration
ECM	extracellular matrix
ER	oestrogen receptor
ERK	extracellular signal-regulated kinases
et al.	Et alia: and others
EUR	European Union Regulation
FBS	foetal bovine serum
FGFs	fibroblast growth factors
GtPP	green tea polyphenols
H_2O_2	hydrogen peroxide
hFOB	human foetal osteoblast
HRT	hormone-replacement therapy
HT	hydrolysable tannin
IGF	insulin-like growth factor
IL	interleukin
IV	intravenous
MDA	malondialdehyde

MEPE	matrix extracellular glycophosphoprotein
Mg	milligram
Ml	millilitre
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
MSCs	mesenchymal stem cells
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
$Na_2S_2O_3$	sodium thiosulfate solution
OD	optical density
OS	oxidative stress
Osx	Osterix
PBM	peak bone mass
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
Pi	inorganic phosphate
PPAR-γ	peroxisome proliferator-activated receptor γ
Pre-OBL	pre-Osteoblast
PTH	parathyroid hormone
PTH1R	type 1 parathyroid receptor
QI	*Quercus infectoria*
RA	retinoic acid
RANK	nuclear factor kappa-B ligand
RANKL	nuclear factor kappa-B ligand
RB	netinoblastoma

ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
Runx2	runt related transcription factor-2
SATB2	special AT-rich sequence-binding 2
SERMs	selective oestrogen receptor modulators
STAT1	signal transducer and activator of transcription 1
TA	tannic acid
TAZ	transcriptional co-activator
TNF-α	tumour necrosis factor-α
WHO	World Health Organization
Wnt	wingless-int
ZPF521	zinc-finger protein 521

ABSTRAK

AKTIVITI PEMBEZAAN DAN PROLIFERASI TURUNAN SEL FETAL OSTEOBLAS MANUSIA (hFOB 1.19) YANG DIRAWAT DENGAN POLIFENOL, ASID TANIK SENDIRIAN ATAU GABUNGAN DENGAN PAMIDRONATE

ABSTRAK

Tulang sentiasa melalui proses pembentukan semula tulang dan ketidakseimbangan dalam proses tersebut mampu menyebabkan penyakit tulang, osteoporosis. Asid tanik (TA) adalah polifenol dengan kandungan antioksidan yang mungkin meningkatkan metabolisme tulang. Sinergisme adalah salah satu dari kesan farmakologi yang berlaku apabila gabungan banyak drug digunakan. Oleh itu, kajian ini dijalankan untuk memerhati kesan TA sendirian dan gabungan antara TA dan pamidronate terhadap pembezaan, proliferasi, pemineralan dan juga morfologi sel fetal osteoblast manusia (hFOB 1.19). Sel hFOB 1.19 dirawat dengan TA sendirian dan gabungan antara TA dan pamidronate yang pelbagai pada kepekatan yang berbeza (0.1 hingga 99.0 µg/ml). Kepekatan efektif median (EC_{50}) untuk TA, pamidronate dan nisbah gabungan yang berbeza (25:75, 50:50, 75:25) diukur menggunakan ujian MTT. Analisis kesan sinergi turut dijalankan terhadap ratio kombinasi yang berbeza berdasarkan Indeks Gabungan (CI) di mana CI < 1: sinergisme, CI = 1: kesan tambah *(additive)* and CI > 1: antagonisme. Rawatan gabungan dengan nilai EC_{50} yang paling rendah dan CI<1 dipilih untuk kajian yang selanjutnya. Asai proliferasi sel dijalankan untuk membandingkan bilangan kebolehhidupan sel and morfologi sel hFOB 1.19 diperhatikan menggunakan mikroskop songsang dari hari pertama hingga hari ke-7. Berikutnya, penilaian pemineralan kalsium dan fosfat dilakukan menggunakan kaedah pewarnaan histokimia. Pengesanan gen spesifik tulang [*bone sialoprotein* (BSP)

dan *osterix* (Osx)] dilakukan dengan menggunakan ujian tindak balas rantai polymerase (PCR). EC_{50} untuk sel hFOB 1.19 yang dirawat dengan TA sendirian (0.56 ± 0.22 µg/ml) dan gabungan nisbah 75:25 TA dan pamidronate (0.48 ± 0.03 µg/ml) didapati lebih berkesan berbanding pamidronate sendirian (15.27 ± 0.22 µg/ml) dan gabungan yang lain [(25:75; 3.80 ± 0.13 µg/ml); (50:50; 2.25 ± 0.11 µg/ml)]. Analisis sinergi menunjukkan hanya gabungan 75:25 mempamerkan kesan sinergi manakala gabungan lain menunjukkan kesan antagonis. Kesemua kumpulan menunjukkan perubahan ketara apabila dibandingkan antara hari pertama dan ke-7. Bagaimanapun, semua kumpulan kecuali kumpulan gabungan menunjukkan perubahan yang ketara apabila dibandingkan antara hari ke-3 dan ke-7. Apabila perbandingan dibuat pada hari yang sama, iaitu hari ke-7, kedua-dua kumpulan TA dan pamidronate sendirian menunjukkan peningkatan yang ketara untuk proliferasi sel berbanding kumpulan kawalan (tidak dirawat) dan gabungan. Sel hFOB 1.19 yang dirawat dengan kumpulan TA dan kombinasi mempunyai morfologi yang seragam dan bentuk memanjang hingga akhir kajian. Sel yang dirawat dengan TA sendirian mempunyai pembentukan deposit kalsium dan fosfat berbanding kumpulan kawalan, pamidronate dan gabungan. Kesemua kumpulan menunjukkan ekspresi gen BSP dan Osx sehingga hari ke-7. Kesimpulannya, TA sendirian mempunyai kebolehan meningkatkan proliferasi dan morfologi sel yang lebih baik di samping pemineralan osteoblast berbanding kumpulan gabungan TA dan pamidronate.

DIFFERENTIATION AND PROLIFERATION ACTIVITIES OF HUMAN FOETAL OSTEOBLAST CELL LINE (hFOB 1.19) TREATED WITH A POLYPHENOL, TANNIC ACID ALONE OR IN COMBINATION WITH PAMIDRONATE

ABSTRACT

Bone continuously undergoes bone remodelling and imbalance in bone remodelling leads to bone pathogenesis, such as osteoporosis. Tannic acid (TA) is a polyphenol with antioxidant properties which may elevate osteoblast metabolism. Synergism is one of pharmacological effect that occur when drug combination is used. Hence, the aim of this study was to evaluate the effect of TA alone and combination of TA and pamidronate on differentiation, proliferation, mineralisation as well as morphology of human foetal osteoblast cells (hFOB 1.19). The hFOB 1.19 cells were treated with TA alone and combination of TA and pamidronate of different combination ratios at different concentrations (0.1 to 99.0 µg/ml). Half maximal effective concentration (EC_{50}) for TA alone, pamidronate alone and different combination ratios of TA and pamidronate (25:75, 50:50, 75:25) were measured by using MTT assay. Analysis of synergistic effects was also performed on the different combination ratios based on Combination Index (CI) where CI < 1: synergism, CI = 1: additive effect and CI > 1: antagonism. The combination treatment with the lowest EC_{50} and CI<1 was then chosen for the next studies. Cell proliferation assay was conducted to compare the cell viability and morphology of hFOB 1.19 cells was observed by using inverted microscope after treatment from day 1 to day 7. In addition, detection of calcium and phosphate deposits were done by histochemical staining. Detection of bone specific genes [bone sialoprotein (BSP) and osterix (Osx)] was conducted by polymerase chain reaction (PCR) test. The EC_{50} of hFOB 1.19 cells

treated with TA alone (0.56 ± 0.22 µg/ml) and 75:25 ratio of TA and pamidronate (0.48 ± 0.03 µg/ml) were more effective compared to pamidronate alone (15.27 ± 0.22 µg/ml) and other combination ratios treatments [(25:75; 3.80 ± 0.13 µg/ml); (50:50; 2.25 ± 0.11 µg/ml)]. Analysis of synergistic result showed that only 75:25 combination ratio exhibited synergistic effect while the other two combination ratios manifested antagonistic effects. All groups showed significant different when compared between day 1 and day 7. However, all groups except combination group manifested significant different when comparing between day 3 and 7. When comparing on similar day which was day 7, both TA alone and pamidronate alone treated groups showed significantly increased in cell proliferation compared to control (untreated) and combination groups. The morphology of hFOB 1.19 cells treated with both TA and combination groups had uniform and elongated shape until the end of study. Treatment with TA alone demonstrated higher production of calcium and phosphate deposits compared to untreated control, pamidronate alone and combination groups. All groups manifested the expression of BSP and Osx until day 7 of treatment periods. In conclusion, TA alone rather than combination treatment of TA and pamidronate has greater ability in enhancing cell proliferation and morphology besides improved osteoblast mineralisation.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Bone involves in structural support of the body, locomotion, mineral storage and storage of bone marrow (Nam & Kampa, 2013; Florencio-Silva *et al*., 2015). Bone also constantly undergoes two processes throughout life which are modelling and remodelling. Modelling helps bone adapt to changing biomechanical forces while remodelling helps to remove old and micro-damaged bones to new and mechanically stronger bone which brings to reservation of bone strength. Bone modelling usually occurs during growth where bone becomes widen however it may increase in pathophysiology such as hypoparathyroidism, renal osteodystrophy or patient of anabolic agents. Bone remodelling on the other hand, keeps repeating removed the old and damaged bone from birth until death (Clarke, 2008).

Bone remodelling is important in order to replace the infantile bone to secondary bone which is more stable and competent, remove ischemic or micro-fractured bone and amend calcium homeostasis (Rucci, 2008). There are three main phases in bone remodelling; 1) bone resorption by osteoclasts, 2) the transition from resorption to new bone formation or reversal and 3) the bone formation by osteoblasts. The process is regulated by basic multicellular unit (BMU), a temporary anatomical structure formed of osteoclasts, osteoblasts, osteocytes and bone lining cells. During bone formation, osteoblasts secretes collagenous and non-collagenous proteins such as osteocalcin, osteopontin, osteonectin, bone sialoprotein (BSP) and bone morphogenetic proteins (BMPs) (Florencio-silva *et al.,* 2015). *In vitro* data suggest that BSP may initiate hydroxyapatite crystal formation in the bone matrix (Hunter & Goldberg, 1994). Besides, an active osteoblast also highly secretes osterix (Osx) which is one of the earliest bone formation marker which decreases as osteoblasts becomes osteocytes (Zhang *et al.,* 2011).

The disturbance in bone remodelling process can lead to metabolic bone disease and skeletal system disorder such as osteoporosis. The disturbance is caused by oxidative stress which is a condition of overproduction of reactive oxygen species (ROS). ROS causes apoptosis of osteoblasts and osteocytes which leads to imbalance in bone remodelling process by decrease bone formation and favour bone loss by osteoclasts (Domazetovic *et al.,* 2017).

The World Health Organization (WHO) defines osteoporosis as a metabolic and progressive bone disease manifested by reduced bone mass and altered microarchitecture of the bone which increased risk of fracture (Lau & Guo, 2011; Rao & Rao, 2013; Ferdous *et al.,* 2015). Between the periods of 2010 to 2050, osteoporosis is predicted to become major public health threat which will result in 8.1 million fractures in 78% women and 22% men (Lao & Guo, 2011). This metabolic disease usually occurs in elder people as a "silent thief" as there are no noticeable symptoms until it demonstrates as fragility fracture (Jahanian *et al.,* 2016).

Pamidronate (a type of bisphosphonate) is mainly used in the treatment of osteoporosis by inhibiting bone resorption by osteoclasts during bone remodelling (Daroszewska, 2015). Besides their inhibitory effect towards osteoclasts, pamidronate also enhances production of collagen type I which eventually increased bone density and osteoblast differentiation (Koch *et al.,* 2010). However, bisphosphonate is inadequately absorbed, needs to be taken separately from food and has many side effects such as gastrointestinal problems, pain in muscles or joints and headache. Moreover, other treatment such as oestrogen or hormone therapy may cause vaginal bleeding, gallbladder disease and clots in the veins (Ferdous *et al.,* 2015).

In that regard, community nowadays have shift their interests to natural products such as herbal medicines which are now considered important as they are believed to be

free from harmful chemicals and side effects (Sooi & Keng, 2013). This can be seen in Chinese community as Chinese herbal medicine is used as preventive agents or treatment on early stage of osteoporosis (Che *et al.,* 2016).

Plants have phytochemicals which protect them from microbial infections or damage from pests. The phytochemicals also have medicinal values in maintaining human health and disease treatments (Shakya, 2016). Polyphenols are example of phytochemical that widely found in plants which have many medicinal values such as prevention against cardiovascular diseases, cancer, diabetes and osteoporosis (Rajesh *et al.,* 2016). Polyphenols have antioxidant properties which can prevent osteoporosis by scavenging ROS and down-regulating inflammatory mediators and help in bone formation by up-regulating bone formation markers such as runt related transcription factor-2 (Runx2), osteocalcin, Wnt signalling pathway, β-catenin and insulin-like growth factor (IGF)-1 (Hubert *et al.,* 2014; Torre, 2017).

Tannic acid (TA) is a polyphenol classified from tannins group which has antioxidant properties (Horcajada & Offord, 2012; Chung *et al.,* 1998). European Union Regulation (EUR) stated that TA is a food flavouring and has been extensively used as preservatives (Arapitsas, 2012; Hu *et al.*, 2015). Antioxidants activate the differentiation of osteoblasts and prevent the action of ROS directly (Domazetovic *et al.,* 2017). There is currently no specific research has been conducted regarding TA and bone formation or osteoporosis treatment. However, there are many researches had been conducted by using plants which have tannins. Thakur *et al.* (2016) in their study had used *Saraca indica* which has tannins as antiosteoporotic on Wistar rats which had been induced by dexamethasone to develop osteoporosis. They found out that the plant increased bone density and biomechanical strength of the rats. By using *in-vitro* technique, Thu *et al.* (2017) had studied the effects of *Eurycoma longifolia* on proliferation and differentiation

of osteoblast. *E. longifolia* had positive effects on osteoblast proliferation and differentiation with the deposition of calcium and phosphate was significantly expressed at the concentration of 25 µg/ml.

Drug combination studies are usually targeting on achieving synergistic treatment effect, dose and toxicity mitigation as well as to minimise or delay the induction of drug resistance (Chou, 2012). The net effects of drug combination include synergism or additive effect, antagonism or substractive effect and alteration of effect of one or more drugs (Nidhi, 2012). Combination treatment in osteoporosis such as combination of oestrogen and bisphosphonate is often given to the patient of osteoporosis rather than non-combination or single agent as it is more effective in increasing bone mineral density (BMD) (Tella & Gallagher, 2014). Oestrogen helps in treating osteoporosis patient by inducing apoptosis of osteoclasts which decreases resorption of bone and increase osteoblast lifespan which enhances bone formation (Imai *et al.,* 2009; Khosla *et al.,* 2012).

The research which involved combination of herbal medicines and antiosteoporosis drug had been conducted by Ko *et al.* (2012), which studied the pharmacological effects of a Chinese Herbal Formula Epimedii Herba, Ligustri Lucidi and Psoralea Fructus (ELP) and anti-resorptive drugs (alendronate and raloxifene) to prevent osteoporosis in rats. The herbal formula had been shown to increase the promotion of osteogenic differentiation in rat mesenchymal stem cells by elevating alkaline phosphatase (ALP) activity and matrix calcium deposition. The herbal formula was found to have different effects on the antiresorptive drugs. It increased the bone protective effect of raloxifene while did not produced synergistic effect when used together with alendronate. Another combination study was also done by Raudhah *et al.* (2018) by using combination of pamidronate and semi-purified fractions of *Quercus infectoria* (QI) which had positive effects on proliferation and differentiation of

osteoblasts. The level of bone formation markers (Runx2 and Osx) were higher in combination treatment compared to single treatment of pamidronate and *Q. infectoria* (QI). In this current study, a single compound of TA was used rather than the crude or semi-purified fractions of plant extract which consists of numerous compounds. For example, liquid chromatography mass spectrometry (LC-MS) analysis of each QI semi-purified fraction reveals the presence of polyphenolic compounds (gallic acid, digallate, ellagic adid, phaseolic acid, syringic acid and theogallin) (Abdullah *et al*. 2018). Moreover, the extraction, purification and isolation procedures are complex and time consuming. A part from that, TA compound can be bought anytime while certain herbs that contains TA may only presence or can be collected during certain times.

Thereby, the TA alone and in combination with pamidronate was used to study the proliferation and differentiation, morphology, mineralisation as well as BSP and Osx genes and proteins expression of osteoblast via various techniques such as microscopy observation, histochemical staining, polymerase chain reaction (PCR) and western blot.

1.2 Problem statement

As life expectancy of Malaysian becomes longer, osteoporosis also becomes a major concern in Malaysia. Osteoporosis may occur in both men and women at any age, however it commonly occurs in older women due to decreased level of oestrogen. Besides, the cost to treat and maintenance of osteoporosis-related morbidity is expensive as osteoporosis is usually detected when the disease had become worsen. Many synthetic drugs are available to treat osteoporosis, however they possess shortcomings in their efficacy. As a result, they need to be taken for long-term treatment. Nonetheless, these osteoporosis medications induce side effects which will get worsen with the long-time usage. This situation causes an urge in research and development to find potential and

safe alternative therapy such as using natural products especially phytochemical to decrease the side effects.

Nowadays, drug discovery of herbal and drug combination therapies is getting spotlight to improve therapeutic efficacy and minimise the adverse effects of the osteoporosis drug through modern molecular biological methods. Although there were several studies conducted by using combination of herbal plant and anti-osteoporotic drug, however, there has been no scientific evidence and research on the effects of a specific polyphenol; tannic acid (TA) alone or in combination with control drug on bone being reported. Therefore, in this current study, it is proposed that TA with the presence or absence of control drug (pamidronate) can assist in bone remodelling process especially in relation to osteoblast metabolism. The osteoblast was employed in this study rather than using osteoclast or osteocyte as osteoblast is the main cell involved in bone formation. The bone formation process such as proliferation and differentiation of osteoblast as well as mineralisation were studied in this study by observing the morphological changes, expression of selected bone formation markers (BSP and Osx), along with the assessment of calcium and phosphate levels.

1.3 Objectives

1.3.1 General objective

To evaluate the effect of TA alone and in combination of TA and pamidronate on differentiation and proliferation activities and formation of mineralised deposits (calcium and phosphorus) of human foetal osteoblast (hFOB 1.19)

1.3.2 Specific objectives

1. To determine half maximal effective concentration (EC_{50}) and proliferative activity of hFOB 1.19 cells after treated with TA alone and in combination of TA and pamidronate
2. To determine combination index (CI) of the combination treatment between TA and pamidronate by synergistic analysis
3. To examine the morphological changes of hFOB 1.19 cells after treated with TA alone and in combination of TA and pamidronate using inverted microscope
4. To evaluate the formation of mineralised deposits (calcium and phosphate) of the hFOB 1.19 cells after treated with TA alone and in combination of TA and pamidronate by using histochemical staining (von Kossa and Alizarin Red S)
5. To investigate the expression of osteoblast proliferation markers BSP and Osx of the hFOB 1.19 cells after treated with TA alone and in combination of TA and pamidronate by using polymerase chain reaction (PCR)

CHAPTER TWO

LITERATURE REVIEW

2.1 Bone

2.1.1 Structure and function of bone

There are five general types of bones based on their shapes; long bones, short bones, flat bones, irregular bones and sesamoid bones (Figure 2.1). Long bones include the clavicles, humeral, radii, ulnae, metacarpals, femurs, tibiae, fibulae, metatarsals and phalanges. Meanwhile, short bones include the carpal, tarsal bone, patellae and sesamoid bones. Flat bones comprise of the skull, mandible, scapulae, sternum and ribs. Irregular bones include the vertebrae, sacrum, coccyx and hyoid bones. Lastly, sesamoid bones are bones which placed within a tendon and they can be found in the hand, knee and foot. Their functions are to preserve the tendons and increase the mechanical effect of the tendons (Clarke, 2008; National Register of Personal Trainers, 2018).

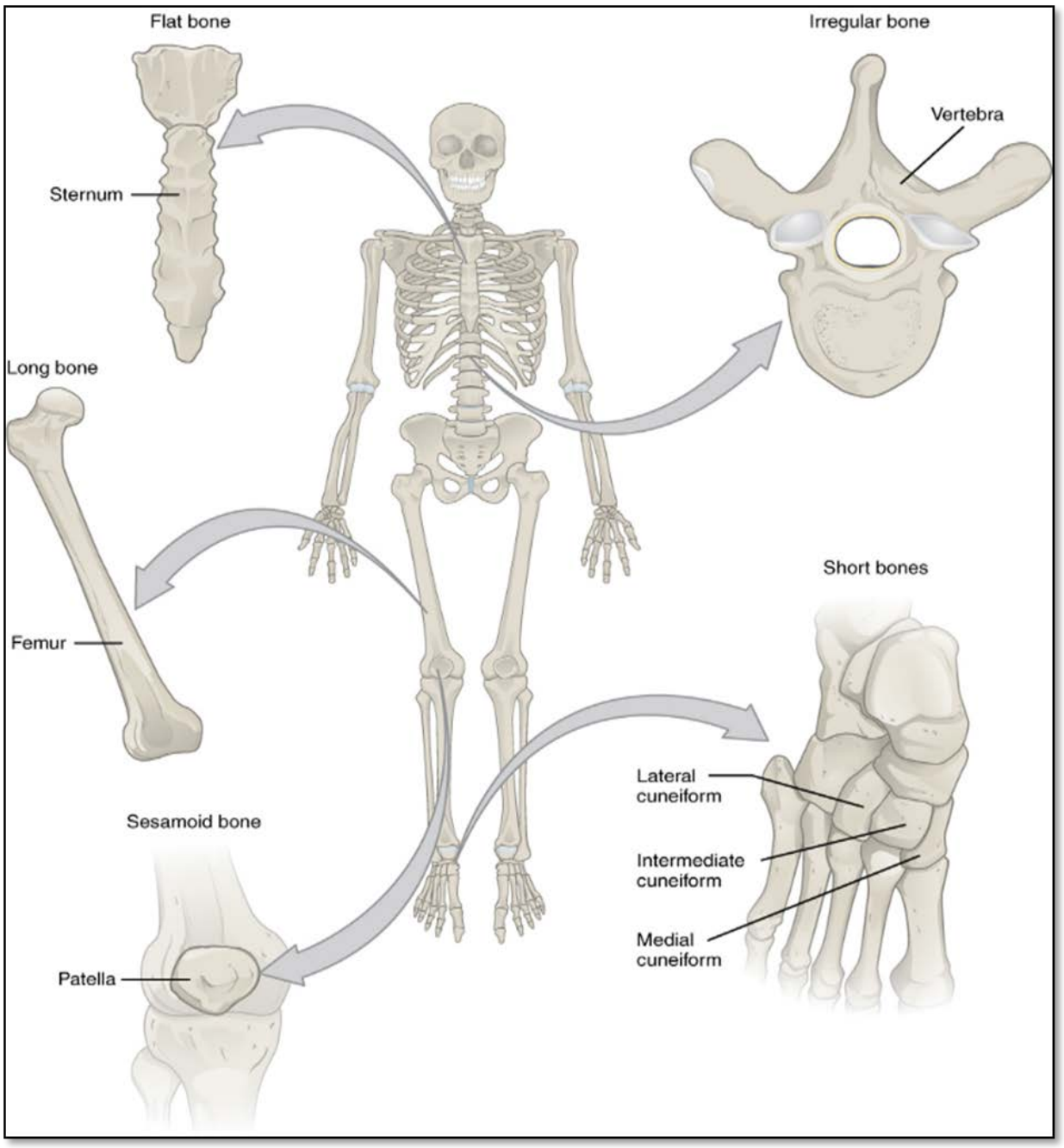

Figure 2.1: Types of bones; long bones, short bones, flat bones, irregular bones and sesamoid bones. (Adapted from Lumen Learning, n.d.)

Bones also divide into immature and mature bone where immature bone tissue is known as primary woven bone tissue while mature bone tissue is known as secondary lamellar bone tissue. Woven bones present in foetal bones at the first-time bones are produced and after fractures in the adult. The bones are later replaced by lamellar bone as woven bone is immature bone or pathologic bone. Woven bones get their name via their woven appearance of fibrous matrix (Figure 2.2) (Singh, n.d.).

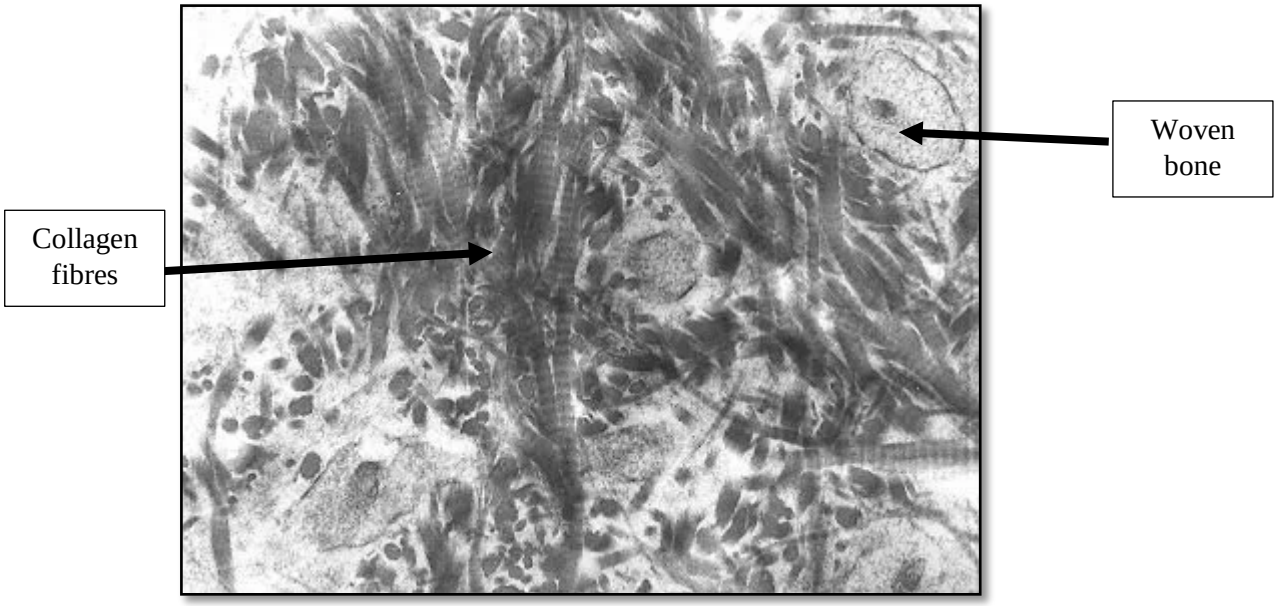

Figure 2.2: Woven bone with presence of abundant fibrous matrix (Adapted from Singh, n.d.)

Remodelling of woven bones produce lamellar bones. Lamellar bones got their name due to arrangement of collagen in parallel form or lamellae form. Different with woven bones which has more osteocytes per unit volume, lamellar bones have lower amounts of osteocytes to surrounding tissue and are filled with many collagen fibres parallel to each other in same layer known as osteons or Harversian system. Among the different layer, the fibres run in opposite directions which creates structural arrangement helps in bone ability to withstand torsion forces (Singh, n.d.).

There are two types of lamellar bone tissue which are the cortical bone (compact bone) and trabecular bone (cancellous bone) (Figure 2.3). Both types of tissue share same matrix composition while having different structure and function. Cortical bone is dense, solid and encloses the marrow space while the trabecular bone is cancellous or spongy bone tissue which is composed of a honeycomb-like network of trabecular plates and rods scattered in the bone marrow compartment (Clarke, 2008; Kini & Nandeesh, 2012; Weatherholt *et al.,* 2012). Cortical bone is made up of osteons which are dense and parallel concentric lamellar units. Each osteon is connected to each other via Haversian system, Volkmann's canals and canaliculi. Cortical bones are covered with connective tissue; periosteum on the outside and by endosteum in the inner surface. Trabecular bones are different compared to cortical bones as they are no vessels like canaliculi within trabeculae. Instead, they are nourished by diffusion from the surrounding bone marrow (Osterhoff *et al.,* 2016). 80% of skeletal is made up of cortical bones. Cortical bones have a slow replace rate and a high resistance to bending and torsion. Thus, they made up the outer part of all skeletal structures as can be seen prominently within the cylindrical shaft of upper extremity long bones as cortical bones form a thick shell or cortex surrounding the medullary canal. As most part of cortical bone is calcified, its function is to give mechanical strength and protection. Besides, cortical bone also takes part in metabolic responses, especially when there is severe and continuous mineral deficit (Hadjidakis & Androulakis, 2006; Weatherholt *et al.,* 2012).

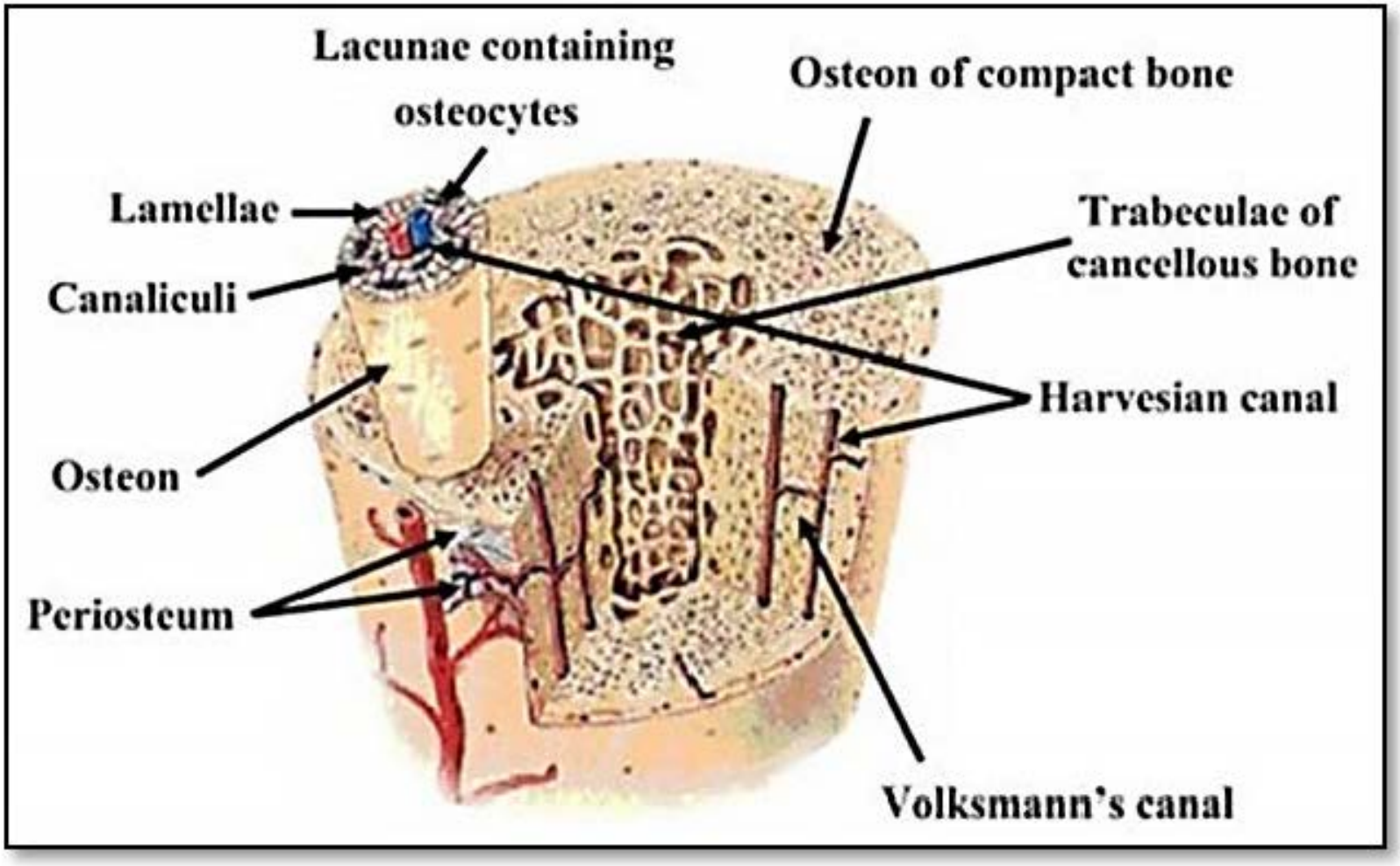

Figure 2.3: Cortical and trabecular bones of lamellar bone (Adapted from Sami, 2015)

Meanwhile, trabecular bone comprises 20% of skeletal structure and it has an outer periosteal surface and inner endosteal surface (Hadjidakis & Androulakis, 2006; Clarke, 2008). The periosteal surface is a fibrous connective tissue sheath that encloses the outer surface of bone except at joints as the bones there are lined by articular cartilage. It has blood vessels, nerve fibres, osteoblasts and osteoclasts. The functions of this outer surface are to protect, nourish and help in bone formation. Besides, it also plays important role in appositional growth and fracture repair. The inner endosteal surface on other hand is a membranous surface surrounding the inner surface of cortical and cancellous bone and the blood vessel canals (Volkmann's canals) in the bone. Trabecular bone also offers mechanical support by having ability to spread load evenly and absorb energy around the joints area (Kini & Nandesh, 2012; Weatherholt *et al.*, 2012).

2.1.2 Histology of bone

Histological composition of the bone tissue includes extracellular matrix, collagen and cells which involve in structural function (Hadjidakis & Androulakis, 2006). There are four different types of cell that make up the bone; osteoblasts, osteocytes, osteoclasts and bone lining cells (Figure 2.4 & 2.5) (Mohamed, 2008). Osteoblasts are mononucleated, cuboidal cells located along the bone surface and make up 4 to 6% of total bone cells (Lau & Guo, 2011; Florencio-Silva *et al.*, 2015). They were named "osteoblast" as they literally produce bone. They are formed from multi-potential mesenchymal progenitors and they present for the whole life with their highest activity are during embryonic skeletal formation and growth (Nam & Kampa, 2013; Rutkovskiy, Stensløkken & Vaage, 2016). The main function of osteoblasts is to produce the components that make up the extracellular matrix such as type I collagen, proteoglycans, and non-collagenous and cell attachment proteins (Mohamed, 2008). As they involve in bone formation process, they

show morphological characteristics of protein synthesizing protein, such as abundant rough endoplasmic reticulum, distinguished Golgi apparatus and variety of secretory vesicles (Florencio-Silva *et al.*, 2015).

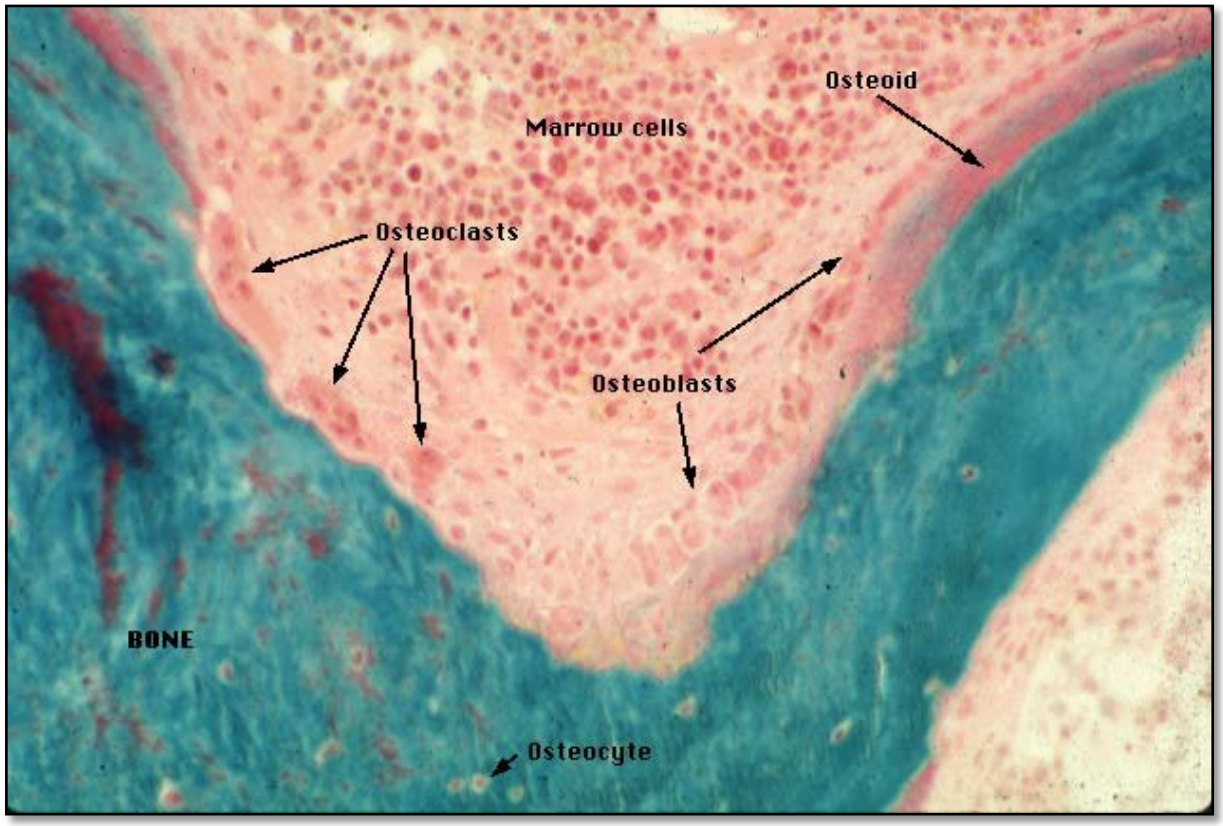

Figure 2.4: Histology of bone shown bone cells (Adapted from Pernick, 2016)

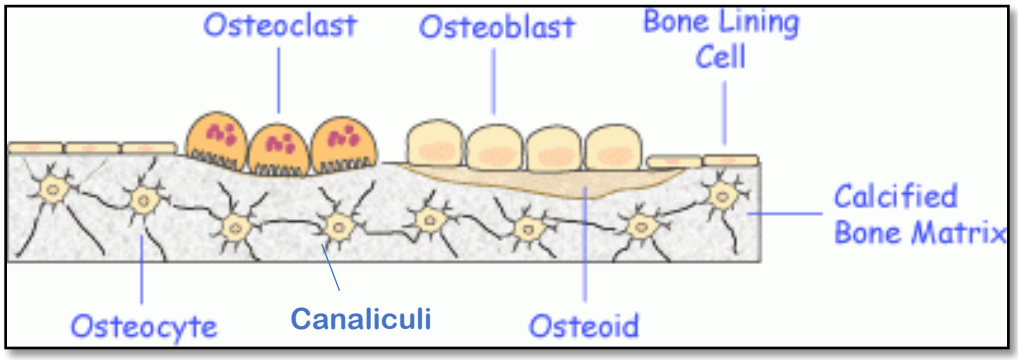

Figure 2.5: Position of different bone cells in bone (Adapted from International Osteoporosis Foundation, 2017)

Osteocytes are produced when osteoblasts are trapped in the bone matrix and made up of about 95% of cells in the mature bone tissue with function of important controller of mechanical and hormonal signals (Mohamed, 2008; Long, 2012). Osteocytes are postproliferative and locate in the lacunae and are regularly distributed as many fine canals called canaliculi emit from them in all directions. The role of the canaliculi is to allow the diffusion of substance via the bone (Figure 2.3). Thus, many processes from the osteocytes go through the canaliculi in all direction, making osteocytes as main cells to detect stresses induced in bone and main mechanoreceptors of bone due to their widespread distribution and interconnections (Mohamed, 2008).

Bone lining cells are inactive osteoblasts that line on the bone surface as a layer of flattened and elongated cells (Mohamed, 2008; Long, 2012). They are also known as periosteal surface which layered along outer cortical bone (Clarke, 2008). Along with osteocytes, they are also postproliferative and protect bone surface from any osteoclast resorptive activity. However, they may be reactivated to form osteoblasts (Mohamed, 2008). They have a thin and flat nucleus, cytoplasm which broading along the surface and a few cytoplasmic organelles such as rough endoplasmic reticulum and Golgi apparatus (Florencio-Silva *et al.*, 2015).

Among all cells that form the bone, osteoclasts are the only cells which able to resorb bone. They are multinucleated cells which derived from monocyte or macrophage lineage (Lau & Guo, 2011). Presence of numerous organelles especially mitochondria show that osteoclasts are actively associated in energy production and protein synthesis, mainly production of lysosomal enzymes. Well defined cell polarity is shown by active bone-resorbing osteoclasts as their plasma membrane can be differentiated into three sections; clear zone, ruffled border and basolateral plasma membrane (Nakamura, 2007). The osteoclasts from long bone marrow are produced faster than in the jaw as the

osteoclastogenic potential of osteoclasts are different depends on the bone site they occupy which is due to cellular composition of the bone-site specific marrow (Florencio-Silva *et al.,* 2015). Life expectancy of the osteoclasts can be from 6 to 10 days depends on location and need (Nam & Kampa, 2013). Oestrogen able to reduce the life expectancy of the osteoclasts by promoting apoptosis (Lau & Guo, 2011).

Inorganic salts and organic matrix make up bones. The extracellular matrix (ECM) is also known as osteoid when it first deposited but not yet mineralised. The ECM starts to mineralise via accumulation of crystalline hydroxyapatite [$Ca_3(PO_4)_2]_3Ca(OH)_2$]. The inorganic salts of the bone are mainly phosphate and calcium ions which form the crystalline hydroxyapatite besides other ions such as bicarbonate, sodium, potassium, citrate, magnesium, carbonate, fluorite, zinc, barium and strontium (Feng, 2009; Long, 2012; Fluorencio-Silva *et al.,* 2015). The bone extracellular matrix is secreted by osteoblasts and at some points mineralized to build strength and hardness (Nam & Kampa, 2013). Collagen I is the main collagen present in the matrix which constitute of approximately 90% of bone organic composition followed by types III and IV and FACIT collagens (Collagen IX, XII, XIV, XIX, XX, and XXI). FACIT collagens are important in organizing and stabilizing the matrix. Non-collagenous proteins such as proteoglycans, glycosylated proteins, glycosylated proteins with potential cell-attachment activities, and γ-carboxylated (gla) proteins also present to help to regulate matrix mineralization (Clarke, 2008; Kini & Nandeesh, 2012).

2.1.3 Physiology of bone formation

Ossification or osteogenesis is the process of new bone formation by osteoblasts. The main components of this process are osteoblasts and bone matrix. This process can be divided into two more specific processes; intramembranous ossification and endochondral ossification (Figure 2.6) (Kini & Nandeesh, 2012). Intramembranous ossification occurs in the flat bones of the skull, facial bones, mandible and clavicle, it involves direct differentiation of embryonic mesenchymal cells the bone-forming osteoblasts while endochondral ossification occurs in other places of the skeleton and involves the replacement of a cartilage model by bone tissue (Mackie *et al.,* 2011; Tortura & Derrickson, 2011). Intramembranous ossification is also present in the healing process of fractures treated by open reduction and stabilization by metal plate and screws. Meanwhile, endochondral ossification presents during fracture healing when treated by cast immobilisation (Kini & Nandeesh, 2012).

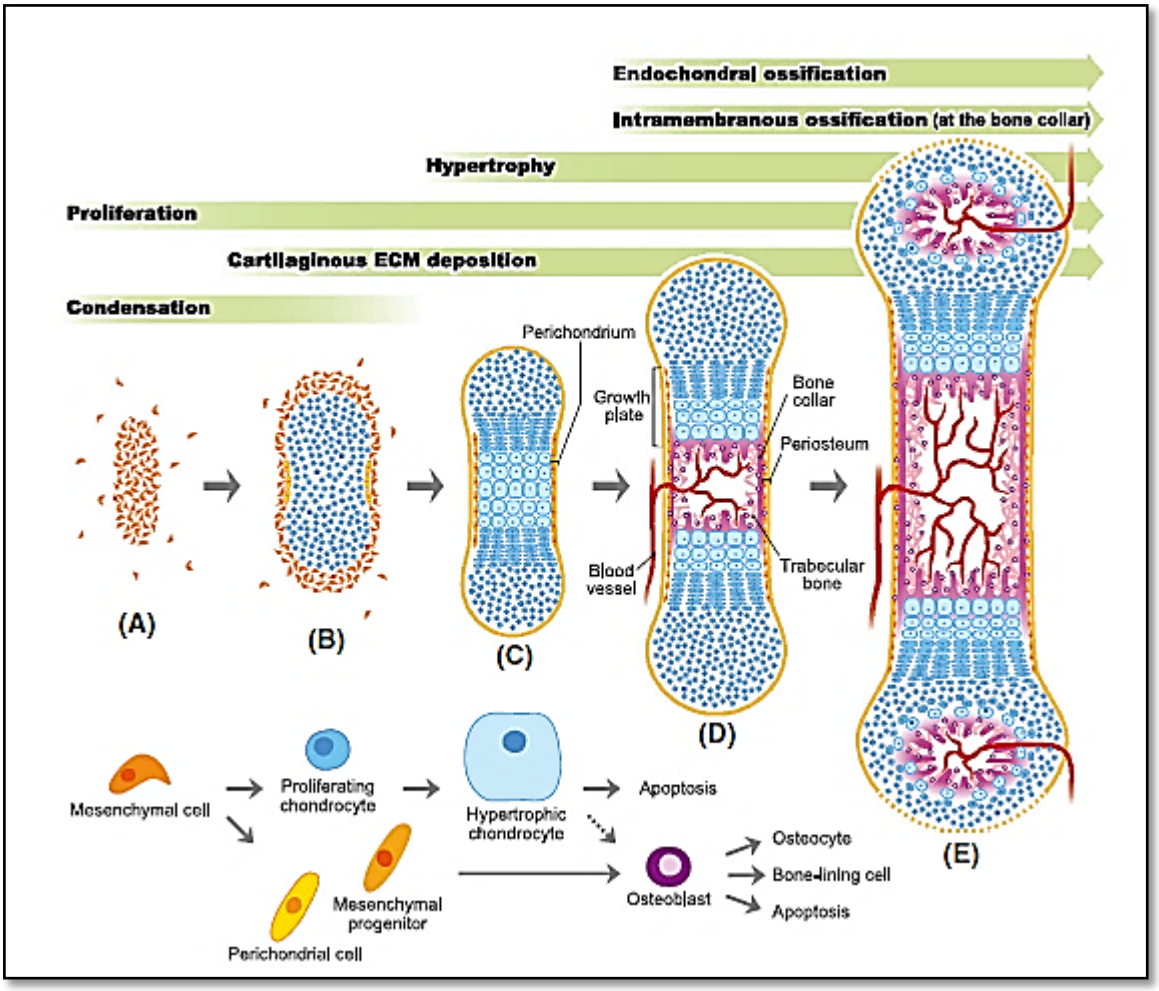

Figure 2.6: Intramembranous and endochondral ossification. A & B) development of cartilage model where mesenchymal cells differentiate into chondrocytes, C) chondrocytes undergo cell division caused growth of cartilage model which covered by perichondrium, D) development of primary ossification centre E) development of secondary ossification centres occurs in the epiphyses of the bone (Adapted from Egawa *et al.*, 2014).

The intramembranous ossification involves four stages; 1) development of the ossification centre, 2) calcification, 3) formation of trabeculae and 4) development of periosteum. During the initial formation of bone, specific chemical messages will be secreted and cause the mesenchymal stem cells (MSCs) to cluster among themselves and differentiate into osteogenic cells and later into osteoblasts. The site where the MSCs cluster is called ossification centre and osteoblasts later continue to secrete the organic ECM until the bone is surrounded by ECM. Osteoid or unmineralised bone matrix is produced by osteoblasts in vesicle forms (Figure 2.3). It serves as a template for the later mineralised bone matrix when hydroxyapatite started to presence in these osteoid vesicles and become hydroxyapatite crystals which grow inside the vesicles and penetrate via the membrane to form mineralised nodules or calcified nodules. The mineralisation of bone matrix also eventually leads to collagen mineralisation when the hydroxyapatite crystals are continued being passage into the nodules and extends along the collagen fibrils. When the secretion of ECM stops, osteocytes start to migrate to canaliculi and scatter to all directions. Then, the calcium and other minerals begin deposited and the ECM calcifies. The ECM later develops to trabeculae and fuse with each other to form spongy bone around the blood vessels and connective tissues that trap in the trabeculae link with blood vessels and differentiate into red bone marrow. At the same time as production of trabeculae, the MSCs condense at the borderline of the bone and form periosteum or bone lining cells. After some time, compact bone replaces spongy bone with spongy bone remains in center (Ozawa *et al.*, 2008; Tortora & Derrickson, 2011).

Another type of ossification is endochondral ossification where the cartilage is replaced by bone. The steps of this ossification are 1) development of the cartilage model, 2) growth of cartilage model, 3) development of primary ossification centre, 4) development of medullary (marrow cavity), 5) development of the secondary ossification

centre and 6) formation of articular cartilage and epiphyseal (growth) plate. In the beginning, the MSCs differentiate into chondroblasts which secrete ECM to produce a cartilage model made up of hyaline cartilage and is covered by perichondrium. The chondroblasts are later embedded in the ECM and is referred as chondrocytes which later undergo cell division along with secretion of cartilage ECM cause the cartilage model to increase in length. This process is known as interstitial or endogenous growth or growth from within. On the other hand, the cartilage model becomes thicker when ECM secreted by chondroblasts deposited on the cartilage surface in the appositional or exogenous growth process which is regulated by insulin-like growth factor (IGF) signalling. As the cartilage model keep on growing, chondrocytes stop dividing but start enlarging to proliferate into hypertrophic chondrocytes. While ECM also continues to calcify due to collagen type X secreted by hypertrophic chondrocytes, it becomes lack of nutrients and causes chondrocytes undergo apoptosis and the spaces left by died chondrocytes become small cavities; lacunae which eventually become vascularised (Tortura & Derrickson, 2011; Egawa *et al.*, 2014).

The primary ossification centre later starts to develop from inward direction with a nutrient artery invades into the perichondrium and calcifies the cartilage model which later trigger MSCs in the perichondrium to differentiate into osteoblasts. The perichondrium starts to form bone and changes into periosteum. The primary ossification begins to form and spread from the centre when periosteal capillaries emerge into the disintegrating calcified cartilage. Osteoblasts also secrete ECM which later forming spongy bone over the calcified cartilage causes the calcified cartilage to be replaced with bone tissue. As the primary ossification forms from the centre to the ends of the bone, osteoclasts dissolve some of the newly produced spongy bone and causes a cavity called medullary or marrow cavity in the diaphysis or shaft of bone. The wall of the diaphysis

is then replaced by compact or cortical bone. The secondary ossification centre is later progress as epiphyseal artery enter the epiphyses region of bone. The bone formation during this process is similar in primary ossification centre except that no medullary cavity is formed as spongy bone remains in the inner side of epiphyses. In addition, the secondary ossification centre grows outward from the centre of epiphyses unlike the primary ossification centre. Lastly, the hyaline cartilage serves as epiphyseal or growth plate which later during adulthood becomes articular cartilage (Tortura & Derrickson, 2011; Egawa *et al.*, 2014).

2.1.4 Osteoblast differentiation and proliferation

Osteoblasts are derived from two different process; intramembranous and endochondral ossification. The multipotent MSCs are capable to differentiate into either of these cells; osteoblasts, adipocytes, chondrocytes, myoblasts and fibroblasts (Figure 2.7). Through the intramembranous ossification, the osteoblasts originate from neural ectoderm MSCs are form directly without any transitional stages that form squamous bones of the skull and face (calvaria) and part of the clavicle. Osteoblasts of the axial skeleton are derived from paraxial mesoderm while from lateral plate mesoderm for the appendicular skeleton. On the other hand, via endochondral ossification, the osteoblasts differentiate from an intermediate class of perichondral cells or directly from hypertrophic chondrocytes (James, 2013; Rutkovskiy, *et al.*, 2016).

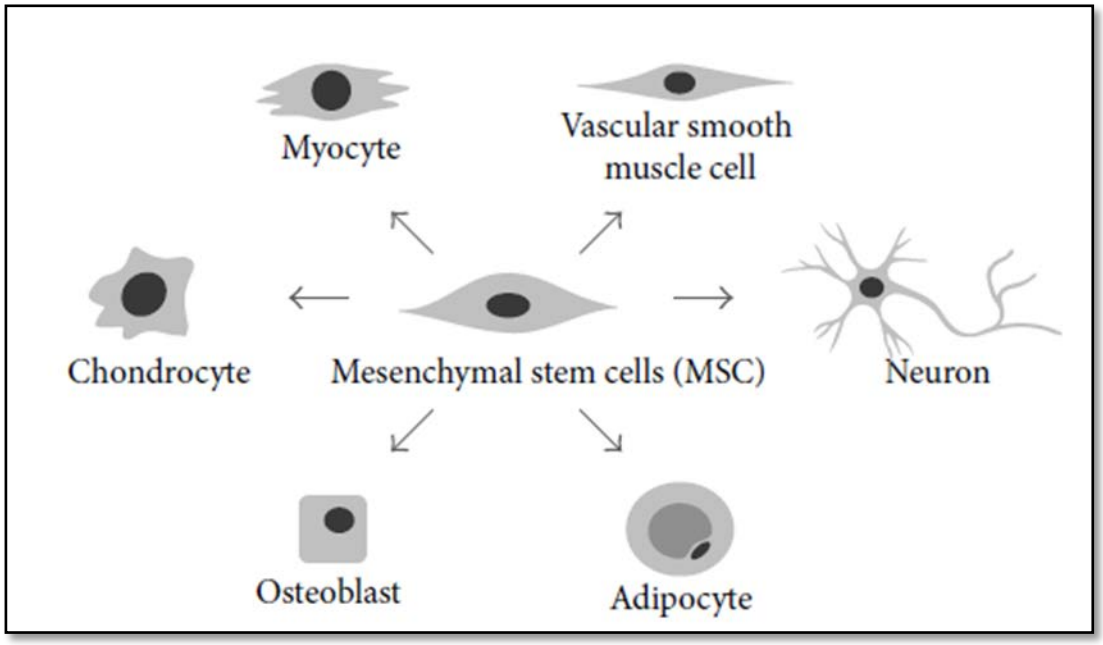

Figure 2.7: Multilineage differentiation of mesenchymal stem or stromal cells (MSC) (Adapted from James, 2013)

In the early stage, Wnt10b from wingless-int (Wnt) pathway involves in differentiation of MSCs into osteoblast or chondroblast progenitor while prevent differentiation of MSCs into preadipocyte via suppression of the adipogenic transcription factors CCAAT enhancer protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPAR-γ) (Figure 2.8). Wnt10b also activates transcription factor Runx2, Msx2, distal-less homeobox 5 (Dlx-5) and β-catenin to promote osteoblastogenesis instead of chondrocyte differentiation. Osterix (Osx) also involves in osteoblastogenesis by promoting osteoblast progenitor into Pre-Osteoblast (Pre-OBL). Pre-OBL is identified as it secretes alkaline phosphatase (ALP), the type 1 parathyroid receptor (PTH1R) and alpha-1 type I collagen (Col1A1) as earliest markers. When the pre-OBL becomes active osteoblast (active OBL), it continues secretes ALP and Col1A1 along with osteopontin, osteocalcin, osteonectin and bone sialoprotein (BSP) (Rucci, 2008).

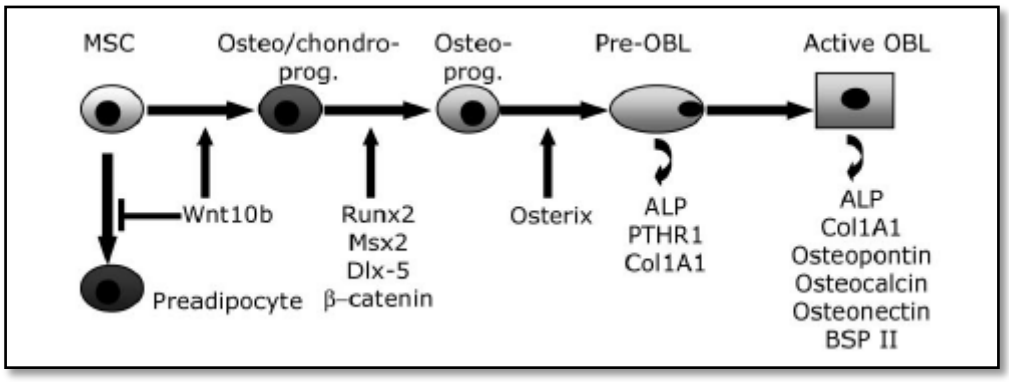

Figure 2.8: Differentiation and proliferation of osteoblast via intramembranous and endochondral ossification and the marker involved (Adapted from Rucci, 2008)

Wnt signalling pathway involves in many important biological processes including tissue homeostasis, stem cell conservation, proliferation of cells, transformation of cancer cells and tumour suppression (Wend *et al.*, 2012). The functions of Wnt family especially Wnt10b had been investigated via *in vivo* and *in vitro*. Longo *et al.* (2004) in their study had proved that under control of FABP4-promoter, transgenic mice which expressed Wnt10b had increased bone mass and strength besides Bennetts *et al.* (2005) found that those mice were resistant to aging or hormonal-related bone loss. Bennett *et al.* (2005) also found that by using *in vitro* technique on bone-marrow-derived St2 cells, Wnt/β-catenin signalling promotes osteoblastogenesis and increases mineralisation. However, the main function of Wnt10b is to trigger the expression of osteoblast transcription factors while suppresses the key adipogenic transcription factors so that the MSCs differentiate into osteoblasts instead of adipocytes (Wend *et al.*, 2012).

Runx2 is in Runx family and essential for both endochondral and intramembranous ossifications which produce osteoblasts. It also important for osteoblasts as matured osteoblasts need Runx2 for production of bone matrix. Runx2 is expressed in the chondrogenic mesenchymal with the help of Sox9 expression. When the