CHAPTER 2

Literature Review

I Bone Homeostasis

2.1 Introduction

The mature skeleton is a metabolically active organ that undergoes continuous remodeling by a process that replaces old bone with new bone. Remodeling is necessary to maintain the structural integrity of the skeleton and to serve its metabolic functions as a store of calcium and phosphorus. This dual function often comes into conflict under conditions of changing mechanical forces or of nutritional and metabolic stress.[6] Osteoblasts that are responsible for bone formation originate from bone marrow stromal precursor cells that then differentiate into mature osteoblasts. Osteoclasts that are responsible for bone resorption originate from haematopoietic stem cells known as monocytes.[6]

The remodeling cycle is finely regulated by a variety of systemic and local factors e.g. oestrogen (E2), parathyroid hormone (PTH), $1,25(OH)_2D_3$ (vit D_3), growth factors and cytokines.[6,34-35] Bone formation and resorption are usually balanced and a constant level of bone mass is maintained. An imbalance between bone formation and resorption causes metabolic diseases such as osteoporosis and osteopetrosis (a family of diseases characterised by increased bone mass due to decreased bone resorption).[6]

2.2 Composition of bone

Bone tissue has three components: an inorganic bone mineral component, an organic matrix, and bone cells. In mature bone, inorganic bone mineral is deposited on a framework of organic support material known as osteoid. The mineral fraction of bone consists of calcium phosphate in the form of hydroxyapatite crystals

(Ca_{10} $(PO_4)_6(OH)_2$), and is responsible for about half of the bone mass. The organic matrix of bone contains 95% collagen. The remaining 5% of noncollagen organic matter, also known as ground substance, consists of a mixture of various proteoglycans, high-molecular-weight compounds comprised of carbohydrate and protein. The combination of organic and inorganic materials is responsible for bone's mechanical strength.[36]

Mature bone comprises two basic bone types, cortical or compact bone and cancellous or trabecular bone. Cortical bone makes up the shafts of long bones as well as the outer envelope of all bones. It has mainly supporting, protective and mechanical functions in the body. Trabecular bone, on the other hand, has a honeycomb structure well suited for a site for bone-forming cells and a large surface area that provides a reservoir for minerals. Trabecular bone is found in the inner parts of the vertebrae and pelvis and the ends of the long bones.[37] Figure 2.1 depicts the basic bone types as seen with an electron microscope.[38]

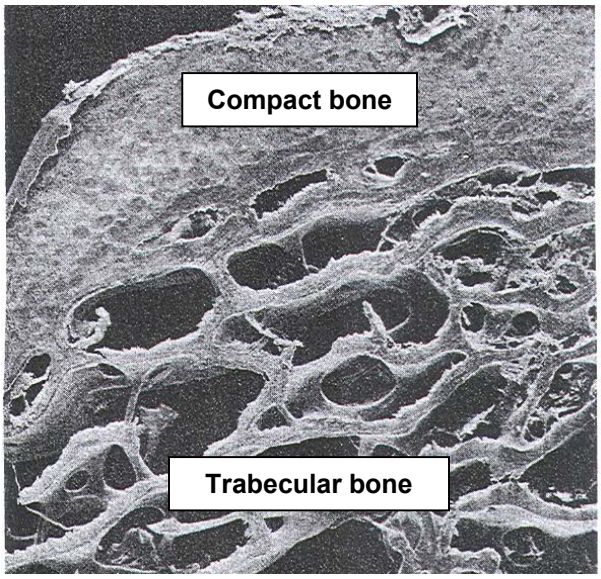

Figure 2.1 A scanning electron micrograph of compact and trabecular bone.
(Reprinted from: Moffet DE, Moffet B, Schauf CL. Human Physiology: Foundations and Frontiers. 2nd ed. St Louis: Mosby-Year Book, Inc; 1993. p. 577[38]) Copyright (1993), with permission from Elsevier.

2.3 Bone cells

There are three types of bone cells, osteoblasts, osteocytes, and osteoclasts. Osteoblasts, located side by side on the surface of bone, synthesise osteoid (organic matrix) and are therefore responsible for bone formation. As the deposition of osteoid and bone mineralisation continues, osteoblasts become surrounded by mineralised bone. At this stage, osteoblasts progressively lose their bone-forming capability and are termed osteocytes. Osteoclasts are multi-nucleated cells that break down osteoid by a process known as bone resorption.[36] Figure 2.2 depicts the location of the different types of bone cells.[39]

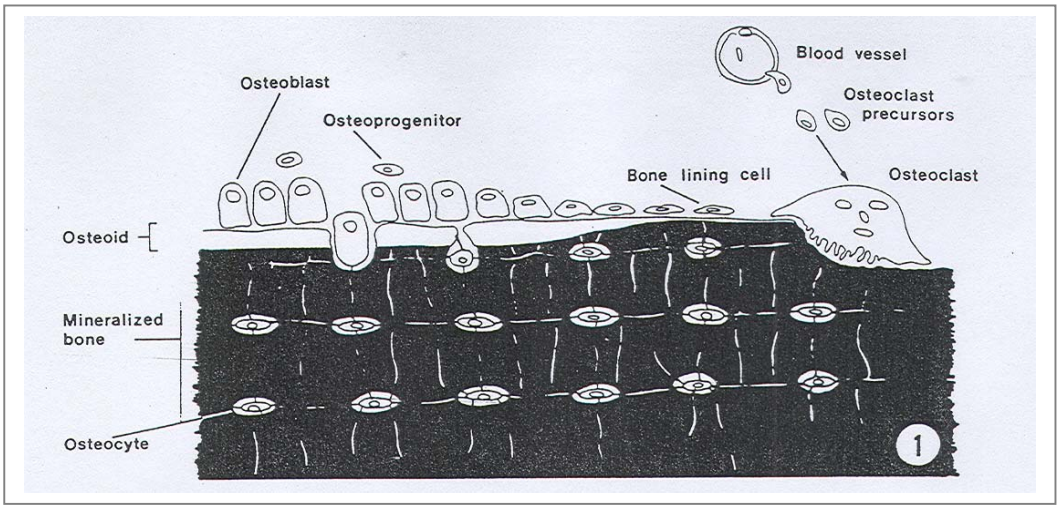

Figure 2.2 The origins and locations of bone cells.

(Marks SC, Popoff SN. Bone cell biology: The regulation of development, structure, and function in the skeleton. Am J Anat 1988;183:1-44)[39] ©1988 (Wiley-Liss, Inc., A Wiley Company) Reproduced with permission of John Wiley & sons, Inc.

2.3.1 Osteoblasts

Osteoblasts actively producing osteoid are cuboid-shaped and exhibit an abundant endoplasmic reticulum and Golgi complex, characteristic of cells synthesising proteins for export. Osteoblasts are responsible for synthesising, secreting, organising, and mineralising the bone matrix, or osteoid. They also produce a variety of regulatory factors including prostaglandins, cytokines and growth factors, some of which are incorporated into the developing matrix.[40] Mature osteoblasts are rich in

alkaline phosphatase that is believed to play an important role during mineralisation.[41]

Osteoblasts, not actively engaged in bone formation have a flatter appearance. Osteoblasts have numerous cytoplasmic processes that bring them into contact with neighbouring osteoblasts.[36] Once the osteoblast has differentiated and completed its cycle of matrix synthesis, it can either become a flattened lining cell on the bone surface, be buried in bone as an osteocyte, or undergo programmed cell death (apoptosis).[6,42]

2.3.1.1 Origin of osteoblasts

Bone marrow contains hematopoietic precursors, their progeny, and stromal cells. Stromal cells include adipocytes, fibroblastic cells, endothelial cells, and mesenchymal stem cells (MSCs). Mesenchymal stem cells are pluripotent and able to differentiate into several distinct cell types, including osteoblasts, adipocytes, fibroblasts and myoblasts.[43-44] The osteoblastic differentiation pathway from mesenchymal stem cells is illustrated in figure 2.3.[6]

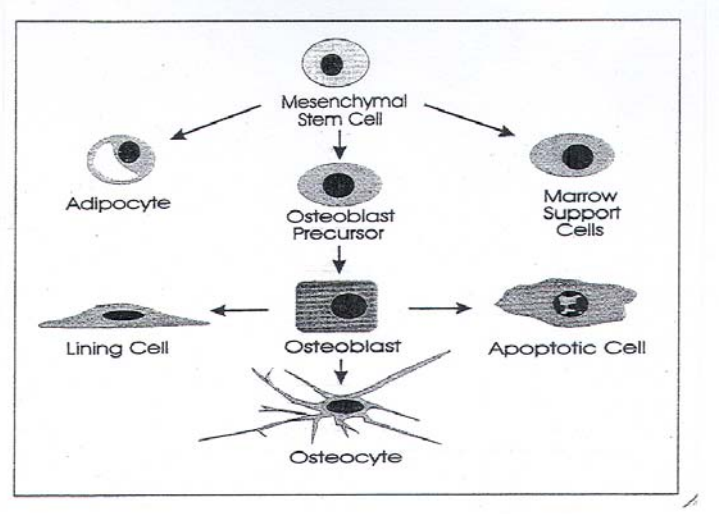

Figure 2.3 Origin and fate of osteoblasts.

The mesenchymal stem cell that gives rise to osteoblasts can also produce cells of other lineages. It is also possible that osteoblast precursors can differentiate into, or derive from adipocytes and marrow support cells. Osteoblasts can be buried as osteocytes, remain in the bone surface as lining cells, or undergo apoptosis. *(Reprinted with permission from Raisz LG. Physiology and pathophysiology of bone remodeling. Clin Chem 1999;45:1353-8)*[6]

2.3.1.2 Transcriptional control of osteoblast differentiation

The commitment of a mesenchymal stem cell to the osteoblastic lineage is regulated by specific transcription factors. Activated transcription factors bind to nuclear DNA and induce the expression of a new set of genes which ultimately change the characteristics of that cell.[45] Core Binding factor α-1 (Cbfa1, also known as Runx-2) is the earliest and most specific marker of osteoblastogenesis.[46-47] In addition to its critical role during osteoblast differentiation, Cbfa1 controls bone formation by differentiated osteoblasts.[46-49] Another transcription factor called Osterix (Osx), acting downstream from Runx/Cbfa1 was recently identified.[49] Figure 2.4 shows a model of the osteoblast differentiation pathway as regulated by transcriptional factors.[49]

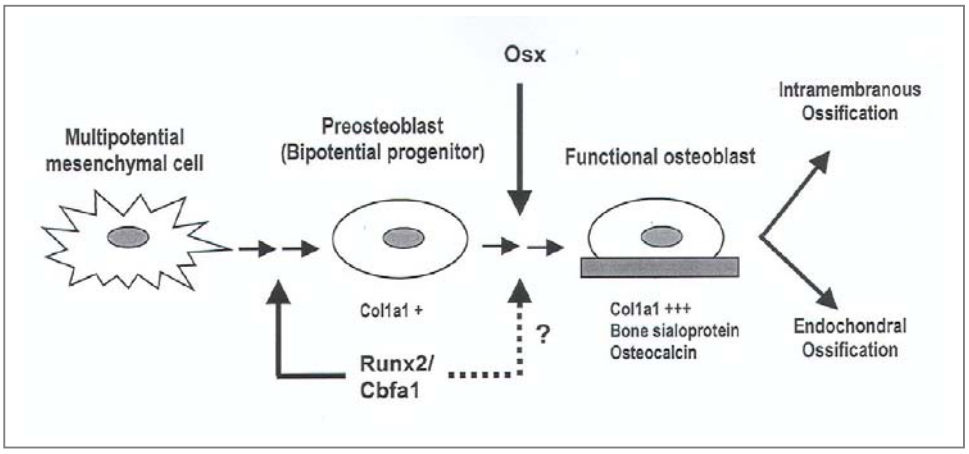

Figure 2.4 Model of the osteoblast differentiation pathway.

Multipotential mesenchymal progenitors first differentiate into preosteoblasts, a process for which Runx2/Cbfa1 is needed. These preosteoblasts are still bipotential; i.e. they have the potential to differentiate into both osteoblasts and chondrocytes. Preosteoblasts do not express osteoblast marker genes, except low levels of Col1a1 typical of mesenchymal cells. Preosteoblasts then differentiate into functional osteoblasts expressing high levels of osteoblast marker genes. This process requires Osx. *(Reprinted from: Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor Osterix is required for osteoblast differentiation and bone formation. Cell 2002;108:17-29.[49] Copyright (2002), with permission from Elsevier.)*

The differentiation potential of osteoblastic precursors is not yet fully understood. One theory is that multipotent bone marrow progenitor cells could differentiate into various phenotypes.[50] Plasticity between cell types has also been reported. For a cell to be converted from one cell type to another there must be suppression of differentiation of the original cell type with promotion of differentiation to the new

type. For example, it has been shown that both adipocytes and preadipocytes obtained from murine bone stromal cells express a number of osteoblastic markers.[51] Skillington *et al* (2002) proved that pre-adipocytes could be converted into fully differentiated osteoblasts in response to bone morphogenetic protein-2 (BMP-2) and retinoic acid signaling.[52] This observation is consistent with the hypothesis that a single progenitor cell gives rise to both adipocytes and osteoblasts and that conversion between these lineages, in response to exogenous growth factors, is possible.[51]

The early step in the commitment of a mesenchymal stem cell to the osteoblastic or the adipocytic lineage depends on activation and expression of Cbfa1, necessary for osteogenesis, and the peroxisome proliferator-activated receptor γ2 (PPAR-γ2) necessary for adipocytic differentiation respectively.[46,53] PPARγ is a member of the nuclear receptor family of transcription factors, a large and diverse group of proteins that mediate ligand-dependent transcriptional activation or repression.[54] The diverse spectrum of activities appears due to cell specificity of PPARγ function and the nature of the ligand.[50,55] The basic action of the nuclear hormone receptors such as PPAR is illustrated in figure 2.5.[54]

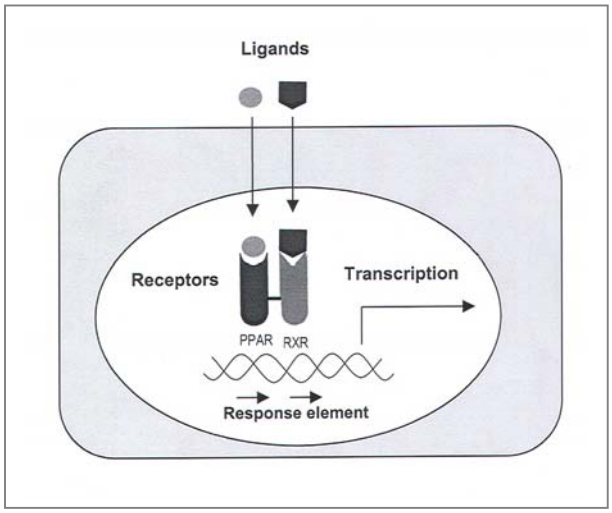

Figure 2.5 Basic action of nuclear hormone receptors.

Nuclear hormone receptors bind to a specific sequence in the promoter of target genes (called hormone response elements), and activate transcription upon binding of ligand. Several nuclear hormone receptors, including PPARγ, can bind to DNA only as a heterodimer with the retinoid X receptor (RXR), as shown. *(Kersten S, Desvergne B, Wahli W. Roles of PPARs in health and disease. Nature 2000;405:421-4).*[54]

With ageing and osteoporosis there is a decrease in osteoprogenitor cells with an accompanying increase in adipocytes in bone marrow.[44] This is due to altered differentiation of the common mesenchymal stem cell.[44,56-58] It has been shown that cells cultured from human trabecular bone are not only osteogenic, but able to undergo differentiation to adipocytes when treated with long chain fatty acids. The formation of differentiated adipocytes was dependent on increased expression of PPARγ2.[57] *In vitro* studies have shown that overexpression of PPAR-γ reduces osteoblastic differentiation of murine MSCs by inhibition of Cbfa1 expression.[59] PPARγ deficiency on the other hand, results in enhanced bone formation with increased osteoblastogenesis from bone marrow progenitors.[60] The pathway to adipocytes is of great importance since cells that have the potential to form osteoblasts can be diverted into the adipocytic lineage and are then no longer available for bone formation. Factors driving MSCs to differentiate down the two lineages therefore play important roles in determining bone density.

Although there is sufficient evidence for the theory that multipotent bone marrow progenitor cells could differentiate into various phenotypes such as osteoblasts and adipocytes, others differ. Bellows and Heersche (2001) for instance, reported that in fetal rat calvaria, the large majority of osteoprogenitors are committed and restricted to the osteoblastic lineage and that the large majority of adipocyte progenitors are committed and restricted to the adipocytic lineage, whilst the common osteoblast/adipocyte progenitor is only present in low numbers. This common osteoblast/adipocyte progenitor is suggested to be the source of the clonal cell lines that have been described to possess both adipogenic and osteogenic potential.[61]

2.3.2 Osteocytes

As the deposition of osteoid and bone mineralisation continues, osteoblasts become surrounded by mineralised bone, progressively lose their bone-forming capability and become osteocytes. Osteocytes form cell networks and can transduce mechanical stimuli from the periphery to the centre of bone.[36] Osteocytes are critical for maintaining fluid flow through bone, and changes in this fluid may provide the signal for the cellular response to mechanical forces such as impact loading.[6]

2.3.3 Osteoclasts

Osteoclasts are multi-nucleated cells that break down osteoid by a process known as bone resorption.[36] Active multinucleated osteoclasts adjacent to bone have a ruffled appearance that increases surface area and allows the cell to perform the task of bone resorption effectively. When stimulated, osteoclasts secrete H^+ and proteolytic enzymes into the extracellular space adjacent to the bone. The acidic environment increases the solubility of bone mineral, while the proteolytic enzymes attack the organic matrix of bone. Together, these two factors promote the process of bone resorption.[36]

2.3.3.1 Origin of osteoclasts

Osteoclasts are derived from haematopoietic cells of the monocyte-macrophage lineage. These stem cell precursors first undergo a phase of *determination,* acquiring the potential to become either osteoclasts or macrophages. The transcription factor PU.1 is required for the commitment of myeloid precursors to macrophage and osteoclast precursors.[62] Macrophage-colony stimulating factor (M-CSF) secreted by osteoblasts, through its action on the *c-fms* receptor on early but already committed progenitors, promotes proliferation, differentiation and survival of these cells.[62] A phase of lineage-specific differentiation follows when the early response gene *c-fos* permits differentiation into the osteoclast-lineage and away from macrophages.[62]

Cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and osteoclast precursors is required for osteoclastogenesis.[7] Recently various research groups have identified some of the proteins involved in the interaction between cells of osteoblastic and osteoclastic lineage. These proteins belong to the families of tumor necrosis factors and receptors. RANKL (Receptor activator of nuclear factor (NF)-κβ ligand), a protein expressed on the osteoblast cell membrane, binds to RANK (Receptor activator of NF-κβ) a receptor located on the osteoclast membrane.[8] This cell-to-cell interaction initiates a signaling cascade downstream of RANK/RANKL. RANKL interacts with RANK to recruit tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF-6), a member of a family of TNF signal transducers. TRAF-6 binds to binding sites in the intracellular domain of the RANK

receptor and then signals downstream through several signaling cascades, most notably those involving NFκβ, mitogen-activated protein (MAP) kinases, extracellular signal-regulated kinase (Erk), Janus N-terminal kinase (JNK), and p38.[62-64] Three distinct variants of RANKL have been identified: 1) a transmembrane cell bound variant,[9] 2) a soluble (cleaved) form,[9,65] and 3) another secreted form produced by activated T cells.[66]

A number of molecular 'markers' of the mature osteoclast, each with important roles to play in osteoclast function, have been identified. These include tartrate-resistant acid phosphatase (TRAP) activity, calcitonin receptors (CTR), vitronectin receptors (αvβ3 subunits) carbonic anhydrase II, cathepsin K and vacuolar proton ATPase (H^+-ATPase). However, the singular defining feature of the active osteoclast is its ability to resorb bone.[67] Figure 2.6 depicts the differentiation pathway of osteoclast progenitors into functionally active osteoclasts.[68]

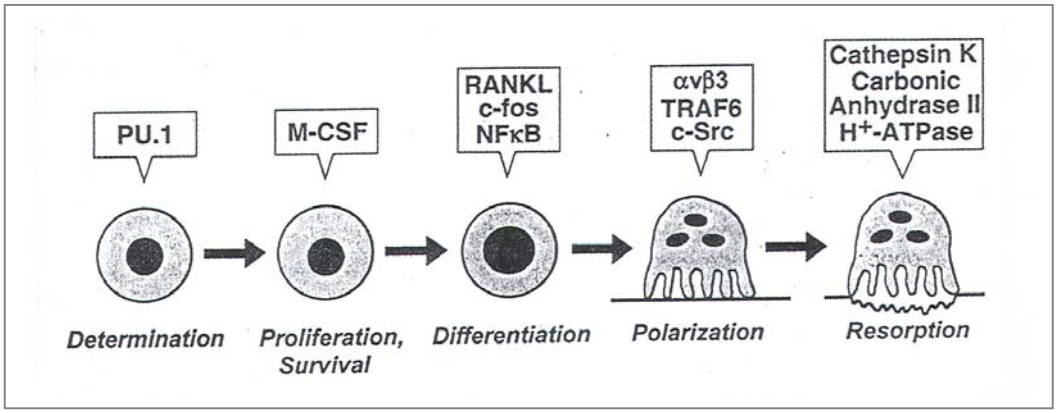

Figure 2.6 Differentiation of osteoclast progenitors into functionally active osteoclasts.

The transcription factors and cytokines required for each step of the pathway is indicated.

(Reprinted with permission from Teitelbaum SL. Bone resorption by osteoclasts. Science 2000;289:1504-8.[68]) Copyright (2000)AAAS.

Experimental data suggest that osteoclasts are not only derived from immature cells but also from mature cells of the monocyte-macrophage lineage when a suitable microenvironment is provided by bone marrow derived stromal cells.[69]

2.3.3.2 Regulation of osteoclast differentiation and activation by the osteoprotegerin-RANK-RANKL system

Cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and osteoclast precursors is required for osteoclastogenesis.[7] When RANKL (expressed on the osteoblast cell membrane) binds to its receptor RANK (located on the osteoclast membrane) it not only stimulates osteoclastogenesis, but also activates these cells to become mature resorbing osteoclasts.[8] Macrophage-colony stimulating factor produced by osteoblastic cells, is also required for osteoclastic proliferation, differentiation and survival.[8,62]

Osteoprotegerin (OPG*),* a secreted member of the tumor necrosis factor receptor family, is produced by cells of the osteoblast lineage.[10] Binding of RANKL and RANK can be prevented by OPG binding to RANKL. If the binding between RANK and RANKL is interrupted by OPG, the osteoclast precursor cannot differentiate and fuse to form mature resorbing osteoclasts. OPG acts as a *decoy* receptor in the RANK-RANKL signaling system inhibiting osteoclast formation.[10] Apart from inhibiting osteoclast formation, OPG is also involved in suppressing osteoclast survival.[11,70] The presence of OPG in the bone microenvironment therefore limits the number of mature osteoclasts and has a determining effect on resorption rate and bone mass. The regulation of osteoclast differentiation and activation by the OPG-RANK-RANKL system is depicted in figure 2.7. [64]

Overexpression of the osteoblast-specific transcription factor Cbfa1 in human osteoblast-like osteosarcoma cells, results in a significant increase in the level of OPG secreted into the culture medium of these cells.[71] These results indicate that Cbfa1, in addition to its role in osteoblast differentiation and osteoblast maintenance, could also inhibit osteoclast formation and activity by stimulating OPG gene expression.

Tissues other than bone (e.g., lung, kidney, thyroid and endothelial cells) also produce OPG.[10,72-73] The physiological functions of OPG in these tissues are not clear yet. OPG is implicated as a potent survival factor for endothelial cells, thereby implicating OPG as a potential regulator of vascular homeostasis.[73]

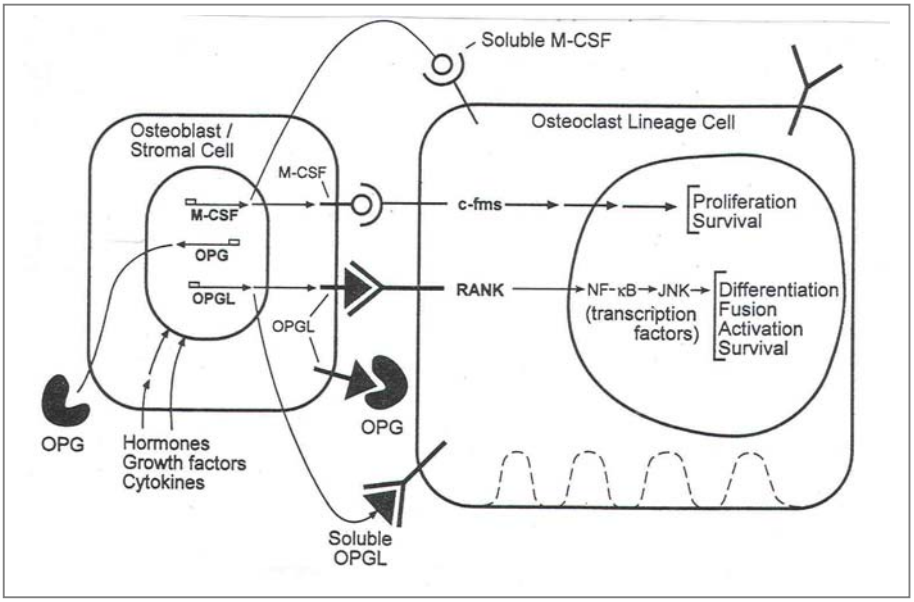

Figure 2.7 Interactions of osteoprotegerin, RANKL (OPGL) and RANK on the differentiation and activation of osteoclast precursors.

M-CSF and its receptor c-fms act as proliferation and survival factors for preosteoblasts. The subsequent differentiation of osteoclasts require RANKL (OPGL). Hormones, growth factors and cytokines, which direct the synthesis of membrane-bound (and secreted) OPGL and secreted OPG from osteoblasts and stromal cells, regulate bone resorption. The relative amounts of OPG and OPGL produced will dictate the osteoclast response. Binding of RANKL (OPGL) to RANK leads to activation of transcription factors. This binding results in the differentiation, fusion, and activation of osteoclasts. OPG serves as a secreted 'decoy' receptor, which opposes the RANKL-RANK interaction. *(Reproduced with permission from Kostenuik PJ, Shalhoub V. Osteoprotegerin: a physiological and pharmacological inhibitor of bone resorption. Curr Pharm Des 2001;7:613-35).*[64]

Local inflammation within bone due to metastasis, infections or fractures, or joint inflammation in arthritis, attracts T cells. It has been shown that activation of T cells might influence bone metabolism as these activated T cells produce RANKL, both in a membrane-bound and secreted form. It is therefore suggested that activated T cells could trigger osteoclastogenesis culminating in bone loss through RANKL.[66,74]

2.3.4 Cell proliferation and cell death

Since osteoblasts and osteoclasts have opposing effects on bone homeostasis, the number of functional osteoblasts and osteoclasts will affect bone's structure and strength. Cell number is dependent on the rate of replication and the number of cells dying through oncosis (toxic cell death) or apoptosis (programmed cell death).

Somatic cells alternate between periods of growth and division (mitosis). In the cell cycle the period of cell growth is known as interphase and is divided into three stages: **G_1**, a period of protein synthesis and organelle production; **S**, the period during which DNA is replicated in preparation for cell division; and **G_2**, a period of protein synthesis and final preparations for cell division. Cells can also enter from the G_1 phase in a rest phase, called the **G_0** phase. Cell replication takes place by means of mitosis (M phase) that is divided into prophase, metaphase, anaphase and telophase.[75] Stimulation of cell proliferation depends on the activity of the cell cycle. Cyclins and cyclin-dependent kinases (cdks) regulate cell cycle progression. Specific cyclin and cdk complexes are responsible for progression through each stage of the cell cycle.[76]

Cell death can either be caused by oncosis or apoptosis. Oncosis is a *passive* process that is induced by lethal chemical, biological or physical events resulting in cells being lysed or cell membranes ruptured with the resultant leakage of cytosol into the surroundings.[77] The escape of the cytosol releases kinins, lysosomal proteases and lipases into the tissue that stimulate inflammation.[78]

Apoptosis, or programmed cell death, is a biological process that eliminates unwanted cells. It therefore represents the *physiological* mode of cell death. The majority of nucleated cells appear to possess the genetic programming to undergo apoptosis.[79] Apoptosis is an *active* process which is controlled from within the cell by a large number of regulatory factors, but can be induced or inhibited by external factors through receptor-mediated mechanisms.[79-80] Wang *et al* (1999) defines apoptosis as "a gene-directed mechanism activated as a suicidal event to get rid of excess, damaged, or infected cells".[81] Apoptosis can be induced by activators such as tumor necrosis factor α (TNFα), oxidants, free radicals and bacterial toxins.[82] Presence or absence of specific growth factors, nutrients and hormones also affects

induction of apoptosis.[77,82] It has been shown that nitric oxide (NO) can promote apoptosis in some cells, whereas it inhibits apoptosis in other cells.[83]

Apoptosis is characterised by a sequence of morphologically recognisable events. Initially, an individual cell becomes detached from its neighbours and shrinks morphologically. Condensing of the chromatin and ruffling of the plasma membrane known as 'budding' then follows.[75,78,84] Cell fragments 'pinch off' as separate bodies (known as apoptotic bodies) that contain the condensed cytoplasmic proteins and intact organelles with nuclear fragments.[78,80] Adjacent cells recognise the apoptotic bodies and rapidly eliminate them through phagocytosis thereby avoiding an inflammatory response. Bratton and Henson (2005) suggested that oxidation of the dying cell's membrane lipids may provide important recognition signals to scavenger receptors on the phagocyte, thereby aiding phagocytosis of the apoptotic remnants.[85] The process of apoptosis occurrs quickly and cells undergoing this form of death disappear within hours without causing damage to surrounding cells or tissues.[78,80] Figure 2.8 shows the morphological events characteristic of apoptosis and oncosis.[84]

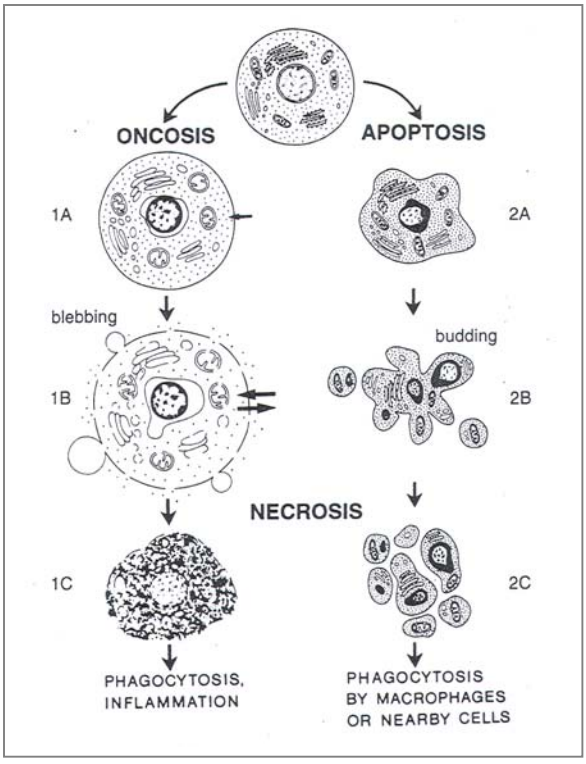

Figure 2.8 Illustration of the morphological events characteristic of apoptosis and oncosis. *(Majno G, Joris I. Apoptosis, oncosis, and oncosis. Am J Pathol 1995;146:3-15.)*[84]

Apoptosis is initiated via two main stimuli. The *extrinsic pathway* signals through a receptor-ligand mediated mechanism while the *intrinsic pathway* is triggered in response to DNA damage and is associated with the release of cytochrome c from the mitochondrial intermembrane space into the cytoplasm.[86] These mechanisms activate cysteine proteases (caspases) which are responsible for the characteristic morphological changes observed during apoptosis.[75] Two opposing mechanisms maintain homeostatic control of apoptosis. Fas ligand promotes cell death by binding to Fas, a cell membrane receptor, initiating a cascade of events leading to apoptosis. In contrast, the *Bcl-2* protein (a member of the *Bcl-2* family of pro- and anti-apoptotic mediators) prevents the release of cytochrome c from the mitochondrial membrane, thereby inhibiting mitochondrial-associated apoptosis.[78]

Jilka *et al* (1998) demonstrated that a large number of both human and murine osteoblasts undergo apoptosis and that growth factors and cytokines in the bone microenvironment can modulate this process *in vitro.*[42] It has been shown that transforming growth factor-β (TGF-β) as well as the cytokine interleukin-6 (IL-6) had antiapoptotic effects on osteoblasts as they were able to counteract the apoptotic effect of serum starvation.[42] Based on these results it was speculated that *in vivo* induction of apoptosis might be attributed to either an increased sensitivity to apoptosis-inducing agents or alternatively to decreased concentrations of anti-apoptotic growth factors and cytokines in the osteoblasts' immediate vicinity.[42] Apoptosis of osteoblasts might also be enhanced as a result of increased concentrations of pro-apoptotic factors such as tumor necrosis factor (TNF) or Fas ligand.[42] Lynch *et al* (1998) suggested that cell death by apoptosis is a fundamental component of osteoblast differentiation that contributes to maintaining tissue organisation.[87]

2.4 Bone remodeling

Bone is remodeled continuously during adulthood through the resorption of old bone by osteoclasts and the subsequent formation of new bone by osteoblasts. Bone remodeling, also known as bone turnover, takes place only on the surface of bone in closely coordinated local packets. As cancellous bone (trabecular bone) makes up

more than 80% of bone's surface, it is more metabolically active and more rapidly remodeled than cortical bone. [37]

Normally, bone remodeling proceeds in cycles in which osteoclasts adhere to bone and subsequently remove it by acidification and proteolytic digestion. Shortly after the osteoclasts have left, osteoblasts invade the area and begin the process of secreting osteoid thereby forming new bone, which is eventually mineralised. Thereafter, a distinct type of differentiated osteoblasts, the lining cells, cover the surface of the bone.[88]

The process of bone remodeling is coupled which means that bone formation is linked to bone resorption. The bone remodeling cycle consists of specific steps known as 1) activation, 2) resorption, 3) reversal, 4) formation and 5) mineralisation.[37] A schematic diagram of the remodeling cycle is depicted in Figure 2.9. [89]

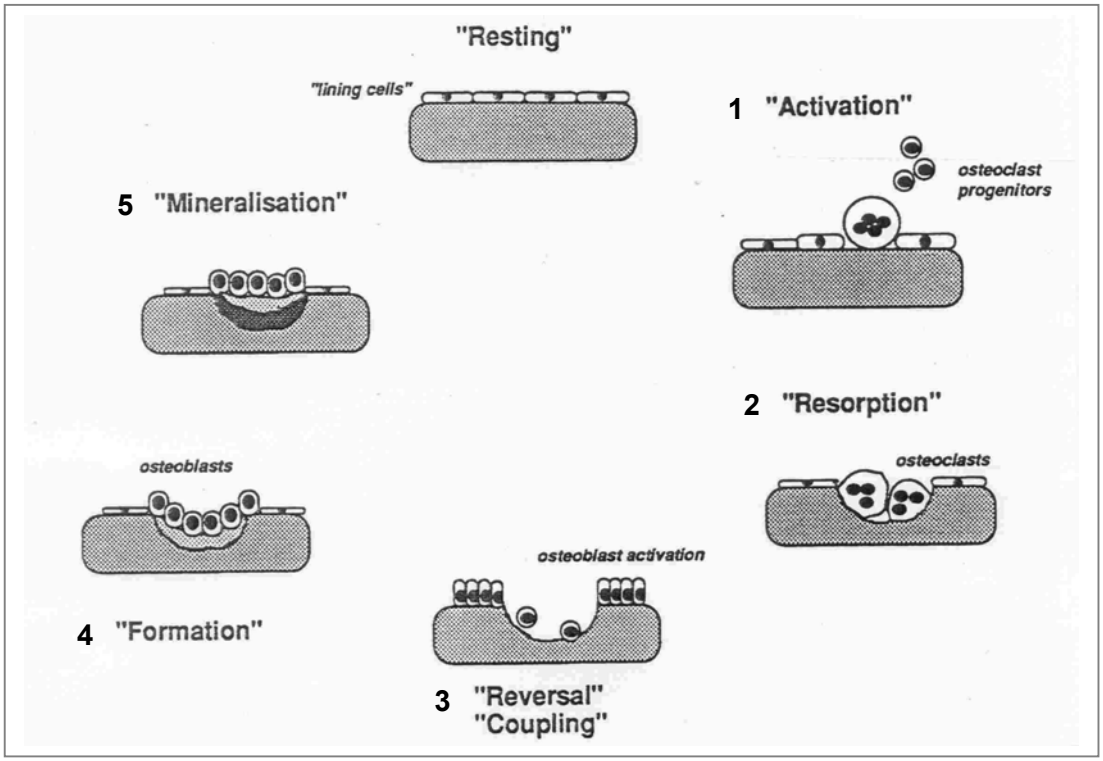

Figure 2.9 Schematic diagram of the bone remodeling cycle.
(Reprinted from Gowen M. Cytokines and cellular interactions in the control of bone remodelling. In: Heersche JNM, Kanis JA, editors. Bone and Mineral Research/8. Amsterdam Elsevier Science. 1994. p. 77-114.[89]) Copyright (1994), with permission from Elsevier.

2.4.1 Resorption

Bone resorption is a multistep process initiated by the proliferation of immature osteoclast precursors, the commitment of these cells to the osteoclast phenotype, and finally, degradation of the organic and inorganic components of bone. The cycle begins with recruitment of osteoclastic precursors from bone marrow monocyte precursors, which attach to the bone surface. Recent research[90] indicates that osteoclast precursors and mature osteoclasts have the capacity to modulate the activity of osteoblasts, and that, yet unknown membrane-bound signaling molecules are essential in inducing retraction of osteoblasts and the subsequent formation of a cell-free area for attachment of mature osteoclasts.

Osteoclast progenitors move from bone marrow to bone either through the circulation or by direct migration from the marrow. Signals targeting osteoclasts to bone and resorption sites are not well characterised and are currently under investigation. Small chemotactic cytokines (also known as chemokines), such as stromal cell-derived factor-1 (SDF-1) seem to be important in attracting osteoclastic precursors to resorption sites. SDF-1 is constitutively expressed at high levels within bone and is a ligand for its receptor CXCR4 that is constitutively expressed on circulating monocytes and pre-osteoclasts.[91]

After activation by the RANK-RANKL mechanism (Refer to Figure 2.7), the differentiated osteoclast polarises on the bone surface. Close physical contact between matrix and osteoclast is required for resorption, and it appears that the recognition of bone by osteoclasts is controlled by transmembrane integrins such as $\alpha v ß 3$ integrin. A ruffled border develops beneath the osteoclast, sealing the space beneath the cell. The osteoclast generates H^+, lactate, and proteolytic enzymes into this subcellular space, which cause a breakdown of the protein matrix and release calcium and other bone mineral constituents.[37,68,92] A recent publication[93] suggests that autocrine nitric oxide (NO) production by osteoclasts might be important in regulating attachment and motility of human osteoclasts *in vivo*. Figure 2.10 depicts bone resorption by osteoclasts.[92]

Figure 2.10. Osteoclastic bone resorption.

Multinucleated osteoclasts adhere to bone through $\alpha_v\beta_3$-integrins. Carbonic anhydrase II (CAII) can generate H^+ and HCO_3^-. HCO_3^- is extruded for Cl^- at the basolateral membrane. H^+ can be secreted through an H^+-ATPase on the apical membrane (ruffled border) into the resorption space and Cl^- is secreted through a Cl^- channel. HCl will dissolve matrix mineral. Phosphatases and cysteine proteinases - notably cathepsin K- will be released from lysosomes and degrade matrix proteins. Metalloproteinases that are released from secretory vesicles will also degrade matrix proteins *(Republished with permission of Nature Publishing Group from Goltzman D. Discoveries, drugs and skeletal disorders. Nature Rev Drug Discov 2002;1:784-96[92]; permission conveyed through Copyright Clearance Center, Inc.)*

2.4.2 Reversal

The regulatory mechanisms that stop osteoclastic activity are poorly understood. Raisz,[6,94] and Teitelbaum(2000)[68] list the following possiblities:

i) Osteoclasts have limited life spans. Anti-osteoporosis agents such as oestrogen (E2) could be involved in osteoclast apoptosis;

ii) Accumulation of calcium at high concentrations in the ruffled border area of the osteoclast results in inactivation;

iii) The release of TGF-β or related peptides from the matrix inactivates osteoclasts and attracts osteoblasts;

iv) During the reversal phase, osteoclasts disappear and macrophagelike cells appear on the bone surface. These cells could also release

factors such as TGF-β that inhibit osteoclasts and stimulate osteoblasts.

2.4.3 Formation and mineralisation

After the osteoclasts have excavated a resorption pit or lacuna, osteoblasts are recruited to this site where they synthesise, secrete, organise, and mineralise osteoid. Osteoid is composed predominantly of type I collagen and other noncollagenous proteins, such as osteopontin, osteonectin, and osteocalcin. Following its formation, osteoid normally undergoes rapid mineralisation with calcium and phosphorus.[37] Local and systemic factors control the replication and differentiation of successive waves of osteoblasts that replace the resorbed bone. Local factors that stimulate formation could be derived from osteoclasts, reversal cells, or marrow cells as well as from the bone matrix itself. Systemic hormones influence osteoblast replication and differentiation.[94]

2.5 Regulation of bone remodeling

Bone remodeling is regulated by both local and systemic factors, including hormones, growth factors and cytokines.[37,95]

2.5.1 Circulating hormones

Although various hormones are known to affect bone resorption and turnover, the most relevant to this presentation are oestrogen, PTH and $1,25(OH)_2D_3$ and will therefore be discussed.

2.5.1.1 Oestrogen (17β-estradiol)

The main cause of bone loss in postmenopausal osteoporosis is oestrogen deficiency,[96] which results in increased osteoclastogenesis causing an imbalance between bone formation and resorption.[97-98] Oestrogen treatment on the other hand, has long been known to inhibit bone loss in postmenopausal women.[99-100]

The principal *in vivo* effect of oestrogen on bone is a decrease in bone resorption, mostly by indirect actions such as regulation of growth factor and cytokine production in osteoblasts and their precursors, which, in turn, regulate osteoclast differentiation and activity.[101] In mature osteoblasts in culture, oestrogen has been shown to induce synthesis TGF-β, insulin-like growth factor-I (IGF-I), and IGF-binding proteins, and to inhibit synthesis of IL-1, IL-6, and IL-11.[97,101-102] Oestrogen, by regulating the levels of local growth factors and cytokines, indirectly manipulates the bone microenvironment thereby affecting bone metabolism.[98] Oestrogen acts on osteoblasts through high affinity oestrogen receptors (ER) located on the nuclear membrane.[35] The molecular mechanisms of oestrogen action on bone however, are not completely understood.

Recent reports demonstrated the involvement of OPG in oestrogen's paracrine-mediated effects in bone. *In vitro* oestrogen exposure dose- and time-dependently stimulates OPG secretion in human osteoblasts[103-105] and mouse bone marrow stromal cells;[103] OPG levels were highest in osteoblasts expressing the largest number of oestrogen receptors.[105] Oestrogen's protective effect on bone could well be explained by its stimulatory effect on OPG synthesis as OPG is known to be a potent inhibitor of osteoclast formation and activation. A local increase in OPG in the bone microenvironment may therefore be an important mechanism by which oestrogen reduces bone resorption. Oestrogen withdrawal, after a five-day pretreatment, mimicking the event occurring *in vivo* at menopause, dramatically down-regulates OPG expression in mouse bone marrow stromal cells.[103] As OPG specifically blocks RANKL-RANK interaction and therefore inhibits osteoclast differentiation and function,[10] down-regulation of OPG expression upon oestrogen withdrawal could increase osteoclastic bone resorption. Oestrogen, apparently, also

modulates M-CSF and RANK expression,[98,106] further enhancing the effects of OPG on the RANK-RANKL system.

In summary, it has been postulated that oestrogen inhibits bone resorption by inducing small but cumulative changes in multiple oestrogen-dependent regulatory factors.[101,107-109] Figure 2.11 illustrates a model of the interaction and coupling between osteoblasts and osteoclasts via OPG, RANKL (OPGL), and other growth factors and cytokines.[101]

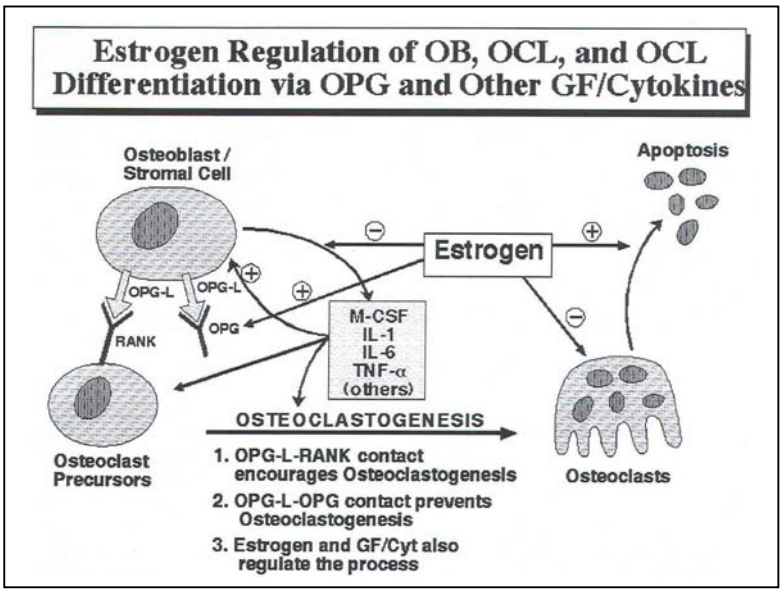

Figure 2.11. Oestrogen regulation of osteoblasts, osteoclasts, and osteoclast differentiation via osteoprotegerin (OPG) and other growth factors and cytokines.
(Spelsberg TC, Subramaniam M, Riggs BL, Khosla S. The actions and intractions of sex steroids and growth factors/cytokines on the skeleton. Mol Endocrinol 1999;13:819-28[101]) Copyright 1999, The Endocrine Society.

Although most of oestrogen's bone protective effects are modulated via osteoclast-mediated mechanisms, oestrogen has also been shown to act on osteoblasts. In a murine bone marrow cell model, oestrogen regulated early differentiation of osteoblastic progenitor cells in an ER-dependent way.[50,110] In another study,[50] oestrogen stimulated mRNA expression of Cbfa1 and down-regulated mRNA expression of PPAR-γ 2 in bone marrow stromal cells, which resulted in increased osteoblast numbers and decreased adipocyte numbers. It therefore seems that oestrogen not only inhibits bone resorption indirectly by regulating osteoclast numbers, but also affects bone formation by stimulating osteoblast differentiation from osteoblastic precursors in the bone marrow.

2.5.1.2 Parathyroid Hormone (PTH)

In humans, PTH is the major calcium-regulating hormone that maintains adequate levels of plasma calcium in times of dietary calcium deficiency by promoting bone resorption and inhibiting renal calcium excretion.[111-112] Calcium-sensing proteins, that register plasma calcium concentrations, are found in the parathyroid gland. When calcium concentrations decrease below normal, PTH is secreted into the circulation and proceeds to the osteoclasts where it enhances bone resorption, thereby releasing calcium. In addition, PTH acts on the proximal convoluted tubule cells of the kidney, thereby increasing the activity of 1α-hydroxylase which catalyses the formation of 1,25$(OH)_2D_3$ (calcitriol), the hormonal form of vitamin D_3 that stimulates intestinal calcium absorption. Apart from PTH's effect on vitamin D_3 hormone activation, it also stimulates the active reabsorption of calcium from the distal renal tubules. As soon as the serum calcium concentration exceeds the set point of the calcium-sensing system, it shuts down the parathyroid gland-induced cascade of events.[112]

PTH has complex effects on bone, depending on the mode of administration.[113-115] *Continuous* PTH administration results in enhanced bone resorption. *Intermittent* PTH therapy, while having a net anabolic effect on bone, stimulates both bone formation and bone resorption.[34,116-118] The mechanisms of these observed effects of PTH are not yet fully understood. However, it has been suggested that it could in part be explained by desensitisation of the PTH/PTH-related peptide (PTHrP) receptors.[119] G protein-coupled receptors (GPCRs) play a key role in regulating bone remodeling. Whether GPCRs exert anabolic or catabolic effects in bone may be determined by the rate of receptor desensitisation in osteoblasts. *Continuous* presence of PTH might attenuate the reponsiveness of GPCRs; in contrast to *intermittent* stimulation of the receptor, which permits prolonged activation of signaling pathways leading to net bone formation.[119] PTH, apparently also induces several growth factor genes, including those for IGF-I, IGF-II, and TGF-β, thereby indirectly affecting the bone microenvironment and bone quality.[120]

Parathyroid hormone effects on osteoprotegerin (OPG) and RANKL

Depending on the model used, disparate results have been reported on the effects of PTH on the OPG/RANKL ratio. Experimental evidence suggests that PTH administration rapidly and transiently inhibits the level of OPG mRNA in bone cells both *in vivo* and *in vitro.*[121-123] Regulation, however, is complex and depends on the differentiation status of the cells as well as the interval after stimulation when they were examined. In murine bone marrow cultures, PTH stimulates RANKL and inhibits OPG expression thereby adversely affecting the OPG/RANKL ratio.[121,122,124-125] Based on experimental results reported by Huang *et al* (2004),[111] it was suggested that PTH might induce a possible switch in the regulatory mechanism of osteoclastogenesis where OPG is inhibited early and RANKL is increased at later stages of osteoblast differentiation.

Parathyroid hormone effects on osteoblast numbers

The number of functioning osteoblasts available largely determines the *in vivo* bone formation rate. Experimental results reported by Jilka *et al* (1999) suggests that the increased osteoblast number, bone formation rate, and bone mass caused by daily PTH injections to mice is caused by an anti-apoptotic effect of this hormone on the osteoblasts.[126]

Using cultured murine marrow cells, [117] it was shown that *intermittent* PTH treatment increases mRNA for osteoblastic differentiation markers e.g., Runx2, alkaline phosphatase and type I procollagen. *Continuous* treatment on the other hand, resulted in production of large numbers of mature osteoclasts. Experimental evidence suggests that *intermittent* PTH treatment enhances osteoblast differentiation through an IGF-I dependent mechanism whilst *continuous* PTH treatment enhances osteoclastogenesis through reciprocal increases in RANKL and decreases in OPG.[117]

2.5.1.3 1,25-Dihydroxy vitamin D_3 (calcitriol)

Vitamin D_3 is a prohormone that can be obtained from the diet or formed in skin through ultraviolet irradiation of 7-dehydrocholesterol. It is biologically inert and must be metabolised to 25-hydroxyvitamin D_3 in the liver and subsequently to $1,25(OH)_2D_3$ (calcitriol) in the kidney upon PTH stimulation, as previously described (refer to 2.5.2.2). Calcitriol, the hormonal form of vitamin D_3, acts through a nuclear receptor to carry out its many functions, including active absorption of calcium and phosphorus in the intestine, calcium mobilisation in bone, and calcium reabsorption in the renal tubule.[112,127]

In vivo bone effects of calcitriol depend on its dose levels. It has been shown in rats as well as in an ovariectomised (OVX) mouse model that pharmacological doses of this hormone are required to induce bone resorption.[128-129] These high doses of calcitriol increase *in vivo* serum Ca^{2+} and expression of RANKL in the presence of PTH. Physiological doses of calcitriol on the other hand, do not stimulate bone resorption but rather inhibit bone resorption by inhibiting PTH-induced expression of RANKL mRNA.[128] At cellular level, it was shown that calcitriol accelerates *in vitro* osteoclastogenesis by upregulating RANKL gene expression in human osteosarcoma osteoblastic cells[130] and bone marrow stromal cells.[131] However, Hofbauer *et al* (1998)[132] reported that calcitriol up-regulated OPG mRNA expression as well as OPG protein synthesis in a human fetal osteoblastic cell line (hFOB) and normal trabecular osteoblastic cells.

2.5.2 Prostaglandin E_2

Osteoblasts produce prostaglandins from fatty acid precursors. Prostaglandins, especially PGE_2 derived from its precursor arachidonic acid, have pronounced effects on bone. Prostaglandins are likely to be local mediators *in vivo* because they do not circulate in significant amounts.[134] Depending on the concentration and experimental model, both anti-resorptive and pro-resorptive effects of prostaglandins have been reported.[134]

PGE_2 stimulates osteoclast formation in bone marrow cultures (10^{-10} to 10^{-5} M),[135-137] increases expression of mRNA for RANKL (10^{-6} M)[138] and down-regulates OPG in cultures of primary human bone marrow stromal cells (10^{-10} to 10^{-6} M).[139] These reported effects of PGE_2 on OPG and RANKL will ultimately have a detrimental effect on the OPG/RANKL ratio in the bone microenvironment and could ultimately lead to a decrease in bone mass as previously described.

Inflammatory conditions are associated with increased PGE_2 levels.[134,140] One could therefore speculate that the effects of PGE_2 on OPG (downregulation) and RANKL expression (upregulation) might be the cause of increased bone loss adjacent to inflammatory tissues, as is observed in rheumatoid arthritis and other diseases. Non-steroidal anti-inflammatory drugs (NSAIDS) e.g. indomethacin have been shown to inhibit bone loss *in vivo* and bone resorption *in vitro*, and this is associated with a loss of osteoclasts from the bone surface.[141] Increased OPG secretion was reported after PGE_2 inhibition by indomethacin in mouse calvaria *in vitro*.[142]

Prostaglandins have dual effects on bone formation. Several *in vivo* animal studies proved that PGE_2 administration increases bone formation.[143-145] In organ cultures, stimulation of DNA, collagen, and noncollagen protein synthesis is observed with low concentrations of PGE_2 whilst high PGE_2 concentrations inhibits collagen synthesis.[146] The stimulatory effects of prostaglandins may depend on their ability to stimulate endogenous growth factors such as IGF-I, bone morphogenetic protein-7 (BMP-7) and BMP-2.[147-149] For a detailed description of prostaglandins' effects on bone metabolism see 2.11.

2.5.3 Growth factors and cytokines

Growth factors are proteins that serve as signaling agents for cells. They are synthesised by osteoblasts, nonosteoblast skeletal cells, and marrow cells and function as part of a vast cellular communications network that influences critical functions such as cell division, matrix synthesis, and tissue differentiation.[150] Regulation of bone volume may in part depend on local growth promoting activities of these bone growth factors. Apart from IGFs, human bone cells in culture produce

IGF-binding proteins (IGFBPs), which have been shown to modulate IGF actions in bone.[45,151]

Large amounts of growth factors, of which IGF-II and TGF-β are the most abundant, are deposited in the mineralised matrix of bone. When these growth factors are released again during osteoclastic bone resorption, they may act on preosteoblasts thereby allowing for a site-specific replacement of bone that is lost to resorption. Systemic hormones such as PTH, oestrogen, progesterone and vit D_3 may modulate local bone formation, at least in part, through regulation of synthesis and release of bone growth factors.[6,94,101,151]

The mechanisms whereby growth factors exert their effects are not clear but Jilka *et al* (1998) presented evidence that growth factors such as TGF-β and IL-6 type cytokines prevent osteoblast apoptosis which suggests that osteoblast survival is regulated by factors produced in the bone microenvironment.[42] Growth factors have also been reported to impact on the OPG-RANKL system. Hofbauer *et al* (1998), for instance, have shown that BMP-2 increases OPG production in human osteoblast lineage cells.[132] Experimental data have shown that low TGF-β levels stimulate osteoclast differentiation by affecting the RANKL/OPG ratio while high TGF-β levels repress osteoclast differentiation by multiple mechanisms independent of the RANKL/OPG ratio or M-CSF expression regulation.[152]

Cytokines are extracellular protein messengers that regulate immune responses. TNF-α, TNF-β and IL-1 stimulate osteoclast recruitment and are potent stimulators of bone resorption.[94,153-155] At least part of the effects of IL-1 on bone resorption is prostaglandin mediated, since the prostaglandin synthetase blocker indomethacin partially inhibits them.[133,153,155] Hofbauer *et al* (1998) reported stimulation of OPG production by IL-1ß and TNF-α in osteoblast lineage cells,[132] which could ultimately inhibit osteoclastogenesis and osteoclast activation.

Interleukin-6 (IL-6) is a multifunctional cytokine, which, apart from its immunomodulatory effects, affects osteoclastic bone resorption. IL-6 is produced in nanomolar quantities by both stromal cells and osteoblastic cells and production thereof is stimulated by systemic hormones as well as various bone resorptive agents such as PTH, PGE_2, IL-1, and TNF.[88,156-158] IL-6 has been shown to stimulate

osteoclastogenesis in organ culture systems[133,156] as well as mesenchymal cells.[156] Evidence has recently been provided for cross-talk between PGE_2 and IL-6 signaling that enhance osteoclast differentiation via effects on the OPG/RANKL/RANK system in bone cells.[159]

2.6 Summary

Although bone is constantly being remodeled throughout life, the process of bone remodeling is normally tightly regulated. Two distinct types of bone cells mediate remodeling; the multinucleated osteoclast reponsible for bone resorption and the osteoblasts, which are bone forming cells. The process of bone remodeling is coupled which means that bone formation is linked to bone resorption. The process of bone remodeling proceeds through a number of steps, e.g. activation, resorption, reversal, formation and mineralisation that are summarised in figure 2.9.[89]

It is well known that cell-to-cell contact between osteoblasts (or bone marrow stroma cells) and osteoclast precursors are required for osteoclast formation. However, the precise mechanism by which pre-osteoblastic/stromal cells control osteoclast development, activation and subsequently bone resorption was unknown. The discovery of the RANK-RANKL-OPG system has solved this long-standing question in bone biology (summarised in Figure 2.7). As reported in the literature review, many bone-active agents that regulate osteoclastic bone resorption do so indirectly by controlling the production of RANKL or OPG by osteoblasts thereby affecting osteoclast maturation and activation. The balance between the osteoclast-promoting RANKL and the osteoclast-inhibiting OPG can therefore regulate the number and activity of osteoclasts. Figure 2.12 summarises some of the effectors of osteoclast formation, function, and apoptosis in the bone microenvironment.[109]

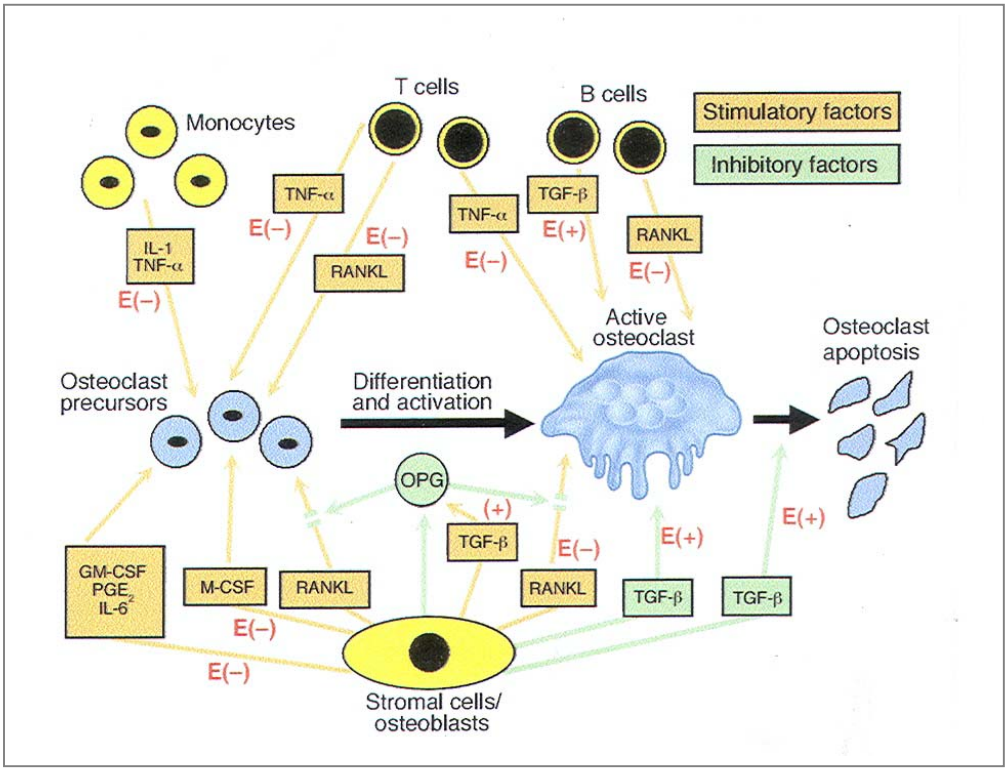

Figure 2.12. Regulation of osteoclast formation, function, and apoptosis by cytokines produced by bone marrow cells, osteoblasts, monocytes, T cells, and B cells.

Stimulatory factors are shown in orange and inhibitory factors in green. The effects of E (oestrogen) to enhance (+) and inhibit (-) the factors are shown in red. *(Republished with permission of The journal of Clinical Investigation Organisation from Bell NH. RANK ligand and the regulation of skeletal remodeling. J Clin Invest 2003;1120-2*[109]*) Permission conveyed through Copyright Clearance Center, Inc.*

In addition, Hofbauer *et al* (2000) proposed a *Convergence Hypothesis* for the regulation of osteoclast functions by cytokines.[14] According to this hypothesis, the regulation of RANKL and OPG by various systemic hormones, growth factors as well as cytokines are due to convergence at the level of RANKL and OPG, which then function as the final effector system to modulate differentiation and activation of osteoclasts. For example, the stimulation of RANKL by PTH and PGE_2,[124,138] and the inhibition of OPG by these same agents[125,139] may mediate the pro-resorptive effects of these agents. Figure 2.13 depicts this *Convergence Hypothesis* as proposed by Hofbauer *et al* (2000).[14]

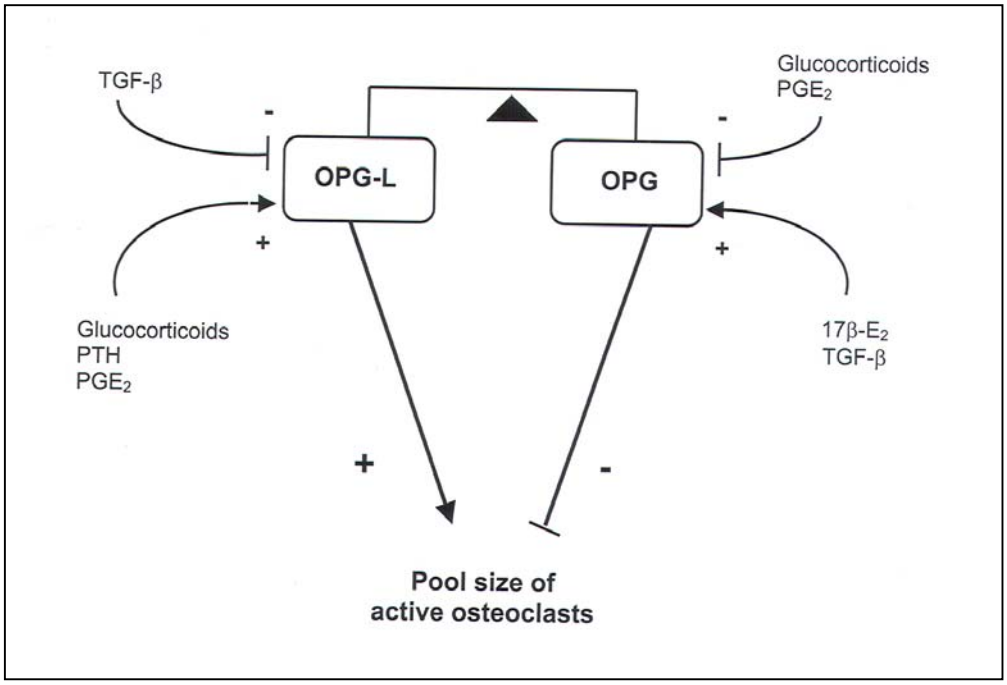

Figure 2.13 The 'convergence hypothesis' for the regulation of osteoclast functions by cytokines.

This hypothesis proposes two levels of regulation of osteoclast functions. A variety of "upstream" cytokines and hormones alter the pool size of active osteoclasts by converging at the level of OPG-L (RANKL) and OPG. These two "downstream" factors serve as the final effectors for osteoclastogenesis and also affect osteoclast activation and osteoclast apoptosis. At steady state, there is a "balance" of levels of OPG-L (RANKL) and OPG levels that maintain a pool size of active osteoclasts that supports normal levels of bone resorption. When a change in one or more upstream factors tilts the balance toward a functional excess of OPG-L (RANKL), the pool size of active osteoclasts increases; when the balance tilts toward a functional excess of OPG, the pool size decreases. 17β-E_2, 17β-estradiol; PGE_2, prostaglandin E_2; PTH, parathyroid hormone; TGF-β, transforming growth factor β.
(From: Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res 2000;15:2-12.[14]) With permission of the American Society for Bone and Mineral Research.

II Polyunsaturated Fatty Acids

2.7 Types of polyunsaturated fatty acids (PUFAs)

Fats contain fatty acids, which vary widely in the number of carbons and the number of double bonds in the carbon chain. Saturated fatty acids have no double bonds, whereas cis-unsaturated fatty acids have one (monounsaturated) or more (polyunsaturated) double bonds. Lengthening of the chain and the introduction of additional double bonds beyond the first one occur from the carboxyl-terminal of the fatty acid. Based upon the position of the first double bond, polyunsaturated fatty acids (PUFAs) are further classified in families: omega-3, or n-3, fatty acids have the first double bond between carbon atoms three and four (counting from the methyl end) whilst omega-6, or n-6, fatty acids have the first double bond between carbon atoms six and seven.[160]

2.8 Metabolic pathways of essential fatty acids

Two PUFAs, linoleic acid (LA) (18:2 c n-6) and α-linolenic acid (ALA) (18:3 c n-3) are considered essential fatty acids (EFAs), as they cannot be synthesised by humans and must be provided in the diet. Human biosynthetic enzymes can only insert a double bond at the n-9 position or higher; but not in any position closer to the methyl end such as position 6 or 3 as in the case of LA and ALA.[22] LA is found in seeds of most plants except coconut, cacao, and palm and ALA is found in linseed and chloroplast of green leafy vegetables.[22] These two fatty acids are converted via a series of desaturation and elongation steps by the same enzyme systems to different fatty acids, which serve as precursors for the eicosanoids.[40,161] Generally, the desaturation steps are slow and rate limiting, while the elongation steps usually proceed rapidly (Figure 2.14).[23]

The n-3 and n-6 PUFAs are competitive inhibitors of each other's metabolism. Competition is not as apparent in the elongation steps, which are rapid and allows large amounts of both series of PUFAs to be metabolised by the same common enzyme systems but is apparent at the slow desaturation steps, where a large

amount of one type of PUFA will interfere with the metabolism of the other. It has been found that n-3 PUFAs are more effective at inhibiting desaturation of n-6 PUFAs than vice versa.[162]

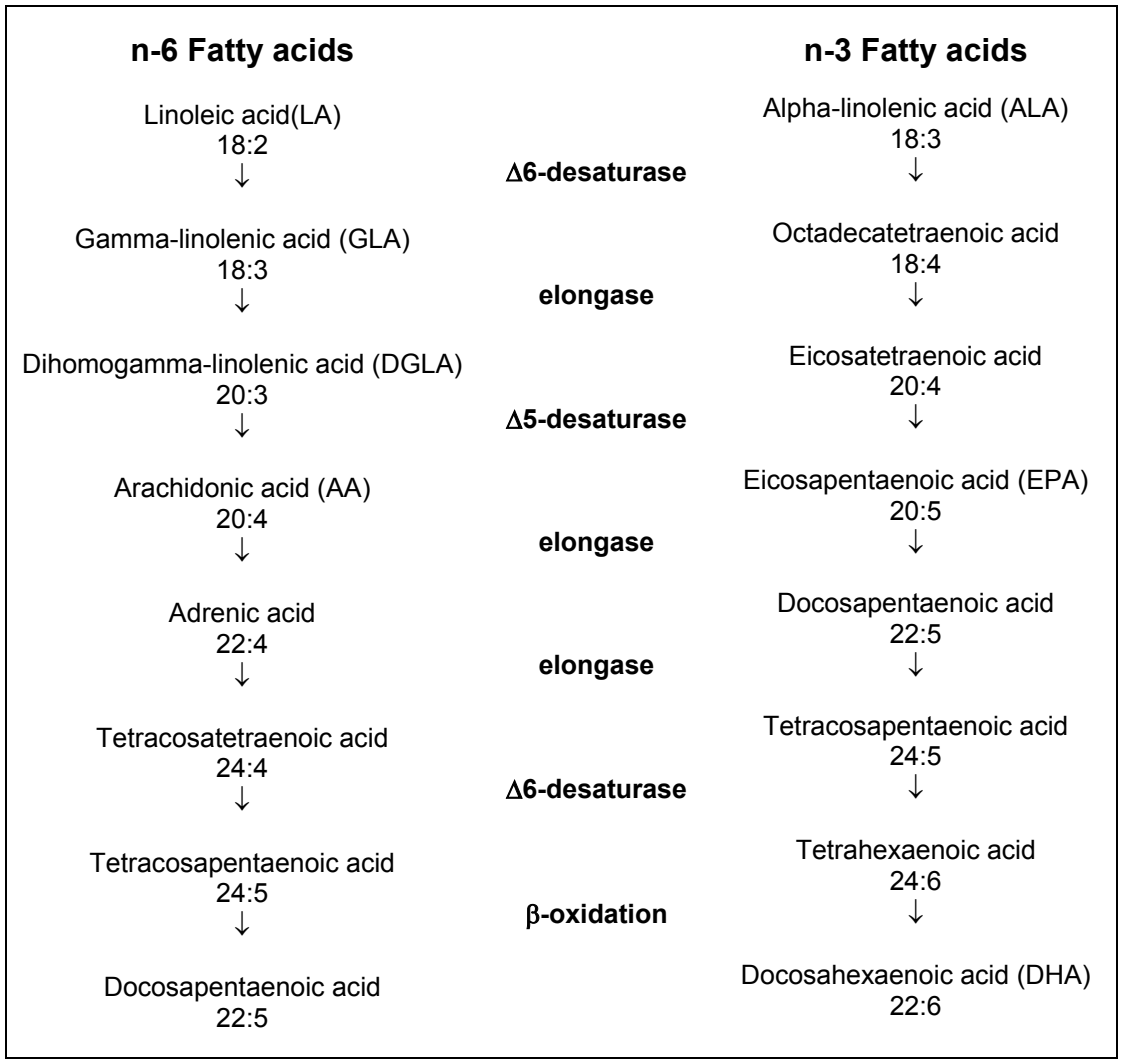

Figure 2.14. The elongation and desaturation pathways for n-3 and n-6 fatty acids.
(Reprinted from Kruger MC, Horrobin DF. Calcium metabolism, osteoporosis and essential fatty acids: a review. Prog Lipid Res 1997;35:131-51.[23]) Copyright (1997), with permission from Elsevier.

2.9 Cellular functions of polyunsatutated fatty acids

Dietary fatty acids can be oxidised to provide energy, stored in adipose tissue, or selectively incorporated into cell membranes. PUFAs have two fundamental physiological functions; they are present as structural phospholipids in high

concentrations in all membranes and are the primary precursors of eicosanoids. In addition to these roles, PUFAs can also affect cell function either by modulating intracellular signal transduction, modulating cell-cell interaction or modulating gene transcription.[22,163] These actions are initiated by phospholipases such as PLA_2 that releases PUFAs thereby enabling them to be metabolised to PUFA derivatives such as eicosanoids.[22]

2.9.1 Composition of membranes

Biological membranes surrounding cells and subcellular organelles exist primarily as lipid bilayers that are mainly composed of phospholipids and free cholesterol, which interface with a variety of proteins functioning as receptors, transporters, enzymes and ion channels.[161] Phospholipids contain a diverse range of PUFAs and manipulation of dietary lipids readily modify the fatty acid composition of membranes in both experimental animals and humans.[161,164-167] The presence of specific PUFAs may determine the biological properties of the membranes and the way cells respond to various stimuli. Because of unsaturation of the PUFAs they affect membrane properties such as fluidity, flexibility, and permeability which in turn affect functioning of such proteins as receptors, enzymes such as ATPases or ion channels.[162,163] It has been shown, for instance, that supplementation of the diet with evening primrose oil or fish oil containing considerable quantities of n-3 PUFAs increases the unsaturation index of intestinal brush border membrane vesicles and significantly enhances calcium transport.[168] A later study by Haag *et al* (2003) showed that n-3 PUFAs are involved in multiple signaling effects that affect ATPases in the basolateral membrane thereby enhancing calcium absorption.[169]

2.9.2 Eicosanoid synthesis

Essential fatty acids are the precursors of the eicosanoids, prostaglandins, leukotrienes, and other oxygenated derivatives, derived predominantly from the 20-carbon polyunsaturated fatty acids dihomogamma-linolenic acid (DGLA) (1 series prostaglandins), arachidonic acid (AA) (2 series prostaglandins) and eicosapentaenoic acid (EPA) (3 series prostaglandins). Eicosanoids are produced

via a cascade of steps starting with the cyclooxygenase (COX) or lipoxygenase (LO) enzymes. The main COX products comprise the classical prostaglandins, prostacyclin and the thromboxanes, while the main lipoxygenase products are leukotrienes (Figure 2.15).[163] Eicosanoids are produced locally as and when they are needed and have effects that are usually confined to the immediate vicinity of the cells in which they are produced.[160,162,170]

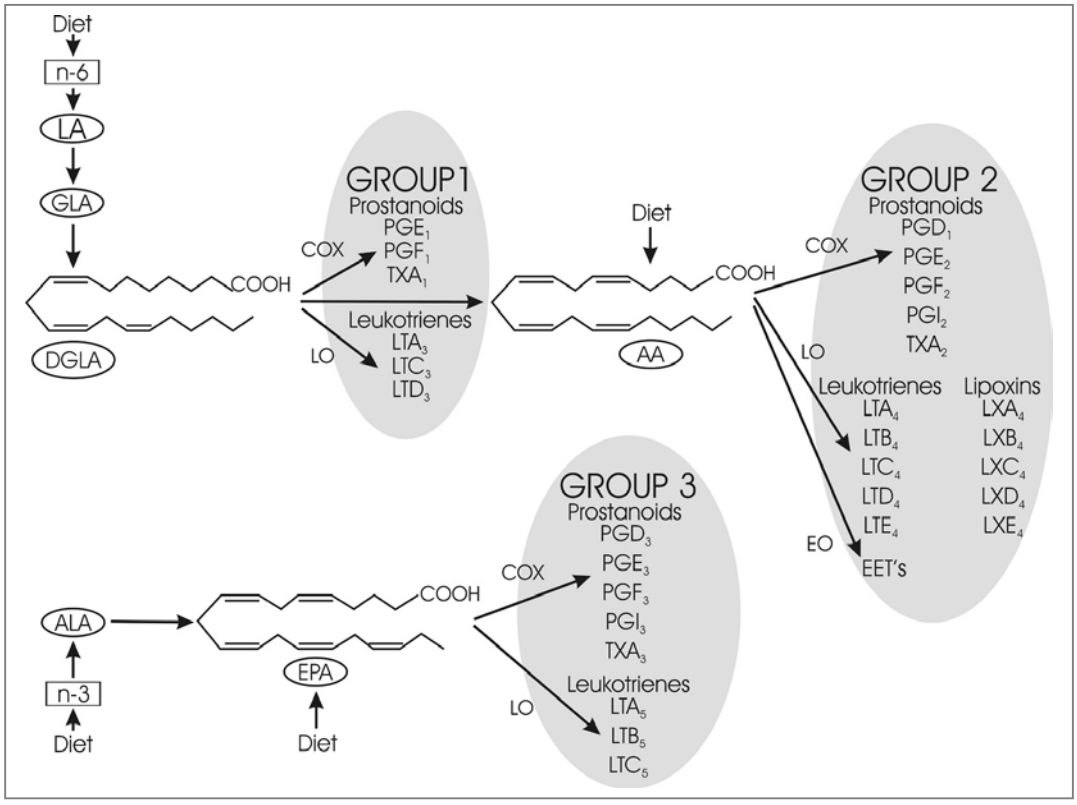

Figure 2.15. The synthesis of eicosanoids from polyunsaturated fatty acids.

LA: linoleic acid; GLA: gamma-linolenic acid; DGLA: dihomo-gamma-linolenic acid; AA: arachidonic acid; ALA: alpha-linolenic acid; EPA: eicosapentanoic acid; COX: cyclo-oxygenase,; LO: lipoxygenase;
EO: epoxygenase; PG: prostaglandin; TX: thromboxane; LT: leukotriene; PGI: prostacyclin; LX: lipoxin; EET: epoxyeicosatrienoic acid. *(Reprinted with permission from Haag M. Poly-unsaturated fatty acids: Their cellular role and clinical applications (Part 1). The Medicine J (SA) 2001;43:13-17.[163]) Copyright (2001), Medpharm Publications.*

Prostaglandins are considered fast-acting local hormones, often displaying biphasic properties. PGE_2, which is derived from AA, is thought to contribute to pro-inflammatory processes and high concentrations may inhibit bone formation.[171] Varying the ratio of the precursor fatty acids in the diet is an effective way to modify prostaglandin production in the body. Increasing the dietary content of the n-3

PUFAs EPA and DHA will inhibit the synthesis of 2 series eicosanoids derived from AA by inhibiting AA release from membranes by PLA_2 and its cascade through the cyclooxygenases and lipoxygenases.[30,160,172-176] Apart from replacing AA in cell membranes, EPA can be utilised as substrate for the synthesis of PGE_3 that is regarded equally potent as PGE_2 in bone resorption. EPA, however, is only one-tenth as effective for PGE_3 synthesis as AA for PGE_2 synthesis,[177,178] suggesting that replacement of AA by EPA could be beneficial. It has also been reported that PGE_3 has milder inflammatory effects compared with PGE_2.[22] PGE_2 synthesis can also be reduced by provision of the n-6 PUFA gamma-linolenic acid (GLA). Available evidence suggests that GLA increases the synthesis of DGLA but not AA, probably due to limited activity of Δ-5-desaturase.[171] In addition to reducing PGE_2 synthesis, dietary GLA can enhance production of PGE_1, which has anti-inflammatory effects.[160,171]

2.9.3 Second messengers

Membrane lipids not only serve a fundamental role in the structure of membranes but also play a critical role in the processes of signal transduction and cell regulation.[179] Phospholipase A_2 that is controlled by hormones and other signals, liberates fatty acids from the sn-2 position of phospholipids, and these can subsequently be used as precursors for eicosanoids. These lipid-soluble molecules can diffuse out of the cell and combine with receptors on neigbouring cells to exert a paracrine function. In addition, free fatty acids can also interact with a number of cellular proteins including phospholipases, G-proteins, ion channels and protein kinases.[18,23,179] PUFAs and their metabolic products have also been shown to be part of most of the second messenger signaling systems within the cell.[180] Reviews by Khan *et al* (1995),[179] Kruger and Horrobin (1997)[23] and Haag (2003)[18] listed the following:

1. PUFAs are regulators of protein kinase function and hence phosphorylation and activation.
2. DGLA, EPA and AA are substrates for oxygenated derivatives including eicosanoids such as the prostaglandins, leukotrienes, thromboxanes and hydroxy fatty acids, which perform a wide range of second messenger signaling functions. The different effects of the prostaglandins are due to

their effects on different signaling systems: prostaglandins of the n2-family transduce signals via a G_s protein, thus elevating cAMP levels, whereas those of the n-3 family use a G_I protein, which have the opposite effect. Prostaglandins of the 1 family use a phosphoinositide signaling system.

3. PUFAs are important constituents of the diacylglycerols (DAG) released from P-inositol during the course of inositol signaling. The diacylglycerols have been shown to be regulators of protein kinases and calcium signaling.
4. Cyclic nucleotide synthesis is under control of various PUFA-derived molecules, especially the prostaglandins.

Figure 2.16 presents the most important membrane second-messenger mechanisms with the red numbered circles showing the locations where PUFA effects have been demonstrated.[18]

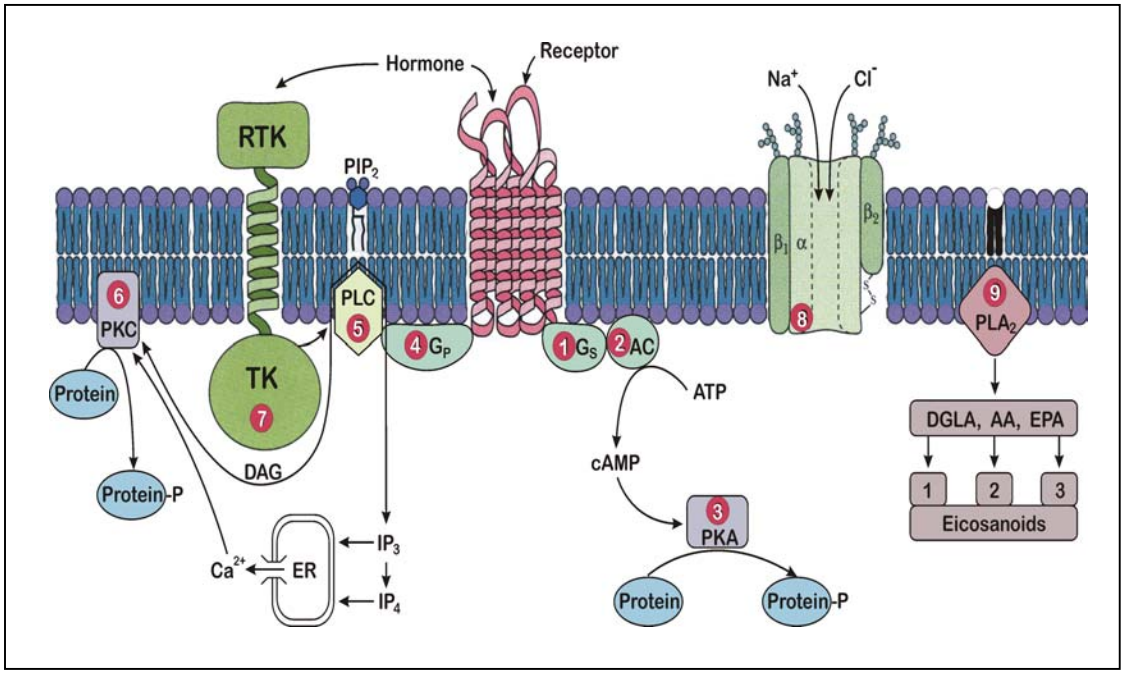

Figure 2.16. Role of polyunsaturated fatty acids in signal transduction.

1: G_s protein; 2: adenylate cyclase; 3: protein kinase; 4: G_p protein; 5: phospholipase C; 6: protein kinase C; 7: tyrosine kinase; 8: ion channel; 9: phospholipase A_2.
(Abbreviations: AA : arachidonic acid; DGLA: dihomo-gamma-linolenic acid; EPA: eicosapentaenoic acid; PL: phospholipid; PIP_2: phosphatidylinositol pyrophosphate; DAG: diacyl glycerol; ER: endoplasmic reticulum; IP_3 and IP_4: inositoltris- and tetrakosphosphates.) *(Reprinted with permission from Haag M. Essential fatty acids and the brain. Can J Psychiatry 2003;48:195-203.)*[18]

Differential effects of the n-6 and n-3 PUFAs on signal transduction have been reported.[180,181] Mirnikjoo *et al* (2001) reported that n-3 PUFAs inhibited the *in vitro*

activities of cAMP-dependent protein kinase, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II, and mitogen-activated protein kinase (MAPK). They concluded that one mechanism by which n-3 fatty acids could affect cellular function is by inhibition of these second messenger-regulated protein kinases.[181]

2.9.4 Modulation of gene transcription

Ingestion of PUFAs will lead to their distribution to virtually every cell in the body with effects on membrane composition and function, eicosanoid synthesis, and signaling as well as the regulation of gene expression.[182] Dietary PUFAs and their derivates such as eicosanoids can act as signaling molecules involved in the regulation of gene expression by interacting with specific nuclear receptors within the cell's nucleus.[183] These nuclear receptors control the rate of gene transcription by binding to DNA at specific responsive elements. Depending on the nature of the transcription factor and its binding substrate e.g. PUFAs or their derivatives, genes associated with the production of functional proteins can either be stimulated or repressed.[160]

Peroxisome proliferator activated receptors (PPAR) are examples of nuclear receptors that may utilise long-chain PUFAs or their derivatives as substrates.[184,185] At present, three isoforms of PPAR have been cloned (PPARα, -β, and -γ) with tissue specific expression, ligand-specific activation, and the ability to heterodimerise with retinoid X receptors (RXR).[183] (Refer to figure 2.5).[54] PPAR can be found in all tissues of the body, but especially in the liver (PPARα), where they control the synthesis of lipids and in adipose tissue (PPARγ) where they control the differentiation of adipocytes.[160] PPARα is often considered a 'master switch' transcription factor[28] as it plays a role in the regulation of an extensive network of genes involved in glucose and lipid metabolism including fatty acid transport, fatty-acid-binding proteins and fatty acyl-CoA synthesis.[186] Various fatty acids have been shown to bind to and activate PPARs including n-6 PUFAs such as GLA, AA, LA and linolenic acid; n-3 PUFAs such as EPA and DHA; mono-unsaturated fatty acids such as oleic acid and elaidic acid; and saturated fatty acids e.g., palmitic and stearic acid.[184,187,188]

Whereas PPARα operates in the catabolism of fatty acids in the liver, PPARγ influences the storage of fatty acids in adipose tissue.[188] PPARγ is also part of the adipocyte differentiation program that induces the maturation of pre-adipocytes into adipocytes.[54,189] Activated PPARγ induces lipoprotein lipase and fatty acid transporters and enhances adipocyte differentiation as well as inhibiting NFκβ function and cytokine and COX-2 expression.[186] Apart from fatty acids themselves, PUFA metabolites such as prostaglandins have also been shown to be direct ligands for PPARγ and inducers of adipogenesis[190,191]

Osteoblasts derive from marrow stromal cell progenitors, which are capable of differentiating into several different cell types, including adipocytes.[43,44] Two forms of PPARγ are expressed in subclones of marrow-derived cell lines, PPARγ1 and PPARγ2.[59] Activation of PPARγ2 has been shown to induce adipogenesis in these cell lines but PPARγ1 does not have this function. In addition, PPARγ2 suppresses the expression of genes such as Cbfa1 involved in osteoblastogenesis.[55,59] Transcriptional activation of PPARγ2 is potentiated by various lipid-like compounds, including naturally occurring PUFAs.[189] Apart from osteoblast progenitors, PPARγ is also expressed in osteoblasts and activation of PPARγ by fatty acids, as well as various linoleic acid peroxidation products such as 9,10-epoxyoctadecenoic acid can induce transdifferentiation of these cells into cells expressing the adipocytic phenotype *in vitro*.[55,56]

The binding of free fatty acids to steroid hormone receptors can also modulate gene expression. It has been shown that free fatty acids and steroid hormones are involved in an intertwined regulatory loop: Free fatty acids can interact with cytoplasmic or nuclear steroid hormone receptors to modulate the binding of steroid hormones positively or negatively.[192] In turn, the steroid hormone, either bound to the receptor or unbound, can intervene in the synthesis and activities of different enzymes responsible for the release, reincorporation or the synthesis of fatty acids.[192]

The effects of fatty acids on gene expression have received considerable attention because it represents a direct route for fatty acids to regulate gene function.[182]

Omega-3 PUFAs for instance, have been shown to have rapid effects on gene expression such as the PPARs[28] and changes in mRNAs encoding several lipogenic enzymes can be detected within hours of feeding animals diets enriched with n-3 PUFAs.[182] These effects are sustained for as long as the n-3 PUFAs remain in the diet.[182]

2.10 Effects of polyunsaturated fatty acids on bone

Polyunsaturated fatty acids (PUFAs), especially the omega-3 (n-3) PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in fish oil, are of paramount importance for health and disease prevention. The n-3 PUFAs, especially, have been shown to be beneficial in the prevention and treatment of a variety of medical conditions such as cardiovascular diseases, neurological disorders, inflammatory diseases, some cancers and rheumatoid arthritis.[18-20]

During the past two decades, the effects of dietary PUFAs on bone health received considerable attention.[16,21,22] It has been suggested for instance that PUFAs of the n-3 series, as well as the n-6 fatty acid GLA, may prove beneficial when consumed in appropriate amounts.[27] In addition, it has been shown that a reduction of the n-6/n-3 PUFA ratio could result in increased bone strength in animals[30-31] and in humans.[32] Changes in dietary PUFAs are reflected in the composition of various tissues, including bone cells such as the osteoblasts.[20,33] As PUFAs are substrates for different prostaglandins, some of the effects of PUFAs have been attributed to modulation of prostaglandin synthesis in bone.[40]

2.10.1 Essential fatty acid deficiencies and bone

Because EFAs are widely distributed in plant products in the diet, EFA deficiency is rare in humans. However, following dietary EFA deficiency, pathological fractures were reported in newborn rats.[193] Borland and Jackson (1931) reported that EFA-deficient animals were found to develop severe osteoporosis coupled with increased renal and arterial calcification.[194] Early studies dating back to 1946

reported that individuals with osteoporosis frequently also had ectopic calcification in other tissues, particularly intervertrabal discs, arteries and kidneys.[195] In osteoporosis, calcium is not simply lost from bone and from the body but some of the calcium is deposited in arteries and kidneys, where it is harmful.[196] Treatment of animals with EPA and GLA attenuated ectopic calcification, thereby suggesting that EFAs might be benificial for treatment of this condition.[197,198]

2.10.2 Nutritional *in vivo* human and animal studies

It has been shown in various animal and human models that supplementation of the diet with n-3 PUFAs such as EPA and DHA have beneficial effects for bone.[21,174,199-201] A controlled clinical study, for instance, found that supplementation of calcium, γ-linolenic acid and EPA in the diets of elderly women enhances calcium absorption, reduces calcium excretion, and have overall positive effects on bone mineral density.[27] Reinwald *et al* (2004) have shown that n-3 deficient weanling rats exhibited a marked increase in n-6 PUFAs and a corresponding decrease in n-3 PUFAs in bone. Diminished structural integrity was observed when mechanical properties of bone in these rats were measured.[202] Repletion with dietary n-3 PUFAs, however, restored the (n-6)/(n-3) PUFA ratio in the bone compartments of these animals and reversed the compromised bone modeling as well.[202] In a study designed to investigate the effects of varied amounts of dietary AA, while keeping DHA status and the total (n-6)/(n-3) ratio constant, it was shown that supplementation of the diet with AA (n-6) enhanced bone mass in piglets.[203]

A large number of studies reported the effects of dietary PUFAs on young growing animals. Norrdin *et al* (1990), however, cautioned that it should be taken into account that the growing skeleton is usually more responsive due to its greater activity concerned with lengthening of bone at the cartilaginous growth plates where bone modeling mainly depends on bone formation.[204] In the mature animal, on the other hand, cellular activity is primarily concerned with bone remodeling where bone resorption and bone formation alternate at specific bone sites and are responsible for internal turnover of the matrix.[204]

It has been shown that lowering of the dietary (n-6)/(n-3) fatty acid ratio could be beneficial for bone in animals and humans.[25,29,30,173,192,205] Weiss *et al* (2005) reported findings of a population-based cohort study, known as the Rancho Bernardo study, in which a large number of older, middle class residents in California participated. In this study, dietary data were obtained between 1988 and 1992 through food-frequency questionnaires. In addition, bone mineral density of the participating subjects was determined. Results from this study have shown that a higher n-6/n-3 PUFAs ratio is associated with lower bone mineral density at the hip in both sexes. These findings suggest that the relative amounts of dietary PUFAs might play a vital role in preserving skeletal integrity in older age.[32]

The mechanisms underlying the response of bone to dietary fatty acids are not fully understood. So far, increased calcium absorption and decreased urinary calcium loss[168,192,206] have been reported as well as alterations of eicosanoid metabolism[30,173,174,207] and growth factors such as insulin-like growth factor I.[173,174,205,207] In addition, the n-3 PUFAs have been shown to posess anti-inflammatory qualities that could also protect bone, especially in inflammatory conditions.[208,209]

2.10.2.1 Effects of dietary polyunsaturated fatty acids on calcium balance and bone status

Dietary fats could influence bone health by affecting intestinal calcium absorption and renal calcium excretion. Calcium absorption is increased and excretion decreased when standard diets are supplemented with specific oils. Addition of the PUFAs GLA and EPA to the diets of healthy[192,206] or ovariectomised rats[25] suppressed bone resorption and enhanced bone mass. Supplementation of the diet with evening primrose oil or fish oil high in n-3 PUFAs has been shown to increase the unsaturation index of intestinal brush border membrane vesicles, resulting in significantly enhanced calcium transport by these membranes.[168] Van Papendorp et al (1995)[26] supplemented the diet of osteoporotic patients with evening primrose and fish oil or olive oil (control) for 16 weeks. Patients supplemented with the PUFA rich oils showed an improvement in calcium absorption and stimulation of osteoblastic

activity indicated by a rise in osteocalcin and procollagen, both markers of bone formation.[26]

Sun *et al* (2003) investigated the action of n-6 and n-3 PUFAs on bone resorption by feeding ovariectomised mice diets containing 5% corn oil (rich in n-6 PUFAs) or fish oil (rich in n-3 PUFAs).[29] Apart from measuring bone mineral density, they also measured RANKL expression in activated spleen lymphocytes from these animals. Analysis for RANKL showed increased $RANKL^+$ T cells in corn-fed mice whereas fish-oil fed mice showed no change in $RANKL^+$ T cells. The increased $RANKL^+$ T cells in corn-fed mice correlated closely with bone mineral density loss, whereas fish oil decreased bone loss by preventing changes in RANKL surface antigen on T cells, therefore demonstrating a bone protective effect of n-3 PUFAs.[29] The mechanism by which n-3 fatty acids prevent activation of RANKL is not known.

A few studies reported negative effects of n-3 supplementation on bone metabolism. Judex *et al* (2000) showed that 10% fish oil supplementation in the presence of modest vitamin E supplementation can lead to substantial degradation of morphological and mechanical properties of cortical bone of rapidly growing rabbits. [210] This observation indicates that supplementation of fish oil in large quantities, had detrimental effects on the skeleton of these animals.[210] In another study, feeding fish oil to weanling male rats showed no effect on biomechanical strength properties of femurs and vertebrae but in female rats reduced length growth and a lower vertrebral peak load was observed.[211]

2.10.2.2 Effects of dietary polyunsaturated fatty acids on prostaglandin secretion and bone status

Prostaglandins are metabolised from PUFAs and are considered fast-acting local hormones often displaying biphasic properties. PGE_2 derived from the n-6 PUFA AA, is the major prostaglandin in bone and has been shown to be a potent modulator of bone remodeling, affecting both bone resorption[136,137] and formation.[143,149] Excessive production of PGE_2 may affect bone modeling adversely, whereas a lower level of PGE_2 is believed to stimulate bone formation in animals fed diets containing moderate levels of n-6 PUFAs. [171] (Refer to 2.8)

Varying the ratio of the precursor fatty acids in the diet is an effective way to modify prostaglandin production in the body. Since n-3 and n-6 fatty acids serve as substrates for the same enzymes along the conversion pathways but are metabolised at different rates,[23] lowering the dietary (n-6)/(n-3) PUFA ratio can reduce PGE_2 production.[30,174,205,212] The n-3 PUFAs are precursors to PGE_3 that is equally potent to PGE_2 in bone resorption.[177] However, conversion is less effective than for PGE_2 from n-6 PUFA resulting in lower PGE_2 levels.[177,178] PGE_2 production can also be reduced by provision of the n-6 PUFA GLA.[171] In addition to reducing synthesis of PGE_2, dietary GLA can enhance production of PGE_1, which has anti-inflammatory effects that could also benefit bone.[160,171]

Weiler's group conducted several fatty acid nutritional studies on growing piglets.[175,212-215] In a short-term study (21-days) it was shown that modulation of the (n-6)/(n-3) PUFA ratio alters the bone fatty acid profile in piglets; however, the bone mass of these animals was not affected.[212] Higher plasma DHA levels were paralleled with lower bone resorption rates as assessed by urinary N-telopeptide. Furthermore, bone formation as indicated by plasma osteocalcin, was suppressed in these piglets with an elevated *ex vivo* PGE_2 release from bone.[212] In another study, Lucia *et al* (2003) compared the effects of dietary PUFAs with that of low dosage exogenous PGE_2 on bone metabolism in piglets.[175] Results from this study indicated that PGE_2 enhances osteoblast activity as indicated by increased plasma osteocalcin and reduced urinary calcium excretion. On the other hand, dietary PUFAs provided as AA and DHA resulted in reduced bone resorption as indicated by urinary N-telopeptide. It was concluded that dietary PUFAs and exogenous PGE_2 could both lead to enhanced mineral content in this growing piglet model, but through distinct mechanisms.[175]

Watkins *et al* (1996) have shown that feeding chicks menhaden oil (high in n-3 PUFAs) resulted in a higher serum ALP activity and an increase in the bone formation rate compared to those chicks given soy-bean oil (high in n-6 PUFAs).[207] The effect of PUFAs might be exerted via modulation of PGE_2 synthesis. It has been shown that PGE_2 inhibits ALP activity, as inhibition of PGE_2 synthesis resulted in higher ALP activity.[205,207] PGE_2 is thought to contribute to pro-inflammatory processes, and high concentrations thereof may inhibit bone formation. Watkins *et al*

(2001) suggested that by lowering the dietary (n-6)/(n-3) PUFA ratio, PGE_2 production could be reduced and bone formation therefore enhanced.[176]

Changes in the PUFA content of the diet have been shown to not only alter the experimental animal's tissue fatty acid profile but also that of its offspring.[216] Liu and Denbow (2001) have demonstrated that supplementation of quail hens' diet with fish oil high in n-3 PUFAs, significantly lowered *ex vivo* PGE_2 production of tibiae in newly hatched quail compared to those from hens fed control diets.[216] These results suggested that maternal dietary lipids might have the potential to influence bone metabolism of embryos by modifying the fatty acid composition of this tissue.[216]

2.10.2.3 Effects of dietary polyunsaturated fatty acids on insulin-like growth factor (IGF-I) and insulin-like growth factor binding proteins (IGFBPs)

It has been shown that PGE_2 at moderate levels may increase the production of bone-derived growth factors such as insulin-like growth factors (IGFs) in osteoblasts.[147,217] IGFs, especially IGF-I, are major bone-derived growth factors and are believed to function as both systemic and local growth factors for bone tissue. Once secreted and deposited in bone matrix, IGFs are released during osteoclastic bone resorptive activity, acting in an autocrine or paracrine fashion to stimulate new bone cell formation and matrix production.[218] IGF-I acts as a regulator of bone cell function as it stimulates the proliferation of pre-osteoblasts, thereby increasing the number of cells capable of producing bone matrix. In addition, IGF-I increases collagen expression while decreasing collagen degradation, causing an anabolic effect in bone tissue.[219,220] In osteoblasts, hormones such as growth hormone, PTH and oestrogen modulate IGF-I expression.[220] Apart from IGF-I, osteoblasts also synthesise extracellular high affinity IGF-binding proteins (IGFBPs), which modify the interaction of IGF-I with its receptors by prolonging IGF stability and by influencing ligand-receptor interaction.[45,221]

It has been suggested that dietary PUFAs, depending upon the type and amount ingested, may up-regulate or down-regulate IGF-I production in bone via their ability to modulate local concentrations of PGE_2.[174] PGE_2, produced from AA by

osteoblasts, stimulates IGF-I synthesis[147,174,220] as well as the expression of various IGF-binding proteins,[217,219,221] suggesting that PGE_2 could keep IGF available for stimulation of osteoblasts at a later phase of bone remodeling.[219] McCarthy *et al* (1991) suggested that the ability of PGE_2 to enhance osteoblastic IGF-I synthesis could explain its anabolic potential, and furthermore suggests a role for PGE_2 in coupled bone remodeling.[147] The anabolic effects of PGE_2 may occur through stimulation of endogenous IGF-I production by osteoblasts[94] or by increased bone cell responsiveness to IGF-I.[222] It has been shown by Li *et al* (1999) that feeding a fish oil-enriched diet to rapidly growing male rats increases the serum concentration of IGFBP-3, an important modulator of IGF-I and overall bone growth and development.[174]

2.10.3 Effects of polyunsaturated fatty acids on bone cells

Although a large number of studies of dietary PUFA effects on bone homeostasis have been published,[16,21,22] the cellular mechanisms of these fatty acids on bone have not been well investigated.

2.10.3.1 Effects of polyunsaturated fatty acids on early osteoblastic differentiation

Atkinson (1997) demonstrated that DHA feeding of weanling male Fisher rats had a substantial bone marrow enhancing activity, resulting in a two-fold increase in bone marrow cell number over n-6 PUFA fed animals.[33] Bone marrow contains various precursor cells including mesenchymal stem cells that are pluripotent and able to differentiate into several cell types including osteoblasts and adipocytes.[43,44] One could therefore speculate that higher numbers of bone marrow cells could increase the potential for osteoblastogenesis, provided the required transcription factors are expressed. Commitment of a mesenchymal stem cell to the osteoblastic lineage is regulated by specific transcription factors of which Cbfa1 has been identified as the earliest and most specific marker of osteogenesis.[46,48]

Watkins *et al* (2003) reported regulatory effects of PUFAs on Cbfa1 expression in fetal murine calvarial osteoblasts.[223] AA, EPA and LA stimulated Cbfa1 expression but conjugated linoleic acid (CLA) decreased protein levels for Cbfa1 after 14 days of treatment.[223] This preliminary study suggests that fatty acids may affect Cbfa1 expression. It was speculated that the observed stimulatory effects of AA and LA in this experimental model could be mediated by elevated PGE_2 production.[223] The possible involvement of PGE_2 in Cbfa1 expression is supported by findings of Zhang *et al* (2002) who reported significant inhibition of Cbfa1 expression in a COX-2 knockout mouse model, which was reversed by the addition of PGE_2.[224] In addition, it has been shown that PGE_2 induces expression of Cbfa1 as well as bone morphogenetic protein-2 (BMP-2) through activation of the EP_4 prostaglandin receptor.[149] Bone-morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily and are considered important regulators of the differentiation of uncommitted mesenchymal cells into osteoblasts during both embryonic development and bone repair.[52,225] Zhang *et al* (2002) speculated that PGE_2 might induce BMPs and/or cooperate with BMPs to increase Cbfa1 and osterix, two essential transcription factors required for bone formation.[149] However, whether dietary PUFAs could affect BMP-2 expression via modulation of PGE_2 synthesis, is not known.

Osteoblastic precursors not only express Cbfa1 but also PPARγ, a transcription factor responsible for adipocyte differentiation.[46,53] It has been shown that activation of PPARγ, by fatty acids as well as a various linoleic acid peroxidation products, can induce adipogenesis and inhibit osteoblastogenesis in some osteoblastic precursors *in vitro.*[55,56] The modulation of osteoblast precursors is complicated and depends not only on the provision of PUFA metabolites but also on the expression of specific subscription factors in these cells.

2.10.3.2 Effects of polyunsaturated fatty acids on osteoclastogenesis

Sun *et al* (2003)[29] investigated the effects of selected n-3 and n-6 PUFAs on *in vitro* osteoclastogenesis by culturing primary murine bone marrow cells in the presence of $1,25(OH)_2D_3$ and examining TRAP (tartrate-resistant acid phosphatase) activity which is considered to be a marker of osteoclast maturation.[29] Compared to $1,25(OH)_2D_3$ alone, both EPA and DHA (n-3 PUFAs), alone or in combination,

caused a significant decrease in osteoclast maturation compared with the n-6 PUFAs linoleic acid (LA) and AA.[29] These results demonstrate an inhibitory effect of n-3 PUFAs on osteoclastogenesis *in vitro.* It was therefore concluded that inhibition of osteoclastogenesis might be one of the mechanisms by which dietary n-3 PUFAs reduce bone loss in OVX mice.[29]

2.10.3.3 Effects of polyunsaturated fatty acids on cytokine expression

Cytokines, such as IL-1 mainly regulate immune responses; however, they have also been shown to stimulate osteoclast recruitment and are potent stimulators of bone resorption.[152] Priante *et al* (2002) conducted an experiment to investigate the effects of different fatty acids on the expression of cytokines involved in bone remodeling.[226] Osteoblasts were exposed to AA, EPA and oleic acid and cytokine mRNAs determined in MG-63 osteoblasts. The results showed that AA (25 to 100 μM) stimulated expression of IL-1α, IL-1β, TNF-α and M-CSF. EPA and oleic acid (25 to100 μM) on the other hand, had no stimulatory effects, but instead caused a significant inhibition of AA-induced cytokine mRNA expression.[226] Results from inhibitor studies suggested that a protein kinase C-dependent mechanism could account for the effects of AA on cytokine production.[226] Downregulation of resorptive cytokines such as TNF-α and IL-6 by n-3 PUFA in OVX mice was also reported by other research groups.[29,227]

2.10.3.4 Effects of polyunsaturated fatty acids on alkaline phosphatase (ALP) activity

Watkins *et al* (2003) reported increased ALP activity after n-3 PUFA treatment of MC3T3-E1 osteoblasts. It was speculated that EPA might achieve the stimulatory effect of EPA on ALP activity via inhibition of PGE_2 production.[223]

2.11 Prostaglandins in bone

With the exception of the red blood cell, prostaglandins are produced and released by nearly all mammalian cells and tissues, including bone.[170] Prostaglandins are considered local hormones that are not stored in cells but are synthesised and released immediately as required. Compounds of the 2-series of prostaglandins derived from AA are the principal prostaglandins in humans and are considered biologically most significant.[170] (Refer to figure 2.15). Although several prostaglandins are produced by osteoblasts, PGE_2 is the major prostaglandin produced by these cells.[228,229]

AA is stored in cell membrane phospholipids and release is brought about mainly through the actions of the hydrolase phospholipase A_2 (PLA_2). Cytosolic PLA_2 ($cPLA_2$) is constitutively expressed in bone cells, and many agents such as cytokines and growth factors increase levels thereof.[153] After AA has been released from membrane phospholipids, it is converted to prostaglandin endoperoxide G_2 (PGG_2) by prostaglandin G/H synthase (PGHS) in a cyclooxygenase reaction, and then reduced to prostaglandin endoperoxide G_2 (PGH_2) by PGHS in a peroxidase reaction.[170]

Two enzymes for PGH_2 encoded by separate genes have been identified. Cyclooxygenase-1 (COX-1) (also known as PGHS-1), is constitutively expressed in most tissues and performs a 'housekeeping' function to synthesise prostaglandins which regulate normal cell activity.[230] Cyclooxygenase-2 (COX-2) (also known as PGHS-2), is generally only expressed at very low levels in most tissues but can be rapidly and transiently induced to high levels by multiple factors e.g., chronic inflammation,[155] nitric oxide,[231] PGE_2 itself[232] and growth factors and cytokines such as TGF-α and IL-1.[228] Compared to COX-2, COX-1 is only moderately affected when stimulated with hormones and growth factors.[228] It has also been reported that COX-1 requires higher concentrations of AA for its optimal function than does COX-2, implying that the amount of AA supplied by $cPLA_2$ critically influences which COX enzymes are utilised.[233] Although COX-1 and COX-2 are both found in the endoplasmic reticulum as well as the nuclear envelope, COX-2 is more highly concentrated on the nuclear membrane than COX-1.[234]

2.11.1 Prostaglandin receptors

Following their intracellular synthesis, prostanoids exit the cell, act on the parent cell and/or neighbouring cells in an autocrine and/or paracrine fashion through specific prostanoid receptors, thereby affecting changes in the levels of second messengers.[170] PGE receptors belong to the G protein-coupled seven transmembrane domain family of receptors. There are at least four distinct receptors for PGE_2 with differential signaling pathways: EP_1 with Ca^{2+} mobilisation; EP_2 and EP_4 with stimulation of cAMP production; and EP_3 mainly with the inhibition of cAMP production.[235,236]

The expression patterns of PG receptors differ in various cell types, differentiation status of these cells, tissues, and species. MC3T3-E1 mouse osteoblastic cells for instance have been shown to predominantly express EP_1 and EP_4 receptors,[229] primary cultures of murine osteoblasts express EP_2 and EP_4 receptors and human mesenchymal stem cells express EP_4 receptors.[149]

2.11.2 Regulation of prostaglandin production in bone

Prostaglandins are amongst the most important local factors in bone and their production is under the control of many hormones, such as the sex hormones, PTH, glucocorticoids and $1,25(OH)_2D_3$.[237] (Refer to 2.9.2). Other cytokines and local factors such as IL-1, TNF-α, and TGF-β also regulate PG production.[134,204,238,239]

2.11.2.1 Stimulation of prostaglandin production in bone

Many of the important regulators of bone metabolism under both physiological and pathological conditions have been shown to stimulate prostaglandin production:

Systemic hormones

PTH and PTH-related peptide are potent stimulators of PGE_2 secretion in cultured neonatal rat calvaria[237] (refer to 2.5.2) and it has been shown that PTH induces

COX-2 expression with little or no effects on COX-1 or $cPLA_2$.[240] Thyroid hormone and $1,25(OH)_2D_3$ also exhibit stimulatory effects on PGE_2 production.[240-242]

Auto-amplification of prostaglandin E_2

PGE_2 is known to enhance its own production by inducing COX-2 in bone[243-246] and it has been shown by Suda *et al* (1998) that this auto-amplified production is mediated via the EP_1 subtype of PGE receptors in mouse MC3T3-E1 osteoblasts.[232] It was suggested that PGE_2-auto-amplification could be important in extending the otherwise short-lived action of this prostaglandin in certain physiological conditions such as mechanical stress and fracture healing.[232] Recently, Sakuma *et al* (2004) demonstrated that PGE_2 is an inducer of COX-2 in cultured primary murine osteoblasts and attributed it to cAMP-dependent PKA activation involving the activation of both EP_2 and EP_4 receptors in this model.[246]

Cytokines and growth factors

Cytokines such as IL-1 and TNF-α are important local factors in bone metabolism and mediators in inflammatory processes. They are thought to play a role in bone loss associated with oestrogen withdrawal.[247] IL-1, IL-6 and TNF-α have been shown to stimulate PGE_2 production largely by stimulation of COX-2 expression[157,228,248,249] in osteoblastic and stromal cell cultures as well as cultured rat calvaria.[133,152,157,231,239,250] IL-1α is regarded as one of the most potent bone-resorbing factors involved in bone loss that is associated with inflammation. A recent study by Tanabe *et al* (2005) reported that IL-1α stimulates the formation of osteoclast-like cells via an increase in M-CSF and PGE_2 production as well as a decrease in OPG production by osteoblasts.[247]

IL-1 and TNF-α induce both COX and nitric oxide synthase, which results in the release of prostaglandins and nitric oxide (NO), respectively. Kanematsu *et al* (1997) demonstrated that NO could be involved in the increased production of PGE_2 through stimulation of COX pathways in murine MC3T3-E1 osteoblastic cells.[231] These researchers suggested that the interaction between NO and the COX

pathways might play an important role in the regulation of osteoblastic functions under physiological as well as pathological conditions.[231]

Growth factors such as TGF-α and TGF-β stimulate PGE_2 synthesis in neonatal mouse calvarial cell cultures[250,251] and mouse osteoblastic cells.[228] Furthermore, the stimulatory effect of IL-1 on PGE_2 biosynthesis is synergistically enhanced by the presence of fibroblast growth factor-1 in MG-63 cells.[249] It has also been shown that cell density affects PGE_2 production, as subconfluent cells displayed a greater reponse to IL-1 than confluent cultures, which could be associated with reduced IL-1 receptor expression in these confluent cultures.[249]

2.11.2.2 Inhibition of prostaglandin production in bone

Hormones

It has been shown that oestrogen and androgens inhibit PGE_2 production in primary osteoblasts, human osteosarcoma osteoblasts and organ culture,[106] while cortisol inhibits PGE_2 production in neonatal rat calvaria.[134,237] Glucocorticoids inhibit COX-2 mRNA and protein expression, which accounts for much of their effects on PG production in bone and other tissues.[153]

Pharmacological blockers

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin synthesis. A well known example of this class, indomethacin, reversibly inhibits prostaglandin synthesis by competing with the substrate AA for the active site of the enzyme, thereby blocking both COX-1 and COX-2 activity.[230,252] NS-398, on the other hand, is known to selectively block only COX-2 mediated prostaglandin production.[252,253] Steroidal anti-inflammatory drugs, such as bethamethasone block prostaglandin synthesis by inhibiting PLA_2 activity, thereby interfering with mobilisation of AA.[254] It has also been demonstrated that the synthetic glucocorticoid dexamethasone inhibits prostaglandin synthesis.[140,214,255,256]

2.11.3 *In vitro* effects of prostaglandin E_2 on bone

PGE_2 is a potent modulator of bone remodeling and influences both bone resorption[136,137,257] and formation.[143,149,175] The effects of PGE_2 on bone are complicated and depend on the duration of exposure, concentration of PGE_2, and animal model or cell type.

Prostaglandins are believed to exert their divergent actions via different membrane receptors on the surface of the target cells.[236] Pharmacological and morphological data indicate that EP_1 promotes cell growth and suppresses cell differentiation, whilst EP_2 and EP_4 are responsible for decreased cell growth and increased osteoblastic differentiation.[229] In addition, it has been shown that EP_4 stimulates osteoblastogenesis and thereby stimulates *de novo* bone formation.[258] EP_2 receptors apparently have a major influence on biomechanical properties of bone in mice. It has been shown that absence of EP_2 receptors in the murine EP_2 knockout model result in weak bone strength properties compared to wild-type control mice.[259]

2.11.3.1 In vitro effects of prostaglandin E_2 on bone formation

The effects of *in vivo* PGE_2 administration on bone parameters were reported in a variety of animal models as previously described. However, prostaglandins could also affect bone formation through effects thereof on the circulation, tissue metabolism or the formation of intermediary factors.[204] It is therefore important to also investigate *in vitro* effects of PGE_2 on bone.

Prostaglandin E_2 effects on osteoblastic differentiation

Zwang *et al* (2002) investigated mineralisation in bone marrow stromal cultures obtained from COX-$2^{-/-}$ and wild type mice and reported that bone nodule formation was severely reduced in the knockout mice cultures.[224] Addition of PGE_2 to these cultures, however, completely reversed the defective osteogenesis, thereby demonstrating that COX-2-mediated PGE_2 synthesis is required for mesenchymal cells to differentiate into mineralising osteoblasts.[224] A recent report indicated that

activation of the EP_4 receptor by PGE_2 enhances differentiation of osteoblast progenitor cells.[260] Importantly, Zwang *et al* (2002) also suggested that the transcription factors Cbfa-1 and osterix are regulated by COX-2 via PGE_2, and that decreased expression of these transcription factors, necessary for bone formation, may contribute to defective bone repair in COX-2 knockout mice.[224] The importance of PGE_2 on osteoblastic differentiation was confirmed by demonstrating that PGE_2 activation of the EP_4 receptor enhances bone formation through induction of both Cbfa-1 and BMP-2 expression.[149,258]

Prostaglandin E_2 effects on osteoblast proliferation

Depending on the model and concentration of PGE_2 used, disparate results were reported on the effects of PGE_2 on osteoblastic proliferation. It has been shown that prostaglandins stimulate proliferation in less differentiated bone cells such as preosteoblasts.[261] In a later study, Woodiel *et al* (1996) reported an anabolic effect of PGE_2 on replication and differentiation in cultured fetal rat calvarial cells and concluded that these effects were likely to be mediated by an EP_2 receptor, which stimulates cAMP-dependent activation of PKA.[262] Exposing human osteoblastic osteosarcoma cells and primary neonatal mouse calvarial osteoblasts to relatively low PGE_2 concentrations inhibited proliferation of these cells.[263-265]

Biphasic growth effects of PGE_2 have also been reported. In MC3T3-E1 cells, lower PGE_2 concentrations inhibited proliferation while higher concentrations were shown to stimulate proliferation slightly.[266] In contrast, a dose-related biphasic effect has been reported in cultured human bone cells with stimulation at 10^{-9} M and inhibition at 10^{-6} M.[267] It was suggested that the stimulation of proliferation by low doses of PGE_2 in this model is mediated by an enhancement of phospholipase C, which results in both an increase in PKC activity and an increase in intracellular calcium influx.[267]

Prostaglandin E_2 effects on in vitro bone formation

Biphasic effects of PGE_2 on bone formation have been reported in cultured fetal rat calvariae. Doses of PGE_2 equal to, or greater than 1 μM inhibit collagen synthesis whilst physiological concentrations have stimulatory effects on bone formation.[146] Raisz and Fall (1990) showed that addition of PGE_2 reversed the inhibitory effects of cortisol on collagen synthesis to levels above untreated cultures and suggested that PGE_2 could be regarded as a local stimulator of bone formation, which could mediate responses to local stress.[146]

The mechanisms whereby PGE_2 affect bone formation are not clear but it has been shown in organ cultures of fetal rat calvariae and neonatal mouse calvariae that PGE_2 stimulates bone formation by increasing osteoblast numbers.[268] Scutt and Bertram (1995) demonstrated the existence of two populations of osteoblastic precursors, one highly adherent and the other non-adherent, in a rat bone marrow cell model.[269] They concluded that the transition between the non-adherent and adherent phenotypes could be PGE_2-mediated thereby explaining some of the anabolic actions of PGE_2 on bone.[269] PGE_2 has also been shown to enhance the production of local growth factors such as IGF-I, BMP-7 and BMP-2 in bone.[147-149] Paralar (2002) concluded that induction of bone formation upon systemic treatment with PGE_2 could in part, be due to local induction of growth factors.[148]

Prostaglandin E_2 effect on osteoblastic alkaline phosphatase (ALP) activity and mineralisation

Alkaline phosphatase (ALP) is a membrane-bound ectoenzyme that can hydrolyse organic phosphates on the outer surface of the cell.[41] An increase in ALP activity reflects the maturation from an earlier to a more mature stage of osteoblast differentiation. The level of ALP is therefore used in *in vitro* experiments as a marker of osteoblast differentiation and bone formation.[41] Divergent effects of PGE_2 on mineralisation properties have been reported, often in the same cell line. It has been shown that when endogenous PGE_2 was blocked by indomethacin in MC3T3-E1 cells, the maximal ALP activity was significantly increased, suggesting that PGE_2 suppresses ALP activity.[226,270,271] The impact of PGE_2 on ALP activity is confirmed

by the observation that exogenous PGE_2 significantly suppresses ALP activity in rat osteoblastic cells[272] as well as murine MC3T3-E1 osteoblastic cells.[231,266,271]

Biphasic effects of PGE_2 on ALP activity and bone formation have also been reported.[146,229] Low PGE_2 concentrations have been shown to stimulate ALP activity, whilst higher concentrations inhibit ALP activity and it was concluded that this effect could probably be due to the presence of multiple EP receptors.[229] Kanematsu *et al* (1997) speculated that low PGE_2 concentrations might stimulate ALP activity through EP_4 whilst high PGE_2 concentrations inhibit ALP activity through EP_1.[231]

Exposing cultured adult rat calvarial cells to PGE_2 stimulated the formation of mineralised bone nodules.[273-275] It was concluded that PGE_2 inhibits proliferation and stimulates differentiation of these calvarial osteoblasts by elevating the $[Ca^{2+}]_I$ through the activation of a phosphoinositide turnover.[275] Ho *et al* (1999) showed that PGE_2 stimulated ALP activity and type I collagen synthesis in rat osteoblasts in culture during the early stages of differentiation, implying that PGE_2 may be involved in the earlier stages of bone matrix maturation and subsequent bone mineralisation.[265]

2.11.3.2 Prostaglandin E_2 effects on bone resorption

PGE_2 is the most abundant prostanoid among prostaglandins in bone and has been believed to be the most potent bone resorber.[134,238] The ability of several hormones and cytokines to regulate bone resorption is to some degree dependent on endogenous prostaglandin synthesis.[231,237,239,276,277]

It has been demonstrated that prostaglandins, especially PGE_2 and PGE_1, stimulate resorption by recruitment of osteoclasts.[135,136] In addition, PGE_2 stimulates osteoclast-like cell formation and bone resorbing activity in mouse bone cell cultures, presumably through mechanisms involving osteoblasts.[240,257,277] It has been shown that PGE_2 stimulates osteoclast formation in bone marrow cultures,[135-137] increases expression of mRNA for RANKL[138] and down-regulates OPG in cultures of primary human bone marrow stromal cells.[139] These reported effects of PGE_2 on OPG and RANKL will ultimately have a detrimental effect on the OPG/RANKL ratio in the bone

microenvironment and could ultimately lead to a decrease in bone mass as previously described. Furthermore, it has been shown that COX-2 expression and the associated PGE_2 production are necessary for maximal resorption responses to $1,25(OH)_2D_3$ and PTH in marrow cultures from COX-$2^{-/-}$ knockout mice.[240,277]

PGE_2 exerts its actions via different PGE receptors on the surface of the target cells. Making use of different EP knock-out mouse models it has been shown that PGE_2 stimulates the formation of osteoclast-like cells *in vitro* and subsequent bone resorption by a cAMP-dependent mechanism via the EP_2[278] and EP_4 receptors.[279] These results were confirmed by others, who employed EP agonists[280] and antagonists[278,281] to show that PGE_2 acts on mouse calvaria cultures mainly via the EP_2 and EP_4 receptors to induce cAMP and expression of RANKL in osteoblastic cells.

The effects of PGE_2 on osteoclastogenesis, however, are complicated and it has been shown that prostaglandins may modulate the process of bone resorption in three ways; 1) through osteoblasts via RANKL, as previously described 2) through osteoclast precursors and 3) through mature osteoclasts.[240] A study by Wani *et al* (1999) showed that apart from the osteoblast-mediated effect of PGE_2 on osteoclastogenesis, PGE_2 also synergises with RANK in inducing osteoclastogenesis in cultures not containing osteoblasts probably through a direct action on the *osteoclastic haemopoietic precursors*.[282] This response depended on the presence of exogenous soluble RANKL, as PGE_2 alone had no effect.[282] It has recently been shown that the direct PGE_2-mediated osteoclastogenic effect is brought about mainly through EP_2 and EP_4 receptors on osteoclast precursors.[283] The effect of PGE_2 on *mature osteoclasts* might be an inhibitory mechanism that could oppose the activating effect of increased osteoclastogenesis.[257] This suggestion has been supported by the discovery that PGE_2 directly stimulates outwardly rectifying Cl^- channels by activation of a cAMP-dependent pathway through EP_2 and, to lesser degree EP_4 receptors, in rat osteoclasts. This pathway has been shown to contribute to the reduction of osteoclast cell area and loss of osteoclast motility, which is likely to reduce bone resorption.[284] Figure 2.17 represents a schematic diagram of the putative roles for prostaglandins in bone resorption.[240]

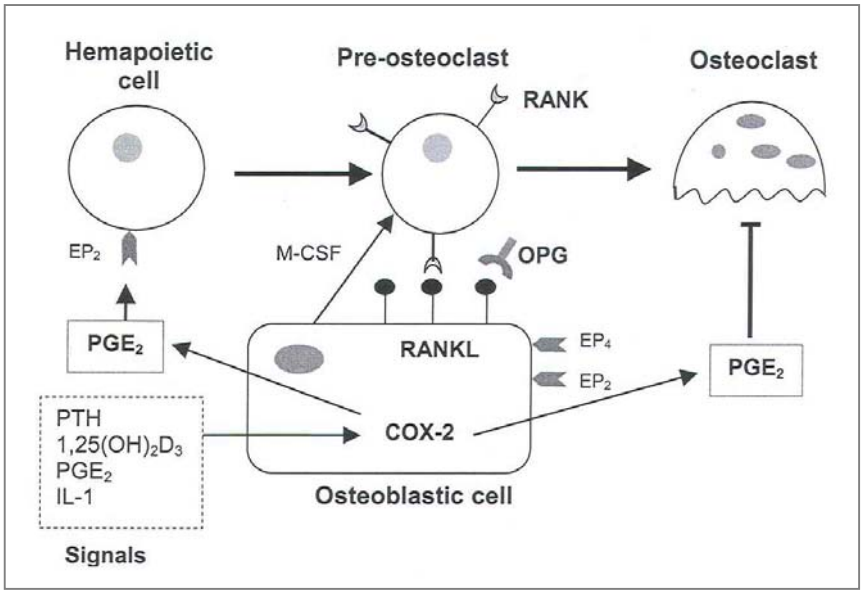

Figure 2.17. Schematic diagram of the putative roles of prostaglandin E_2 in bone resorption.

Prostaglandins have positive effects on formation of osteoclasts but may inhibit osteoclastic activity. Osteoblastic stromal cells express both COX-2 and RANKL. Interaction of RANKL with RANK, which is expressed by osteoclastic precursor cells, is required for precursor cells to differentiate into mature osteoclasts. COX-2 derived prostaglandins stimulate expression of RANKL, and enhance the stimulation of RANKL by other agonists. Prostaglandins can have a transitory effect on the activity of isolated mature osteoclasts. *(Reproduced with permission from Okada Y, Pilbeam C, Raisz LG, Tanaka Y. Role of cyclooxygenase-2 in bone resorption. J UOEH 2003;25:185-95.)*[240] *Copyright (2003) the UOEH.*

2.12 Summary

Systemic hormones and local factors such as eicosanoids, growth factors and cytokines, produced by bone, regulate the activity of bone formation and bone resorption. *In vitro* and animal data suggested that the effects of the PUFAs on bone could largely be mediated through modulation of PGE_2 production. Evidence from experimental studies have suggested that PGE_2, that derives from AA, may have a biphasic, dose-dependent effect on bone formation; stimulatory at low concentrations but inhibitory at higher concentrations.[40,146] It has also been shown that PGE_2 at low levels, may increase the production and action of major bone-derived growth factors such as IGFs[147] that are known to be powerful growth stimulators for bone.[151] High concentrations of PGE_2 on the other hand, have been shown to be associated with bone resorption.[257,277] Dietary supplementation of n-3 PUFAs such as EPA and DHA

inhibits PGE_2 synthesis[40] thereby protecting bone from the effects of high PGE_2 concentrations and could therefore be beneficial for bone. Watkins *et al* (2001) diagrammatically summarised the effects of PUFAs and PGE_2 on bone loss (Figure 2.18).[40]

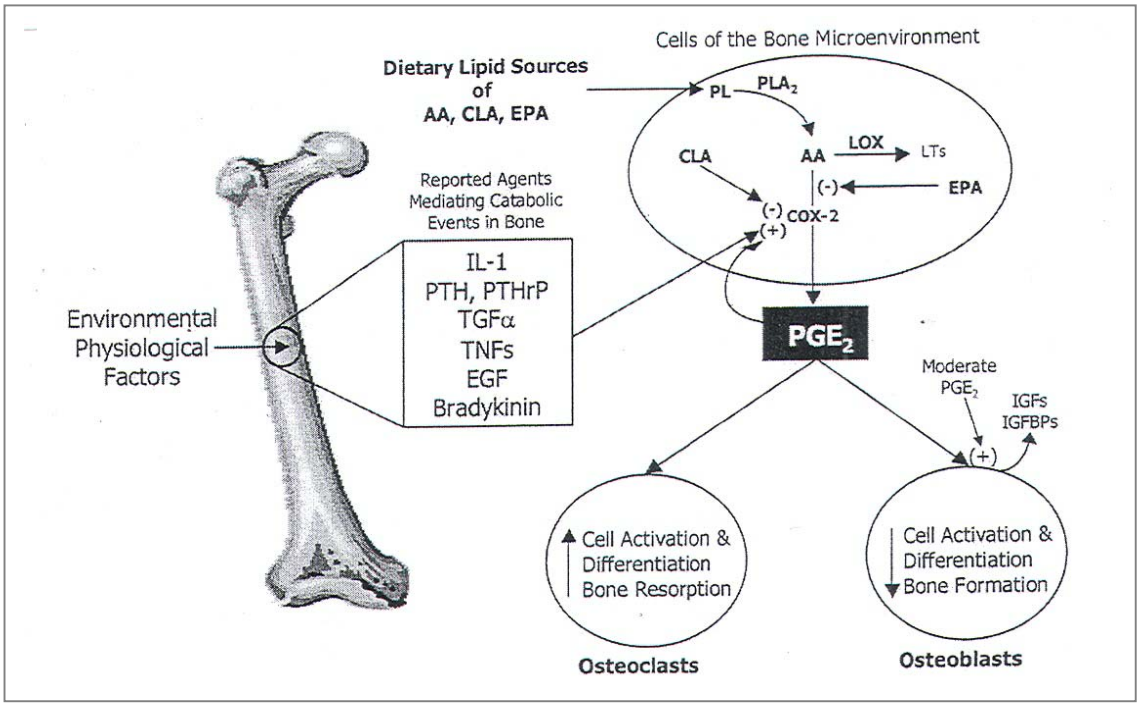

Figure 2.18. Speculative illustration of the possible effects of polyunsaturated fatty acids on bone loss.

This figure illustrates how CLA and EPA can decrease PGE_2 biosynthesised as a result of COX-2 induction by bone resorbing signals. The dietary fatty acids CLA and EPA offer novel opportunities to control potential detrimental effects of excess COX-2 derived PGE_2 on bone metabolism. (Abbreviations: CLA, conjugated linoleic acids; DGLA, dimomo-γ-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; COX-2, inducible cyclooxygenase; IGF/IGFBP, insulin-like growth factors/ IGF binding proteins.) *(Reprinted from Watkins BA, Lippman HE, Le Bouteiller L. Li Y, Seifert MF. Bioactive fatty acids: role in bone biology and bone cell function. Progr Lipid Res 2001;40:125-48.[40]) Copyright (2001), with permission from Elsevier.*