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## Review Article

# Regulatory Mechanisms of Bone Development and Function

is relatively low. Woven bone is later remodelled into lamellar bone, which has better mechanical properties. Formation, remodelling, and repair of the structural elements require the coordinated action of the bone cells: osteoblasts, osteocytes, lining cells, and osteoclasts [3-5].

Bone strength is defined by the parameters mass, geometry, material properties, and microstructure. The bone mass accounts for about half of the decrease in bone strength in the elderly. The diameter of the corticalis is a parameter of bone geometry. Material properties are modulated by the mineral crystals and the correct synthesis of the single components of the bone matrix. The diameter of the trabeculae as well their horizontal and vertical interconnectivity are defined as bone strength [6,7] (Figure 1).

## The bone cells

The osteocytes are cells that are embedded in the mineralized matrix of both woven and lamellar bone. They emerge at the end stage of osteoblast differentiation and become trapped within the mineralized matrix. The osteocytes reside singly in small lacunae but are connected to neighbouring cells through cytoplasmic protrusions, which form a dense network of canalicular. This syncytium permits direct communication between neighbouring osteocytes, as well as with lining cells and osteoblasts on the bone surface. Nutrition and oxygen transport within this network is limited to the diffusion distance, which is approximately 100  $\mu$ m which is equivalent to the wall thickness of trabecular structures and the outer diameter of an osteon [8].

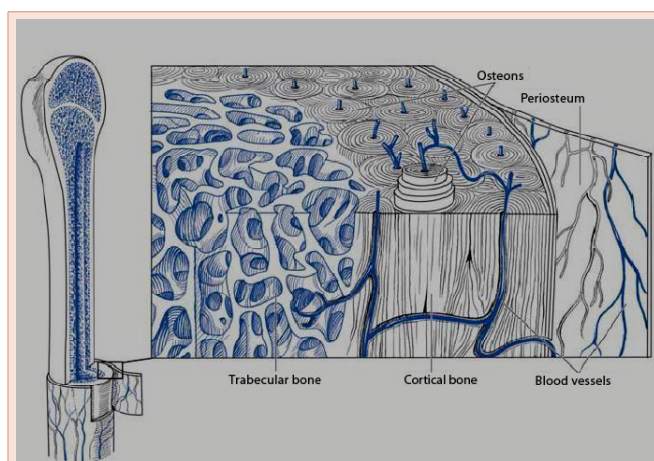
The osteocytes probably act as mechanosensors that signal

## The Structural Organization of Bone

Bone is metabolically active organ where 10% of it is normally and constantly replaced. Bone constitutes the trabecular or spongy bone (25%) and cortical or compact bone (75%). Trabecular bone is metabolically active and forms the internal supportive elements of the bone. The outer capsule is made up of cortical bone and is particularly present in the shafts of long bones. At the structural level, two different forms of bone can be distinguished, i.e. cortical or compact bone—which forms the diaphysis of long bones and provides protection for the medullary cavity— and trabecular, cancellous, or spongy bone, which is found at the ends of the long bones (epiphyses). The total skeleton comprises about 20% of trabecular bone. Trabecular bone has a porosity of 50%–90%, while cortical bone of 10% [1].

The high surface-to-volume ratio of the trabecular bone involves its metabolic function, whereas the cortical bone has mainly a structural and protective role. The outer bone surface is in contact with the surrounding soft tissue via the periosteum. The inner bone surface faces the medullary cavity and is covered with the endosteum. Both the periosteum and endosteum are connective tissues organized in layers. The cambium layer is in intimate contact with the mineralized structure and provides a pool of mesenchymal cells with the potential to differentiate into the chondrogenic and the osteogenic lineage. Trabecular bone and cortical bone are composed of the same microstructural elements: Cells, Organic Matrix, Crystalline Inorganic Matrix and Soluble Factors [2].

The calcified matrix of adult bone is built up in multiple layers of oriented collagen fibres, giving rise to the typical structure of lamellar bone. The lamellae are arranged parallel to each other, as on the surface of flat bones and trabecular structures, or in concentric layers around blood vessels and nerves, forming the Haversian system. The Haversian canals are connected to the periosteum and the endosteum by Volkmann's canals. Blood flow in bone totals 200–400 ml/min in adult humans, indicating that bone is a highly vascularized tissue. Under conditions in which rapid formation of new bone is required, e.g. during skeletal growth in early childhood and in periods of bone regeneration, or in particular metabolic bone disorders, instead of lamellar bone, immature “woven” bone is formed, in which the collagen fibres are randomly oriented and the degree of mineralization



**Figure 1:** Structure of bone: Cortical bone together with its vascular system surrounds the trabecular network [41].

the need for bone remodelling for adapting the bone to functional load according to Wolff's law [8] and remodelling for repairing microstructural changes within the bone matrix. The osteocytes can detect changes in the levels of hormones, such as oestrogen and glucocorticoids that influence their survival rate. Since the osteocytes form a network spanning the skeletal system, they may play– through their residual metabolic activity– a role in bone turnover [5,9-11].

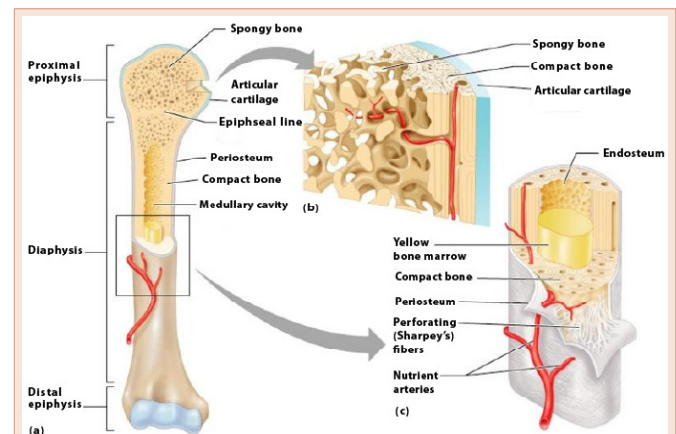
The osteoblasts are mesenchymal cells located on the surface of the mineralized matrix and are responsible for formation of new bone; i.e., they synthesize and regulate the deposition and mineralization of the extracellular matrix. The osteoblasts form a dense monolayer clustering at each bone-forming site. They exhibit a prominent Golgi apparatus and a well-developed rough endoplasmic reticulum. The osteoblasts secrete mainly type I collagen as well as a large number of non-collagenous proteins. The layer of unmineralized bone matrix, termed osteoid, serves as a template for the initiation and propagation of mineralization. During development and the early stages of bone regeneration, woven bone is produced by pinching of matrix vesicles from osteoblasts similar to mineralized hypertrophic cartilage [12].

The osteoblasts lay down lamellar bone onto the previously-formed woven bone [13]. After ceasing matrix-forming activity, the osteoblasts can undergo apoptosis, or remain in inactive form as so called lining cells on the bone surface [5]. In addition, endosteal osteoblasts provide the microenvironment, or niches, for haematopoietic stem cells, which give rise to cells of the myeloid lineage, including osteoclasts [14].

The osteoclasts are large polykaryon cells containing between 3 and 30 nuclei, and are considered to be the exclusive bone resorbing cells. This cell type contains large numbers of lysosomes, mitochondria, and an extensive Golgi complex. The osteoclasts are located at bone surfaces within How ship's lacunae, also called resorption lacunae. Under normal conditions, the osteoclasts are rarely found in bone, but they appear in increased numbers at sites of high bone turnover, such as in the metaphysis of growing bone or in trabecular bone in postmenopausal osteoporosis. A characteristic feature of active osteoclasts, which distinguishes them from polynuclear macrophages, is the development of a ruffled border surrounded by an organelle-free area, termed the "sealing zone". Through the finger-like structures of the ruffled border, the osteoclasts secrete proteolytic enzymes, such as cathepsin K and matrix metalloproteinase nine, as well as hydrogen ion. The acidic pH of approximately 4.5 provides the environment for the mobilization of hydroxyapatite and optimal conditions for the proteolytic enzymes to degrade the bone matrix. The degraded matrix components are released in the periosteoclastic environment by transcytosis [5,15-17] (Figure 2).

### The organic matrix

The organic matrix contains approximately 90% type I collagen; the remaining 10% consist of non-collagenous components. Type I collagen serves as the main structural element which has the ability to bind a large number of non-collagenous proteins. Type I collagen is composed of two  $\alpha_1$  chains and one  $\alpha_2$  chain, which share a common amino acid sequence consisting of Glycine (Gly-X-Y repeats). X represents a proline residue, Y is in most cases modified by a post-



**Figure 2:** Schematic diagram of cortical and trabecular bone [41].

translational reaction into hydroxyproline; the latter is essential for stabilization of the triple-helical structure. Before the procollagen molecules assemble to form a triplehelical structure, the N- and C-terminal propeptides are removed by proteolytic cleavage and serve as metabolic markers of bone formation. The single triple-helical structures are interconnected by the formation of covalent cross-links. The corresponding cleavage products, pyridinoline and deoxypyridinoline, as well as the not completely digested N- and C-terminal telopeptides, are metabolic markers of bone resorption [18,19].

Proteoglycans and glycoproteins are the non-collagenous components of the organic bone matrix. Proteoglycans are large macromolecules composed of glycosaminoglycans (GAGs) covalently linked to a core protein from which the molecule derives its name, e.g. decorin, biglycan, versican and fibromodulin. Each GAG, such as chondroitin sulphate or heparan sulphate, is made up of repeating disaccharide units that contain a sulphated amino sugar. Proteoglycans not only serve as anchoring sites for collagen fibres, but also contain binding sites for growth factors [20]. Gene deletion of biglycan in mice causes a phenotype characterized by a reduced growth rate and decreased bone mass [21].

Glycoproteins, which are produced at different stages of osteoblast maturation, are post-translationally modified proteins with N- or O-linked oligosaccharides. Thrombospondin, fibronectin, osteopontin, and bone sialoprotein are glycoproteins which constitute binding domains for cell membrane receptors of the integrin class. Osteocalcin and matrix gamma-carboxyglutamic acid (Gla) protein are characterized by their content of multiple  $\gamma$ -carboxy glutamic acid residues which exhibit high affinity for mineral ions such as  $\text{Ca}^{2+}$ . Mice deficient in osteocalcin show a higher bone mass when is compared to wild-type animals [22]. Mice deficient in matrix (Gla) protein are normal at birth, but develop severe calcification of their arteries [23]. It seems likely that both glycoproteins are involved in the regulation of matrix mineralization. The expression of the genes coding for matrix molecules is linked to different stages of osteogenic differentiation [24].

The extracellular matrix is also an important storage pool of growth factors that are believed to play a role during bone remodelling [20].

### Crystalline inorganic matrix

The inorganic part of bone is made of calcium hydroxyapatite (HA) which has a chemical formula of  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ ; it is mostly responsible for the stiffness of bone material. The plate-shaped apatite crystals include some impurities, mostly of carbonate, which increase the solubility of bone. The solubility is important for mineral homeostasis, and thus for bone adaptation to exercise loading [25,26].

### Soluble factors

Polypeptide growth factors appear to play an important role in the development and growth of osseous tissue. Polypeptide growth factor effects on bone are necessarily divided into two areas of study: exogenously- produced endocrine factors, which act on specific bone target cells, and endogenously- produced “local” factors with possible autocrine or paracrine action. Known growth factors of extraosseous origin have been investigated for their effects on bone in organ culture [27]. Examples of exogenous factors, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF-1) generally stimulate cellular proliferation. EGF and FGF depress formation of the collagenous extracellular matrix, whereas PDGF and IGF-1 (somatomedin-C) stimulate collagen synthesis [27]. EGF, PDGF, and transforming growth factors (TGF $\alpha$ , TGF-8) may also play a role in bone resorption, as they stimulate calcium release by a prostaglandin-mediated mechanism [28].

Examples of Endogenous or local growth factors, bone-derived growth factor, transforming growth factor beta (TGF- $\beta$ ), cationic cartilage-derived growth factor (CDGF), skeletal growth factor (hSGF), bone morphogenetic protein (BMP). Other local factors affecting bone cells include interleukin-1 (IL-1) [29], macrophage-derived factors (MDGF) [30,31], and an endothelial cell-derived growth factor [32].

### Bone Remodelling

Bone remodelling is a dynamic, lifelong process in which old bone is removed from the skeleton and new bone is added. It consists of two distinct stages – resorption and formation – that involve the activity of special cells called osteoclasts and osteoblasts [33].

Usually, the removal and formation of bone are in balance and maintain skeletal strength and integrity. The bone turnover is a cyclic process of destruction and reconditioning of bone as a result of the concerted effects of osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells) [33].

The objective is to replace micro damage and to adapt bone shape and density to patterns of usage and the attendant forces exerted. These cell types occur in areas referred to as bone remodelling units [33].

Bone remodelling is the result of the coordinated action of bone-resorbing osteoclasts and bone-forming osteoblasts. The osteoblasts and osteoclasts interact within a spatial structure known as the basic

multicellular unit (BMU) in cortical bone and in analogy, bone structural units in trabecular bone. BMUs are functional in the developing and growing skeleton during the process of modelling and during remodelling of mature bone. Histological examinations of BMUs demonstrate that osteoclasts excavate a resorption canal in the corticalis and a cavity in the trabecular structures. It is hypothesized that growth factors as bone morphogenic proteins (BMP) released from the bone matrix and the osteoclasts can attract mesenchymal progenitor cells provided by the blood capillaries and stimulate their differentiation into functional osteoblasts [34].

The osteoblasts deposit osteoid onto the previously-resorbed channel or cavity that is later mineralized. In cortical bone, the resorption canal is continuously filled with layers of lamellar bone with a blood vessel remaining in the middle. The end-stage product is the secondary osteon. An estimated one million BMUs exist at any time in the adult skeleton. The life span of a BMU is much longer than the life span of osteoblasts and osteoclasts. Hence, to replace old cells, their respective progenitors have to be recruited and caused to differentiate into their mature phenotypes. Potential sources of progenitor cells are the blood capillaries, centrally located within each BMU. The osteoblast progenitors can originate from pericytes, covering the outer basal membrane of the capillaries, and from a subpopulation of circulating mononucleated cells [35,36].

The osteoclasts arise from circulating cells of the monocytic lineage, which possibly receive a specific “area code” of adhesion molecules expressed by the endothelial cells [37-40].

The birth, life, and death of osteoblasts and osteoclasts within the BMU is fundamental to understand the pathophysiology of skeletal diseases such as osteoporosis [40]. Osteoblasts, osteoclasts, and their respective progenitors are exposed to a variety of systemic hormones and local factors that regulate the tight balance of bone remodelling. Systemic hormones as PTH, thyroid hormone and sex steroids are brought into the BMU by means of blood capillaries and cells release local factors in an autocrine/paracrine mode of action. Local and systemic factors can influence the activation frequency, which defines the number of BMUs at a given time point, in addition to the remodelling balance of the cells within each BMU [41].

The primary cells partaking in the formation of bone include the osteoclasts, derived from blood (haematopoietic) stem-cell precursors and osteoblasts which differentiate from stromal-cell precursors. The role of osteoblasts is to manufacture a complex extra-cellular matrix, which is capable of undergoing mineralization. Bone strength is a combination of the amount of bone, bone structure, and other aspects of bone quality, which include localized material properties, non-mineralized matrix proteins, and bone turnover [41].

Changes in the bone remodelling rate can be monitored by biochemical parameters. Bone mineral density imaging with Dual X-ray absorptiometry (DXA) provides the current definition of osteoporosis by the world health organization (WHO). However, biochemical parameters and DXA’s usefulness for making predictions about the fracture risk is limited [42].

High-resolution imaging techniques such as computed tomography (CT) and magnet resonance imaging (MRI) that take the



three-dimensional bone architecture into account are evaluated to assess the fracture risk. Moreover, finite element analysis comprises mathematical models where the 3D structural information provides the basis for calculating how much load bone can bear [43].

### Biochemical markers of bone remodelling

A variety of biochemical assays that reflect the activity of osteoblasts (the bone forming cells) and osteoclasts (the boneresorbing cells) have been developed for clinical use. They have helped increase our understanding of the bone remodelling cycle, the pathogenesis of skeletal disorders, and the response of these disorders to therapy [44-46].

Biomarkers of bone formation and resorption reflect the overall osteoblastic and osteoclastic activity in the skeleton and in some situations may serve as surrogates for histologic examination of bone [47].

### Biochemical markers of bone formation

Markers of bone formation are measured in serum. Some are enzymes or other proteins secreted by osteoblasts; others are byproducts of type I collagen deposition [48].

The osteoblasts are mononuclear cells that attach to bone surfaces and form new bone, most commonly at sites that recently underwent resorption. They produce type I collagen and other matrix components of osteoid, and they also mineralize the osteoid with hydroxyapatite [49] (Table 1).

### Biochemical markers of bone resorption

Markers of bone resorption are measured in serum or urine. The most direct indicators are fragments of bone collagen produced by osteoclast activity [48].

The osteoclasts are multinucleated cells that resorb bone. They initiate bone remodelling and help shape the growing bone and so are more numerous in children (Table 2).

### Bone Regeneration

Bone holds the intrinsic capacity to regenerate without the formation of a scar. The basic mechanisms of bone regeneration are very similar to those during bone development, except that early stages are linked with an inflammatory response. Bone injury– e.g. fractures, reconstruction surgery, placement of dental or orthopaedic implants and tooth extraction, blood vessel disruption – immediately

**Table 1:** Showing the biochemical markers of bone formation.

1	Alkaline Phosphatase	Bone-specific alkaline phosphatase (bAP) is a constituent of osteoblast membrane. Its principal role is phosphate hydrolysis, which permits growth of hydroxyapatite crystals [49].
2	Osteoclastin (OC)	Sensitive and specific marker of bone formation. OC is synthesized by osteoblasts and odontoblasts and is incorporated directly into bone matrix, but some circulates in blood [49].
3	Procollagen I extension peptides	N-terminal (called PINPs) and C-terminal (called PICPs) extension peptides are cleaved during the extracellular processing of type I collagen, prior to fibril formation. Procollagen I carboxy-terminal peptide can be measured in plasma and correlates with growth velocity and with bone mineral acquisition. The level of each of the propeptides in blood is thought to reflect the amount of newly-synthesized collagen [50].

**Table 2:** showing the biochemical markers of bone resorption.

S. NO	MARKER	COMMENT
1	Tartrate-resistant acid phosphatase	Enzyme present in the osteoclast and released during osteoclastic activity; however serum TRAP is not bone-specific [51].
2	Hydroxyproline (HP)	HP is a product of post-translational hydroxylation of proline in the procollagen chain and reflects bone resorption. HP is an amino acid common to– and characteristic of– all forms of collagen, and urinary hydroxyproline excretion is the oldest test of bone resorption. However, this test lacks specificity for bone resorption because excreted hydroxyproline also comes from other tissues, particularly from skin collagen (which can turn over rapidly in certain disorders), from newly synthesized collagen that is not incorporated into tissue, and from dietary collagen and gelatin. Because it is less specific than newer tests, it is no longer widely used [52].
3	Collagen pyridinium cross-links (more specific markers)	Pyridinoline (Pyr) and deoxypyridinoline (Dpyr) are generated from hydroxylysine during post-translational modification of collagen. (Pyr) and (Dpyr) are released during matrix resorption and are excreted in urine. (Dpyr) is more specific for bone [44]. Pyridinolines are cross-linking amino acids that strengthen collagen fibrils in the extracellular matrix. They are found in the main fibril-forming collagens (types I, II, and III) of many tissues. Pyridinoline is the major chemical form, but deoxypyridinoline is also unusually abundant in bone collagen and hence is a relatively selective bone marker. Since pyridinolines are not metabolized and are largely excreted as small peptides when produced by osteoclastic bone resorption. Immunoassays have been developed that selectively measure cross-link-containing peptide fragments in urine and serum. The first was an assay that recognizes N-telopeptide of collagen type I (NTx) in urine [53] and serum [50]. The recognized feature in this sequence is fully generated during the process of osteoclastic proteolysis and so requires no further metabolism by the liver or kidney for its production. Results from second-morning urine collections correlate well with those from 24-hour collections, which simplifies patient evaluation. Several other assays target structural variants of a peptide sequence that originates from the carboxy-terminal cross-linking region of collagen type I (CTx) [54,55]
4	Cathepsin-K enzyme	This is of interest because it is the primary proteolytic enzyme used by osteoclasts to degrade bone type I collagen during resorption. Several studies suggest it may be valuable as a marker of bone resorption [56], but more studies are required to evaluate its performance relative to established bone resorption markers.
5	Receptor activator of nuclear factor kappa (RANK), RANK ligand, and its decoy receptor osteoprotegerin	These are the pivotal regulators of osteoclast recruitment and activity [57]. They may eventually be used as markers of bone metabolism, though the broad role of RANK ligand signalling in the immune system may limit its specificity.

leads to the development of a blood clot that fills the defect site with a provisional fibrin-rich extracellular matrix. Accumulating platelets and the immigrating neutrophils and macrophages provide a source of growth factors such as platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and a broad spectrum of other bioactive molecules that target mesenchymal progenitor cells with their osteochondrogenic potential. Blood capillaries sprout into the blood clot by a process termed angiogenesis [58,59]. In parallel, endothelial progenitor cells that originate from the bone marrow are transported via the blood stream to the site of blood vessel formation where they contribute to capillary sprouting. This process is termed vasculogenesis [60].

Once the blood clot is replaced by the blood vessel-rich granulation tissue, differentiation of mesenchymal progenitor cells into functional osteoblasts is initiated. Low oxygen tension and the instability of a fracture, however, favour the differentiation of mesenchymal progenitor cells into the chondrogenic lineage. Cartilaginous tissue, which stabilizes the defect site, is then replaced by bone via endochondral ossification. Lamellar bone is laid appositionally onto immature woven- and pre-existing bone, as well as onto osteoconductive surfaces of implants and grafting materials [4,61,62].

Bone regeneration also involves the process of adaptive modelling and remodelling. This mechanism is based on the sensing of mechanical extension and mechano-transduction. Conversion of mechanical forces into cellular response involves activation of intercellular signals, which are induced by stretched cytoskeletal molecules and piezoelectric potentials [8,63].

### Regulatory Mechanisms of Development and Function of Bone (Figure 3)

Bone development, remodelling and regeneration basically depend on the same elements: mesenchymal progenitor cells that differentiate into osteoblasts or chondrocytes, haematopoietic progenitors that give rise to osteoclasts, local clues that attract the cells and control their proliferation and differentiation into the mature phenotypes, and a provisional matrix, which defines the area for these dynamic processes.

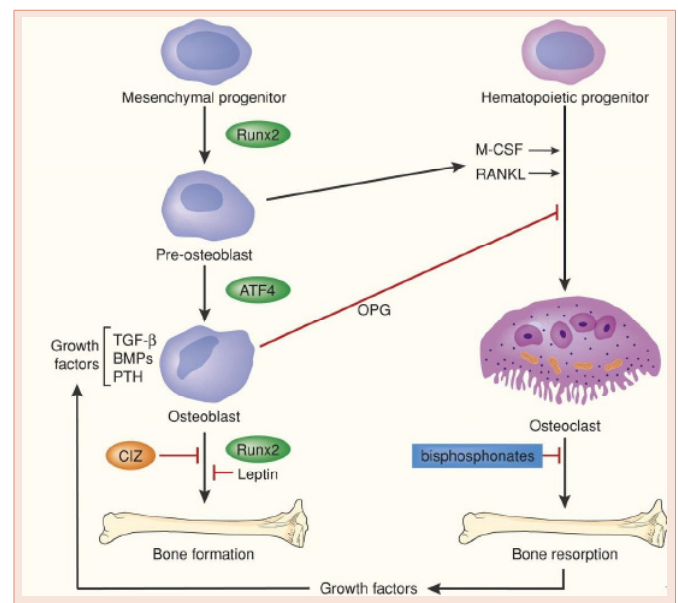
#### Regulatory mechanisms of chondrocytes

Chondrocyte proliferation is positively regulated by the parathyroid hormone-related protein (PTHrP)/Indian Hedgehog (*Ihh*) feedback loop. PTHrP is produced by cells of the periarticular perichondrium and by early stages of proliferating chondrocytes and acts as a mitogen for late proliferating and prehypertrophic chondrocytes. With increasing distance from the PTHrP source, the mitogenic activity fades and prehypertrophic chondrocytes can develop. Prehypertrophic chondrocytes are the main source of the soluble factor *Ihh*, which stimulates the production of PTHrP by an unknown mechanism. *Ihh* is also a mitogen for proliferating chondrocytes directly and through BMP signalling. Thus, PTHrP expression regulated by *Ihh* can control the length of the zone of proliferating chondrocytes and their transition into the prehypertrophic phenotype. Moreover, mice without a functional

*Ihh* gene lack the bony collar because *Ihh* regulates the master gene of osteogenic differentiation, the transcription factors Runx-2 [64]. *Ihh* is not required for osteoblastogenesis in bones formed through intramembranous ossification. Runx-2 is further expressed by chondrocyte condensations, prehypertrophic and hypertrophic chondrocytes. The transcription factor Runx-2 drives differentiation into hypertrophic chondrocytes. Even though in some growth plates hypertrophic chondrocytes can be detected *in vivo* in the absence of functional Runx-2; they fail to be mineralized [65-67].

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor (TGF) superfamily. At least 15 different BMPs have been characterized at the molecular level. During development, BMPs are expressed within mesenchymal condensations (BMP-5), in the perichondrium (BMP-2, BMP-4, BMP-5, BMP-7), and in the future zones of joint formation growth and differentiation factor-5 (GDF-5). BMP-7 is expressed in proliferating chondrocytes and BMP-2 and BMP-6 are found in hypertrophic chondrocytes. Overall, BMPs are mitogenic for chondrocytes and negatively regulate their terminal differentiation [68-70].

BMPs bind to serine-threonine receptors termed BMP-receptor type IA or IB, each of which forms a heterodimer with a type II receptor. BMPRIA controls the pace of chondrocyte proliferation, whereas BMPRIIB is involved in cartilage formation and apoptosis [71]. At the extracellular level, the biologic activity of BMPs is modulated by a number of antagonists known as noggin, chordin, gremlin, and follistatin. Of these, noggin and chordin have been shown to bind to BMP-2 and BMP-4 with high affinity, and nogginnull mutations indicate the involvement of this molecule in patterning the neural tube, somites, and cartilage. Overexpression of noggin in the osteoblastic lineage causes a decreased trabecular bone volume and impaired osteoblastic function [72,73]. During the postnatal



**Figure 3:** Bone formation during development, remodelling and repair [65].

phase, BMPs and their corresponding receptors are expressed during fracture repair [65-67,74-76].

Once the chondrocytes have undergone hypertrophy, they express the key angiogenic molecule vascular endothelial growth factor (VEGF) in response to transcriptional activation by hypoxia-inducible factor-1. Conditional knock out of VEGF in chondrocytes results in delayed blood vessel invasion. Matrix metalloproteinase-9 (MMP-9) highly expressed in chondroclasts and osteoclasts— is involved in the release of VEGF from the mineralized extracellular matrix. Interestingly, *Runx-2* is required for vascular invasion, via regulation of VEGF expression. VEGF also plays a key role in fracture healing [65-67,77].

The transcription factor *Sox-9*, which has a high mobility-group (HMG)-box DNA-binding domain, is required for the transition of mesenchymal progenitors into chondrocytes and their condensation within the skeletal anlagen, as concluded from mouse models and genetic studies of humans. In addition, chondrocytes in *Sox-5* and *Sox-6* double knock-out mice failed to undergo hypertrophy, and consequently columnar organization of the growth plate is affected [65-67].

Genetic studies further suggest the involvement of the functional axis fibroblast growth factor (FGF) - 18/FGF receptor 3 (FGFR-3) in chondrocyte proliferation. Constitutive activation of the FGFR-3 tyrosine kinase receptor causes the virtual absence of prehypertrophic chondrocytes, whereas gene deletion models show larger zones of proliferating chondrocytes. *FGF-18* produced by the perichondrium binds to FGFR-3 on proliferating chondrocytes. *FGF-18* gene ablation similarly results in larger zones of proliferating chondrocytes, suggesting that *FGF-18* is the predicted ligand for FGFR-3, which inhibits chondrocyte proliferation [65-67].

### Regulatory mechanisms of osteoblasts

*Runx-2*, also termed CBFA1/PEBP2αA/AML2, encodes a member of the (runt) family of transcription factors whose expression precedes the appearance of osteoblasts. Null mutation causes the formation of a cartilaginous skeleton without any evidence of bone [78,79]. Heterozygote mutations of *Runx-2* have been detected in mice and humans, correlating with the phenotype of cleidocranial dysplasia, hypoplastic clavicles and nasal bones and retarded ossification of parietal, interparietal, and supraoccipital bones [79-81].

*Runx-2* is also important for the continuing activity of osteoblasts in adults. In addition, the transcription factor plays a role in the transition of proliferating chondrocytes into pre- and hypertrophic chondrocytes [66,82]. *Runx-2* activity is transiently inhibited at the posttranscriptional levels by Twist proteins, basic helix-loop-helix transcription factors, which are expressed in *Runx2*-positive cells throughout the skeleton early in development. The interaction between a C-terminal region of Twist and the Runt domain of *Runx-2* prevents DNA binding and gene activation [83].

*Runx-2* activity is further modulated by co-activator core binding factor β (CBFβ), and other transcription factors such as the Smads, Rb, and MAP kinases and co-repressors [84-86]. Among the factors that control *Runx-2* transcription are BMPs and *Ihh* [64,87]. Moreover,

BMPs and *Runx-2* are also involved in the transition of mesenchymal cells into a phenotype with the potential to support osteoclastogenesis [88-90].

Another transcription factor that controls osteoblast fate is called osterix [91]. Osterix knockout animals express *Runx-2* but not vice-versa, suggesting that osterix acts downstream in the signalling cascade. Osterix-deficient mice show all stages of cartilage development up to mineralized hypertrophic chondrocytes [92].

Distal-less homeobox 5 (*Dlx5*), msh homeobox homologue 2 (*Msx2*) and members of the Fos family are additional transcription factors that regulate bone development [65,93]. Signalling via the Wnt pathway is also involved in the regulation of bone remodelling. Wnt ligands bind to a receptor complex composed of low-density lipoprotein receptor protein (LRP) 5 and LRP6, and a member of the frizzled family of seven transmembrane-spanning proteins. Activation of the frizzled receptor complex causes stabilization of intracellular β-catenin by inhibition of its phosphorylation. Unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus where it associates with LEF/TCF transcription factors and controls gene transcription. Phosphorylated β-catenin is rapidly degraded. Heterozygous gain-of function mutations in the gene coding for LRP5 are associated with a high bone mass [94,95].

The mutation reduced the affinity of LRB5 for the inhibitor Dickkopf-1 [96]. Loss of function mutation causes the osteoporosis pseudoglioma syndrome, which is characterized by low bone density [97]. Both phenotypes could be confirmed in mouse models [97]. Genetic mouse models further indicate that β-catenin is essential in determining that mesenchymal progenitors become osteoblasts and not chondrocytes, regardless of regional locations or ossification mechanisms [98,99].

Animal models based on stabilization and destabilization of β-catenin in osteoblasts showed that these mutations primarily affect bone resorption rather than bone formation [100]. Whether modulation of Wnt signalling, for example by blocking binding of Dkk-1 to LRP-5, will be a therapeutic opportunity in osteoporosis remains to be determined [93,101,102].

Sclerostin is an inhibitor of osteoblast function, causes generalized progressive bone overgrowth termed osteosclerosis. Sclerostin binds LRP5 and 6 and inhibits canonical Wnt signalling, suggesting that increased Wnt signalling is linked with osteosclerosis [103,104]. PTH can inhibit sclerostin transcription [105,106]. Its unique expression by osteocytes and action on osteoblasts suggest that sclerostin may be an osteocyte-derived factor that is transported to osteoblasts at the bone surface and modulates bone formation [107].

### Regulatory mechanisms of osteoclast

Osteotropic hormones such as 1, 25-dihydroxyvitamin D3 [1, 25(OH)2D3] and PTH, and local factors such as prostaglandins or cytokines [interleukin (IL)-1, IL-6, IL-11, tumour necrosis factor (TNF)] can induce the expression of a membrane-associated osteoclast differentiation factor in osteoblasts/stromal cells. Osteoclast precursors recognize this factor through cell/cell interaction, thereby receive the signal necessary for differentiation into mature osteoclasts [108].

Factors that are involved in osteoclast differentiation are members of the TNF/TNF receptor superfamily; the receptor activator of nuclear factor- $\kappa$ B (*RANK*), the *RANK* ligand (*RANKL*), and osteoprotegerin (*OPG*) [109,110]. *RANK* is expressed on mononuclear osteoclast precursors of the monocyte/macrophage lineage, while *RANKL*, which is identical with the fore-mentioned osteoclast differentiation factor, is expressed on stromal/osteoblast cells as well as on activated T lymphocytes.

(Receptor activator of nuclear factor- $\kappa$ B/Receptor activator of nuclear factor  $\kappa$ -B ligand) *RANK/RANKL* interaction and the presence of macrophage colony-stimulating factor (M-CSF) are sufficient to induce the formation of osteoclasts, and are also important for their activation and survival. *OPG*— a soluble member of the TNF-receptor superfamily— acts as a decoy receptor that competes with *RANK* for *RANKL* (Figure 4). This explains why overexpression of *OPG* in mice leads to osteopetrosis, whereas *OPG* knockout mice develop severe osteoporosis. This illustrates the importance of the balance between *RANKL* and *OPG* in the control of bone remodelling.

Other synonyms used in the literature for *RANKL* are osteoclast differentiation factor (ODF), osteoprotegerin ligand (OPG-L) and TNF related activation-induced cytokine (TRANCE). The decoy receptor *OPG* is also known as osteoclastogenesis inhibitory factor (OCIF) and TNF receptor like molecule 1 (TR1) [17,109,110]. *RANKL*, *RANK* and *OPG*, although considered the bottleneck of osteoclastogenesis, represent approximately one-tenth of genes and loci that can control osteoclastogenesis and activation [110].

On the basis of knockout models, it is possible to delineate a genetic cascade that controls osteoclast differentiation. The *PU.1* gene

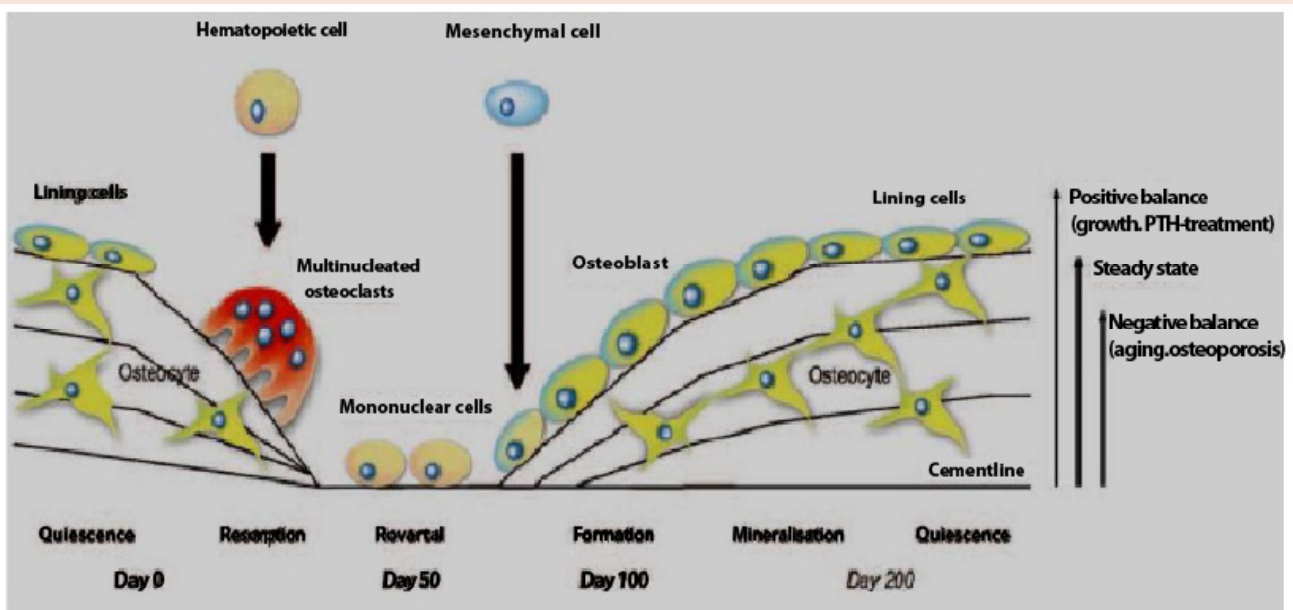
apparently acts at the earliest stages in this cascade, since mice with a loss of function mutation lack both osteoclasts and macrophages. *PU.1* is suggested to regulate the expression of the M-CSF receptor on cells of the monocyte/macrophage lineage [111]. Mice with a severe defect in their *M-CSF* gene (*op/op* mice) generate only immature macrophages and are deficient in osteoclasts [112].

The next steps of osteoclast differentiation are impaired by the absence of cytoplasmic tumour necrosis factor receptor-associated factor (TRAF) - 2, -5, and -6, which interact with the intracellular domain of *RANK*. Among the six major downstream signalling pathways are NFATc1, NF- $\kappa$ B, Akt/PKB, and the three mitogen-activated protein kinases (MAPK): extracellular signal-regulated kinase (ERK), p38, and c-Jun NH (2)-terminal kinase (JNK), which plays distinct roles in osteoclast differentiation, function and survival.

Intracellular signalling pathways can be modulated by interferon ( $\text{IFN}$ )- $\gamma$ , which promotes degradation of TRAF-6, and  $\text{IFN-}\beta$  which down-regulates one of the master genes of osteoclastogenesis, *c-fos*. Activation of the co-stimulatory receptors Fc receptor common gamma chain (Fc $\gamma$ ) and DNAX-activating protein 12 (DAP12), both of which harbor the immune-receptor tyrosine-based activation motif (ITAM), can activate calcium signalling, thereby mediating activation of NFATc1 [113].

Mice in which the genes for either  $\alpha_v\beta_3$  integrin or the tyrosine kinase c-Src were knocked out generate differentiated but functionally-impaired osteoclasts. Finally, failure to produce functional proteins such as  $\text{H}^+$ -ATPase, carbonic dehydratase II, or cathepsin K renders osteoclasts incapable of bone resorption.

Human phenotypes caused by mutation show the same



**Figure 4:** Local factors that regulates bone remodelling.



characteristics; for example juvenile Paget's disease is associated with a mutation in the gene coding for OPG [114], and mutation of carboanhydrase II produces osteopetrosis [115]. Inflammatory cytokines provide co-stimulatory signals that directly modulate osteoclastogenesis, besides their indirect activity *via* increasing the expression of RANKL by stromal cells/osteoblasts. Hence, IL-1 and TNF- $\alpha$ , which are highly abundant at sites of chronic inflammation, can amplify the process of osteoclastogenesis under permissive levels of RANKL [116,117]. Both cytokines are pathologic factors in the progression of bone resorption in rheumatoid arthritis, and neutralization of their bioactivity by intravenous infusion of antagonists or antibodies [118,119].

However, neither cytokine appears to be required for bone development, since disruption of either of the cytokine receptors in mice results in a minimal bone phenotype [120,121]. Bisphosphonates, which are considered first-line therapies for osteoporosis, also target osteoclasts, one of the important examples of bisphosphonates is Zoledronic acid. They are antiresorptive medicines, which means they slow or stop the natural process that dissolves bone tissue i.e. inhibits bone resorption, resulting in maintained or increased bone density and strength.

### Regulation of remodelling

The control of bone remodelling is complex and orchestrated by numerous local and systemic factors, and their expression and release is controlled by regulatory loops. Furthermore, the number and the responsiveness of the bone cells have an impact on bone remodelling. Local and systemic factors can affect bone remodelling by directly or indirectly targeting mature cells and their respective progenitor cells. The following schematics are a brief report on some of the main actions of local factors and a summary of the physiologic function of systemic factors that regulate bone mass (Figures 4, 5).

Members of the BMP family, Wnt family, hedgehog family and growth factors such as transforming growth factor  $\beta$ , platelet-derived growth factor, fibroblast growth factor, and insulin-like growth factor can modulate the various steps of osteoblastogenesis, e.g. commit pluripotent mesenchymal cells to the osteoblastic lineage, stimulate

migration and proliferation and promote their further differentiation into mature cells [122-124].

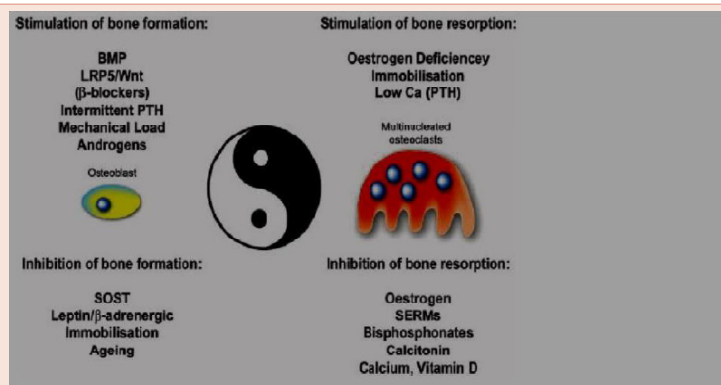
Moreover, local growth factors can also enhance or inhibit osteoclast formation and function. Interleukin-1 (IL-1), IL-3, IL-6, IL-11, leukaemia inhibitory factor (LIF), ciliary neutrophilic factor (CNTF), tumour necrosis factor (TNF), granulocyte macrophagecolony stimulating factor (GM-CSF), M-CSF, and c-kit ligand are among those growth factors that enhance osteoclast activity, whereas cytokines such as IL-4, IL-10, IL-18, and interferon  $\gamma$  inhibit the development of osteoclasts.

Prostaglandins as Prostaglandin (PG)E2 are also centrally involved in bone remodelling and repair and target osteogenic cells as well as osteoclasts via promoting both bone resorption and bone formation [125,126]. Deviations from the coordinated expression of these cytokines and growth factors in various cell types such as osteoblasts/stromal cells, monocytes/macrophages, lymphocytes, and malignant cells have been implicated in several pathologic conditions associated with increased bone loss such as osteoporosis, Paget's disease, and tumour hypercalcaemia. Moreover, it should be clearly stated that most of the local factors influence both the osteoblastic and the osteoclastic lineage [9,15,127].

### Factors regulating bone remodelling

**Parathyroid Hormone:** Parathyroid hormone (PTH) is an 84-amino acid peptide and the main factor responsible for the short-term regulation of calcium homeostasis. Extracellular calcium levels provide the signals for PTH secretion, a process controlled by the calcium-sensing receptor on the parathyroid cell. PTH increases renal tubular absorption of calcium, whereas it inhibits the tubular reabsorption of phosphate. PTH also stimulates the renal synthesis of 1, 25(OH) $_2$ D $_3$ , thereby increasing calcium and phosphate absorption through the intestinal tract. Moreover, PTH enhances bone resorption [128].

**1, 25-Dihydroxyvitamin D $_3$ :** Sources of vitamin D are either dietary or endogenously produced in the skin from 7dehydrocholesterol under the influence of ultraviolet light in the epidermis. Bound to a specific vitamin D binding protein in



**Figure 5:** Systemic factors that regulate bone mass.



the blood, it is transported to the liver where it is hydroxylated at the C-25 position, thus being converted to  $25(\text{OH})_2\text{D}_3$ . A second hydroxylation at the C-1 position takes place in the kidney, resulting in the formation of the most potent metabolite,  $1, 25(\text{OH})_2\text{D}_3$ . In addition, the local production of  $1, 25(\text{OH})_2\text{D}_3$  by the extrarenal 1-hydroxylase has been described. When serum calcium levels are high, the kidney increases the 24-hydroxylase activity, which ensures the synthesis of the relatively inactive metabolites  $24, 25(\text{OH})_2\text{D}_3$  and  $1, 24, 25(\text{OH})_2\text{D}_3$  [129].

A complex of  $1, 25(\text{OH})_2\text{D}_3$  together with the vitamin D receptor (VDR) binds to a vitamin D-responsive element on the DNA, which has been identified in– for example– the promoter region of osteocalcin, 24-hydroxylase and calbindin D28k. Vitamin D metabolites are also considered to have rapid actions mediated via membrane receptors [130]. Since  $1, 25(\text{OH})_2\text{D}_3$  has potent effects on calcium homeostasis, its production has to be strictly regulated. High levels of PTH as a result of hypocalcaemia stimulate 1-hydroxylase activities in the kidney, whereas high levels of calcium and  $1, 25(\text{OH})_2\text{D}_3$  have inhibitory effects. The enzymatic activity of the 24-hydroxylase is reciprocally regulated. The classical target tissues of  $1, 25(\text{OH})_2\text{D}_3$  are bone, intestine, and kidney [131].

**Calcitonin:** Calcitonin is a 32-amino-acid peptide hormone produced by the C cells of the thyroid gland. In contrast to PTH and  $1, 25(\text{OH})_2\text{D}_3$ – both of which increase calcium release from the mineralized matrix– calcitonin is an inhibitor of osteoclast activity. Calcitonin is not secreted until plasma calcium levels reached approximately 9.5 mg/dl in humans. In addition, dopamine, and oestradiol stimulate calcitonin secretion [132].

**Oestradiol:** Oestrogens can regulate calcium homeostasis, which is an essential requirement during fetal development and postnatal lactation. Oestrogen deficiency, after menopause or following ovariectomy is associated with a progressive loss of trabecular bone structure, also increasing the perforation of cortical bone. Oestrogen loss causes high bone remodelling rates, where bone formation cannot compensate for the increased resorption. Also, nongenotropic effects of sex steroids by synthetic ligand can prevent bone loss induced in ovariectomized and orchidectomized mice, without affecting the reproductive organs [133].

Androgen receptor (AR)-deficient mouse models have also increased bone turnover, which is associated with low testosterone levels. Interestingly, substitution of testosterone– but not dihydrotestosterone which cannot be converted to oestradiol by the aromatase– can compensate for orchidectomy-induced bone loss. These findings further suggest that the estrogen receptors (ERs) are of critical importance for bone preservation in male mice [134]. In humans, testosterone is more potently converted into oestradiol by the aromatase expressed in bone and adipose tissue, leading to oestradiol levels of about 30 pg/ml in men [135].

The effects of oestrogen on bone cells is complex and occurs at multiple sites: Oestrogen deficiency is associated with increasing medullary numbers of mesenchymal progenitor cells and progenitors of osteoclasts, in mice [136,137]. Monocytes, T cells and B cells from individuals with postmenopausal osteoporosis have a higher density

of RANKL on their cell membrane [138], and oestrogen can positively regulate OPG expression [139]. Oestrogen levels can also regulate the expression of cytokines of blood and marrow-derived cells, e.g. IL-1 and TNF- $\alpha$ , which in turn can influence the formation, activation and survival of osteoclasts [116,117,140,141]. Cytokines and other bioactive molecules, which are regulated by oestradiol presumably, act in cooperation; they control each other's expression and may have synergistic effects on their target cells [137]. Blocking of each single cytokine, IL-1, IL6 and TNF- $\alpha$ , can counteract bone loss in the ovariectomy mouse model [142,143]. T-cell-derived TNF- $\alpha$  is suggested to be a key factor of bone loss in this model, involving the TGF- $\beta$ -mediated mechanism [144,145].

Also B cells, which provide a subset of osteoclast progenitors and the cytokine IL-7, are considered to be players in the process of ovariectomy-induced bone loss [146,147]. Oestrogen and androgen are also a proapoptotic factor for osteoclasts, implying that steroid deficiency extends the lifetime of the bone-resorbing cell [148,149]. Oestradiol deficiency can lower the lifespan of osteoblasts and osteocytes, thereby signalling the need for increased bone remodelling. Oestradiol can trigger the differentiation of progenitor cells into the osteogenic vs the adipogenic lineage [150] and can stimulate the expression of RUNX-2, which parallels the increased osteogenic differentiation of *in vitro* cell cultures.

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