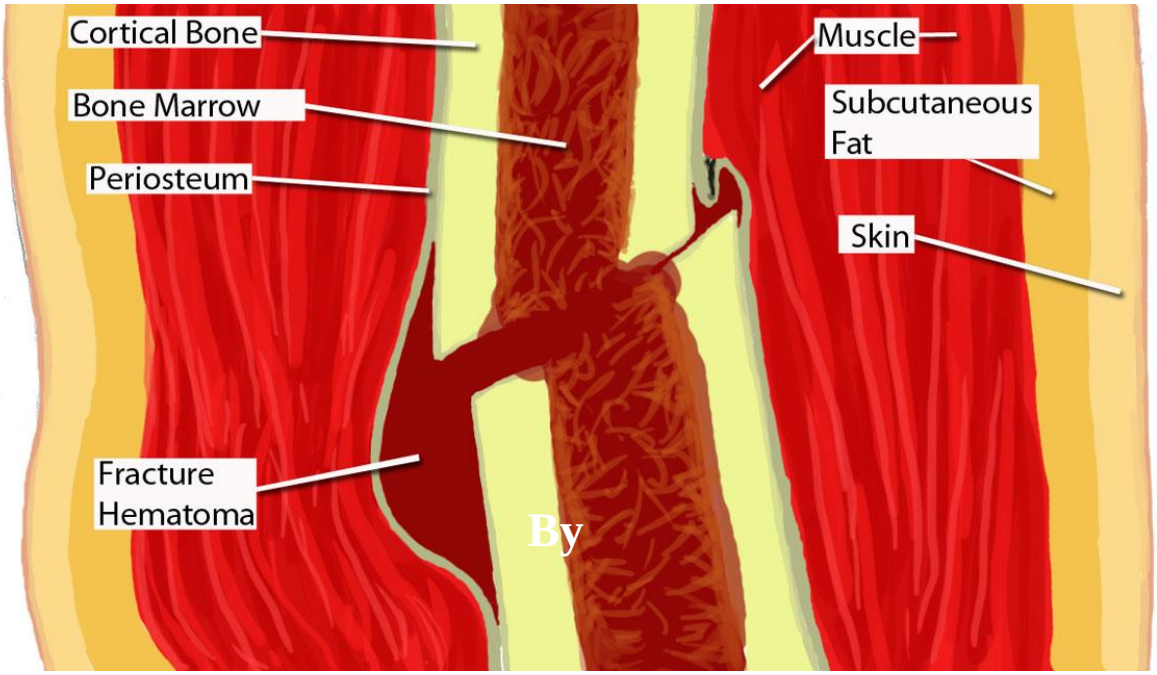
MUSCULO-SKELETAL CHEMISTRY

FOR INNOVATIVE MEDICAL EDUCATION INITIATIVE (NIMEI)



By

BEREKET MIHRETU (M. Sc. Medical Biochemistry)

E-mail: mihretubereket@gmail.com

DEBRE MARKOS UNIVERSITY

MAY, 2012^{EC}

D.MARKOSE ETHIOPIA

Biochemistry of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells

Introduction

Bone is a mineralized connective tissue that exhibits four types of cells: osteoblasts, bone lining cells, osteocytes, and osteoclasts . Bone exerts important functions in the body, such as locomotion, support and protection of soft tissues, calcium and phosphate storage, and harboring of bone marrow. Despite its inert appearance, bone is a highly dynamic organ that is continuously resorbed by osteoclasts and neoformed by osteoblasts. There is evidence that osteocytes act as mechanosensors and orchestrators of this bone remodeling process. The function of bone lining cells is notwell clear, but these cells seemto play an important role in coupling bone resorption to bone formation. Bone remodeling is a highly complex process by which old bone is replaced by new bone, in a cycle comprised of three phases:

(1) initiation of bone resorption by osteoclasts,

(2) the transition (or reversal period) fromresorption to new bone formation, and

(3) the bone formation by osteoblasts.

This process occurs due to coordinated actions of osteoclasts, osteoblasts, osteocytes, and bone lining cells which together form the temporary anatomical structure called basic multicellular unit (BMU). Normal bone remodeling is necessary for fracture healing and skeleton adaptation to mechanical use, as well as for calcium homeostasis. On the other hand, an imbalance of bone resorption and formation results in several bone diseases. For example, excessive resorption by osteoclasts without the corresponding amount of nerformed bone by osteoblasts contributes to bone loss and osteoporosis, whereas the contrary may result in osteopetrosis . Thus, the equilibrium between bone formation and resorption is necessary and depends on the action of several local and systemic factors including hormones, cytokines, chemokines, and biomechanical stimulation. Recent studies have shown that bone influences the activity of other organs and the bone is also influenced by other organs and systems of the body, providing new insights and evidencing the complexity and dynamic nature of bone tissue. In this review we will address the current data about bone cells biology, bone matrix, and the factors that influence the bone remodeling process. Moreover, we will briefly discuss the role of estrogen on bone tissue under physiological and pathological conditions.

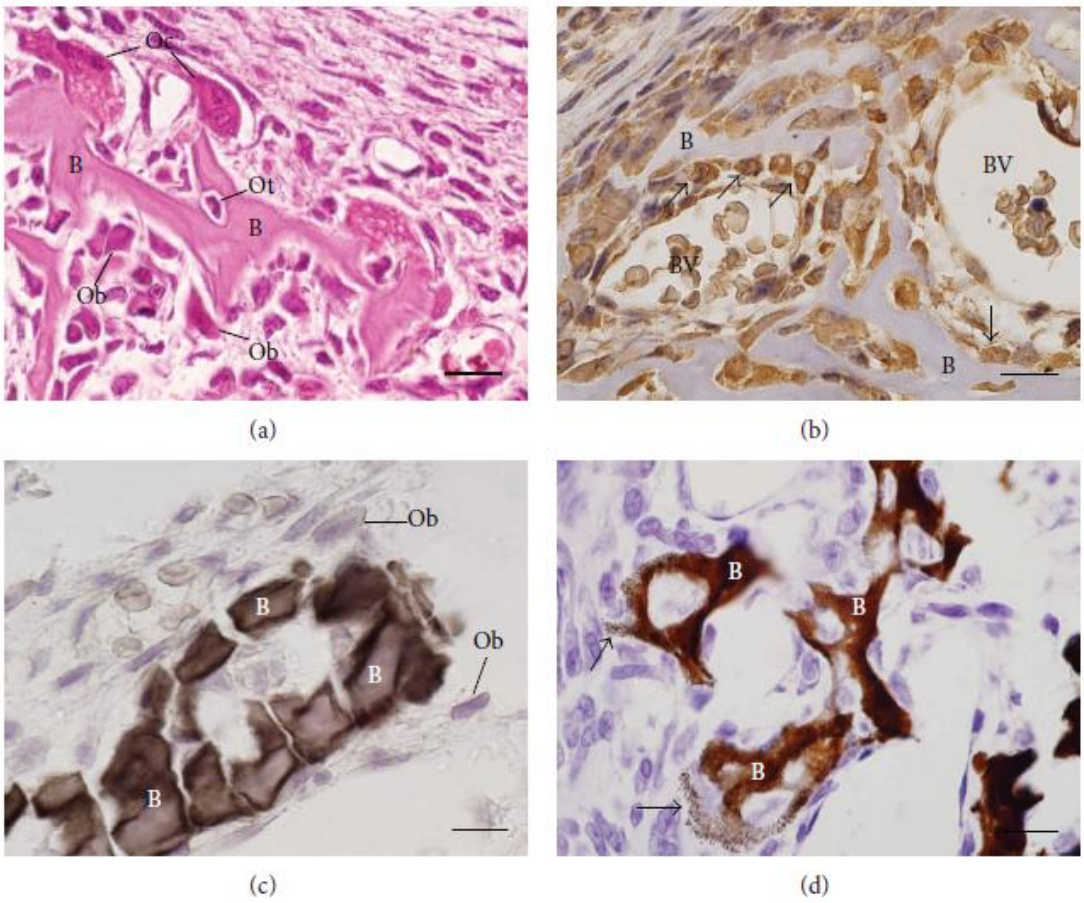


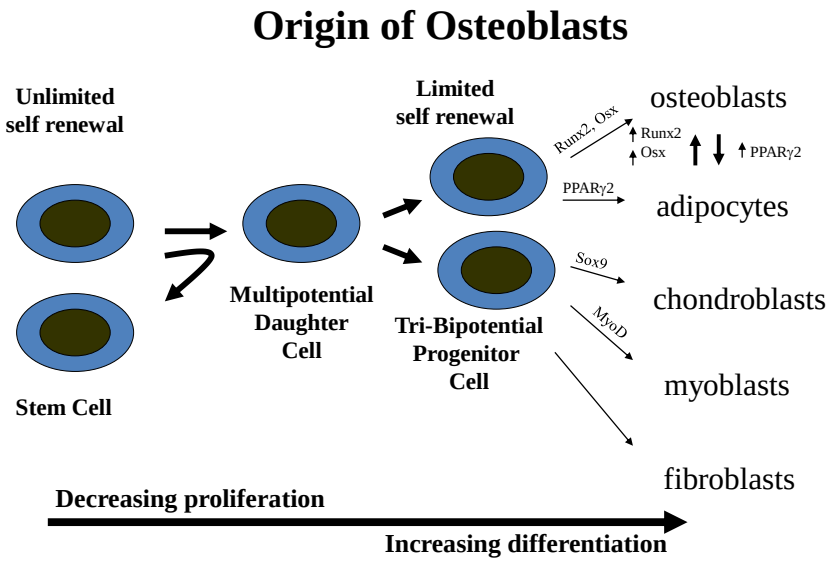
Figure 1: (a)–(d) Light micrographs of portions of alveolar bone of rats. (a) HE-stained section showing a portion of a bony trabecula (B).Polarized osteoblasts (Ob) and giant multinucleated osteoclasts (Oc) are observed in the bone surface; osteocyte (Ot) surrounding bone matrix is also observed. (b) Section subjected to immunohistochemistry for osteocalcin detection and counterstained with hematoxylin. Note osteocalcin-positive osteoblasts (arrows) on the surface of a bony trabecula (B). BV: blood vessel. (c) Undecalcified section subjected to the Gomori method for the detection of alkaline phosphatase, evidencing a portion of bone matrix (B) positive to the alkaline phosphatase (in brown/black). Ob: osteoblasts. (d) Undecalcified section subjected to the von Kossa method for calcium detection (brown/dark color). von Kossa-positive bone matrix (B) is observed; some positive granules (arrow) can also be observed on the surface of the bone trabeculae. Scale bar: 15 μm.

2. Bone Cells

The mesenchymal stem cells and osteoprogenitor cells

Adult mesenchymal stem cells can be isolated from bone marrow, adipose tissues, or amniotic membrane. Mesenchymal stem cells are, by definition, of self-renewal capacity and able to repopulate all the appropriate differentiation lineages. They are multipotent cells that can differentiate into osteoblastic, myoblastic, adipocytic, chondrocytic, endothelial, and neurogenic lineage through a multi-step differentiation sequence as follows: proliferation, commitment,

lineage progression, maturation, and differentiation. For the osteogenic lineage, mesenchymal stem cells sustain a cascade of differentiation steps as described by the following sequence: Mesenchymal stem cell → immature osteoprogenitor → mature osteoprogenitor → preosteoblast → mature osteoblast → osteocyte or lining cells or apoptosis. The later the differentiation stage, the lower the cell self-renewal and proliferation capacity. In bone marrow, osteoprogenitor cells represent a very small percentage (eg <0.005%) of nucleated cell types in healthy adult bone. Embryonic stem cells are also a potential source due to their pluripotentiallity and therefore their ability to differentiate into osteogenic lineage.



Adapted from Primer on the metabolic bone diseases and disorders of mineral metabolism. Lian JB, Stein GS and Aubin JE., Bone formation: Maturation and functional activities of osteoblast lineage cells, ASBMR, 2003

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2.1. Osteoblasts. Osteoblasts are cuboidal cells that are located along the bone surface comprising 4–6% of the total resident bone cells and are largely known for their bone forming function .These cells show morphological characteristics of protein synthesizing cells, including abundant rough endoplasmic reticulum and prominent Golgi apparatus, as well as various secretory vesicles. As polarized cells, the osteoblasts secrete the osteoid toward the bone matrix (Figures 1(a), 1(b), and 2(a)). Osteoblasts are derived from mesenchymal stem cells (MSC). The commitment of MSC towards the osteoprogenitor lineage requires the expression of specific genes, following timely programmed steps, including the synthesis of bone morphogenetic proteins (BMPs) and members of the Wingless (Wnt) pathways . The expressions of Runtrelated transcription factors 2,Distal-less homeobox 5 (Dlx5), and osterix (Osx) are crucial for osteoblast

differentiation. Additionally, *Runx2* is a master gene of osteoblast differentiation, as demonstrated by the fact that Runx2-null mice are devoid of osteoblasts . *Runx2* has demonstrated to upregulate osteoblast-related genes such as *ColIA1*, *ALP*, *BSP*, *BGLAP*, and *OCN*.

Once a pool of osteoblast progenitors expressing *Runx2* and *ColIA1* has been established during osteoblast differentiation, there is a proliferation phase. In this phase, osteoblast progenitors show alkaline phosphatase (ALP) activity, and are considered preosteoblasts . The transition of preosteoblasts to mature osteoblasts is characterized by an increase in the expression of Osx and in the secretion of bone matrix proteins such as osteocalcin (OCN), bone sialoprotein (BSP) I/II, and collagen type I.Moreover, the osteoblasts undergo morphological changes, becoming large and cuboidal cells.

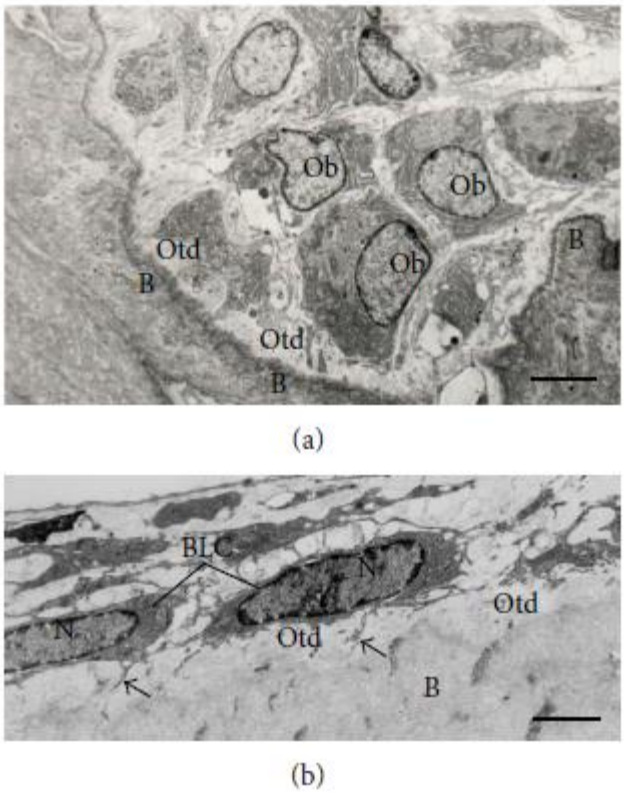


Figure 2: Electron micrographs of portions of alveolar bone of rats. (a) Oteoblasts exhibiting abundant rough endoplasmic reticulum are observed adjacent to the bone (B) surface. A layer of bundles of collagen fibrils situated between osteoblasts (Ob) and calcified bone surface (B) constitutes the osteoid (Otd). Scale bar: 2.7 μm. (b) Bone lining cells (BLC) exhibiting scarce cytoplasm are situated on the osteoid surface (Otd). Bone lining cells (BLC) extend some thin cytoplasmic projections (arrows) towards the osteoid (Otd). Scale bar: 2 μm. N: nucleus.

There is evidence that other factors such as fibroblast growth factor (FGF), microRNAs, and connexin 43 play important roles in the osteoblast differentiation. FGF-2knockoutmice showedadecreasedbonemass coupled to increase of adipocytes in the bone marrow, indicating the participation of FGFs in the osteoblast differentiation. It has also been demonstrated that FGF-18 upregulates osteoblast differentiation in an autocrine mechanism. MicroRNAs are involved in the regulation of gene expression in many cell types, including osteoblasts, in which some microRNAs stimulate and others inhibit osteoblast differentiation. Connexin 43 is known to be the main connexin in bone .The mutation in the gene encoding connexin 43 impairs osteoblast differentiation and causes skeletal malformation in mouse. The synthesis of bone matrix by osteoblasts occurs in two main steps: deposition of organic matrix and its subsequent mineralization (Figures 1(b)–1(d)). In the first step, the osteoblasts secrete collagen proteins, mainly type I collagen, noncollagen proteins (OCN, osteonectin, BSP II, and osteopontin), and proteoglycan including decorin and biglycan, which form the organic matrix. Thereafter, mineralization of bone matrix takes place into two phases: the vesicular and the fibrillar phases. The vesicular phase occurs when portions with a variable diameter ranging from 30 to 200 nm, called matrix vesicles, are released from the apical membrane domain of the osteoblasts into the newly formed bone matrix in which they bind to proteoglycans and other organic components. Because of its negative charge, the sulphated proteoglycans immobilize calcium ions that are stored within the matrix vesicle . When osteoblasts secrete enzymes that degrade the proteoglycans, the calcium ions are released from the proteoglycans and cross the calciumchannels presented in thematrix vesiclesmembrane.

These channels are formed by proteins called annexins. On the other hand, phosphate-containing compounds are degraded by the ALP secreted by osteoblasts, releasing phosphate ions inside the matrix vesicles. Then, the phosphate and calcium ions inside the vesicles nucleate, forming the hydroxyapatite crystals. The fibrillar phase occurs when the supersaturation of calcium and phosphate ions inside the matrix vesicles leads to the rupture of these structures and the hydroxyapatite crystals spread to the surrounding matrix.

Mature osteoblasts appear as a single layer of cuboidal cells containing abundant rough endoplasmic reticulum and large Golgi complex (Figures 2(a) and 3(a)). Some of these osteoblasts show cytoplasmic processes towards the bone matrix and reach the osteocyte processes. At this stage, the mature osteoblasts can undergo apoptosis or become osteocytes or

bone lining cells. Interestingly, round/ ovoid structures containing dense bodies and TUNEL-positive structures have been observed inside osteoblast vacuoles. These findings suggest that besides professional phagocytes, osteoblasts are also able to engulf and degrade apoptotic bodies during alveolar bone formation.

2.2. Bone Lining Cells. Bone lining cells are quiescent flatshaped osteoblasts that cover the bone surfaces,where neither bone resorption nor bone formation occurs. These cells exhibit a thin and flat nuclear profile; its cytoplasm extends along the bone surface and displays few cytoplasmic organelles such as profiles of rough endoplasmic reticulum and Golgi apparatus (Figure 2(b)). Some of these cells show processes extending into canaliculi, and gap junctions are also observed between adjacent bone lining cells and between these cells and osteocytes. The secretory activity of bone lining cells depends on the bone physiological status, whereby these cells can reacquire their secretory activity, enhancing their size and adopting a cuboidal appearance. Bone lining cells functions are not completely understood, but it has been shown that these cells prevent the direct interaction between osteoclasts and bone matrix, when bone resorption should not occur, and also participate in osteoclast differentiation, producing osteoprotegerin (OPG) and the receptor activator of nuclear factor kappa-B ligand (RANKL). Moreover, the bone lining cells, together with other bone cells, are an important component of the BMU, an anatomical structure that is present during the bone remodeling cycle.

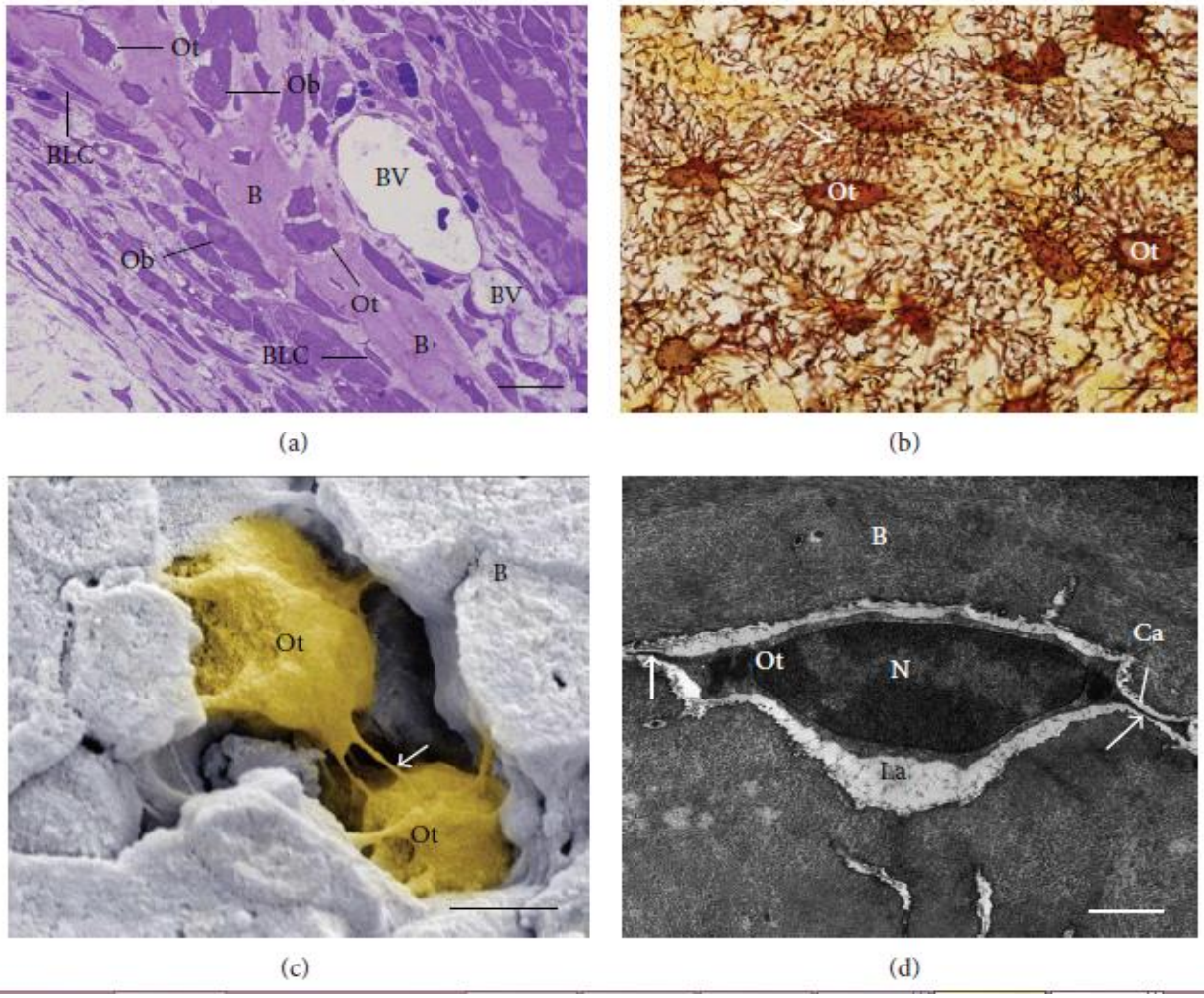


Figure 3: Light (a and b) and electron micrographs of portions of alveolar bone rats. (a) a semithin section stained with toluidine blue showing a portion of a bony trabecula (B). Osteoblasts (Ob) and bone lining cells (BLC) are present on bone surface while osteocytes (Ot) are observed entrapped in the bone matrix. BV: blood vessels. Scale bar: 15μm. (b) Section subjected to the silver impregnation method. Note the cytoplasmic processes (arrows) of the osteocytes (Ot) connecting them with each other. Scale bar: 15 μm. (c) Scanning electron micrograph showing two osteocytes (Ot) surrounded by bonematrix (B). Note that the cytoplasmic processes (arrows) are observed between the osteocytes (Ot) forming an interconnected network. Scale bar: 2 μm. (d) Transmission electron micrograph showing a typical osteocyte (Ot) inside a lacuna (La) in the bone matrix (B), with its cytoplasmic processes (arrows) inside the canaliculi (Ca). Scale bar: 2 μm.N: nucleus.

2.3. ***Osteocytes.*** Osteocytes, which comprise 90–95% of the total bone cells, are the most abundant and long-lived cells, with a lifespan of up to 25 years. Different from osteoblasts and osteoclasts, which have been defined by their respective functions during bone formation and bone resorption, osteocytes were earlier defined by their morphology and location. For decades, due to difficulties in isolating

osteocytes frombone matrix led to the erroneous notion that these cells would be passive cells, and their functions were misinterpreted. The development of new technologies such as the

identification of osteocyte-specific markers, new animal models, development of techniques for bone cell isolation and culture, and the establishment of phenotypically stable cell lines led to the improvement of the understanding of osteocyte biology. In fact, it has been recognized that these cells play numerous important functions in bone.

The osteocytes are located within lacunae surrounded by mineralized bone matrix, wherein they show a dendritic morphology (Figures 3(a)–3(d)).Themorphology of embedded osteocytes differs depending on the bone type. For instance, osteocytes from trabecular bone are more rounded than osteocytes from cortical bone, which display an elongated morphology. Osteocytes are derived from MSCs lineage through osteoblast differentiation. In this process, four recognizable stages have been proposed: osteoid-osteocyte, preosteocyte, young osteocyte, and mature osteocyte. At the end of a bone formation cycle, a subpopulation of osteoblasts becomes osteocytes incorporated into the bone matrix. This process is accompanied by conspicuous morphological and ultrastructural changes, including the reduction of the round osteoblast size. The number of organelles such as rough endoplasmic reticulumandGolgi apparatus decreases, and the nucleus-tocytoplasm ratio increases, which correspond to a decrease in the protein synthesis and secretion.

During osteoblast/osteocyte transition, cytoplasmic process starts to emerge before the osteocytes have been encased into the bone matrix. The mechanisms involved in the development of osteocyte cytoplasmic processes are not well understood. However, the protein E11/gp38, also called podoplanin may have an important role. E11/gp38 is highly expressed in embedding or recently embedded osteocytes, similarly to other cell types with dendritic morphology such as podocytes, type II lung alveolar cells, and cells of the choroid plexus. It has been suggested that E11/gp38 uses energy from GTPase activity to interact with cytoskeletal components andmolecules involved in cell motility, whereby regulate actin cytoskeleton dynamics. Accordingly, inhibition of E11/gp38 expression in osteocyte-like MLO-Y4 cells has been shown to block dendrite elongation, suggesting that E11/gp38 is implicated in dendrite formation in osteocytes. Once the stage of mature osteocyte totally entrapped within mineralized bone matrix is accomplished, several of the previously expressed osteoblast markers such as OCN,

BSPII, collagen type I, and ALP are downregulated. On the other hand, osteocyte markers including dentine matrix protein 1 (DMP1) and sclerostin are highly expressed.

Whereas the osteocyte cell body is located inside the lacuna, its cytoplasmic processes (up to 50 per each cell) cross tiny tunnels that originate from the lacuna space called canaliculi, forming the osteocyte lacunocanalicular system (Figures 3(b)–3(d)). These cytoplasmic processes are connected to other neighboring osteocytes processes by gap junctions, as well as to cytoplasmic processes of osteoblasts and bone lining cells on the bone surface, facilitating the intercellular transport of small signaling molecules such as prostaglandins and nitric oxide among these cells. In addition, the osteocyte lacunocanalicular system is in close proximity to the vascular supply, whereby oxygen and nutrients achieve osteocytes.

It has been estimated that osteocyte surface is 400-fold larger than that of the all Haversian and Volkmann systems and more than 100-fold larger than the trabecular bone surface. The cell-cell communication is also achieved by interstitial fluid that flows between the osteocytes processes and canaliculi . By the lacunocanalicular system (Figure 3(b)), the osteocytes act as mechanosensors as their interconnected network has the capacity to detectmechanical pressures and loads, thereby helping the adaptation of bone to daily mechanical forces. By this way, the osteocytes seem to act as orchestrators of bone remodeling, through regulation of osteoblast and osteoclast activities. Moreover, osteocyte apoptosis has been recognized as a chemotactic signal to osteoclastic bone resorption. In agreement, it has been shown that during bone resorption, apoptotic osteocytes are engulfed by osteoclasts.

The mechanosensitive function of osteocytes is accomplished due to the strategic location of these cells within bone matrix.Thus, the shape and spatial arrangement of the osteocytes are in agreement with their sensing and signal transport functions, promoting the translation of mechanical stimuli into biochemical signals, a phenomenon that is called piezoelectric effect .Themechanisms and components by which osteocytes convert mechanical stimuli to biochemical signals are not well known. However, two mechanisms have been proposed.One of them is that there is a protein complex formed by a cilium and its associated proteins PolyCystins 1 and 2, which has been suggested to be crucial for osteocyte mechanosensing and for osteoblast/osteocyte-mediated bone formation. The second mechanism involves osteocyte cytoskeleton components, including focal adhesion protein complex and its multiple actin-associated proteins such as paxillin, vinculin, talin, and zyxin. Upon mechanical stimulation, osteocytes produce several secondary messengers, for example, ATP, nitric oxide (NO), Ca2+, and prostaglandins (PGE2 and PGI2,) which influence bone physiology. Independently of the

mechanism involved, it is important to mention that the mechanosensitive function of osteocytes is possible due to the intricate canalicular network, which allows the communication among bone cells.

2.4. ***Osteoclasts***. Osteoclasts are terminally differentiated multinucleated cells (Figures 4(a)–4(d)), which originate from mononuclear cells of the hematopoietic stem cell lineage, under the influence of several factors. Among these factors the macrophage colony-stimulating factor (M-CSF), secreted by osteoprogenitor mesenchymal cells and osteoblasts , and RANK ligand, secreted by osteoblasts,

osteocytes, and stromal cells, are included. Together, these factors promote the activation of transcription factors and gene expression in osteoclasts. M-CSF binds to its receptor (cFMS) present in osteoclast precursors, which stimulates their proliferation and inhibits their apoptosis. RANKL is a crucial factor for osteoclastogenesis and is expressed by osteoblasts, osteocytes, and stromal cells. When it binds to its receptor RANK in osteoclast precursors, osteoclast formation is induced. On the other hand, another factor called osteoprotegerin (OPG), which is produced by a wide range of cells including osteoblasts, stromal cells, and gingival and periodontal fibroblasts, binds to RANKL, preventing the RANK/RANKL interaction and, consequently, inhibiting the osteoclastogenesis (Figure 5). Thus, the RANKL/RANK/OPG system is a key mediator of osteoclastogenesis. The RANKL/RANK interaction also promotes the expression of other osteoclastogenic factors such as NFATc1 and DC-STAMP. By interacting with the transcription factors PU.1, cFos, and MITF, NFATc1 regulates osteoclastspecific genes including *TRAP* and *cathepsin K*, which are crucial for osteoclast activity . Under the influence of the RANKL/RANK interaction, NFATc1 also induces the expression of DC-STAMP, which is crucial for the fusion of

osteoclast precursors. Despite these osteoclastogenic factors having been well defined, it has recently been demonstrated that the osteoclastogenic potential may differ depending on the bone site considered. It has been reported that osteoclasts from long bone marrow are formed faster than in the jaw. This different dynamic of osteoclastogenesis possibly could be, due to the cellular composition of the bone-site specific marrow . During bone remodeling osteoclasts polarize; then, four types of osteoclast membrane domains can be observed: the sealing zone and ruffled border that are in contact with the bone matrix (Figures 4(b) and 4(d)), as well as the basolateral and functional secretory domains, which are not in contact with the bone matrix.

Polarization of osteoclasts during bone resorption involves rearrangement of the actin cytoskeleton, in which an F-actin ring that comprises a dense continuous zone of highly dynamic podosome is formed and consequently an area of membrane that develop into the ruffled border is isolated. It is important to mention that these domains are only formed when osteoclasts are in contact with extracellular mineralized matrix, in a process which $\alpha v\beta 3$-integrin, as well as the CD44, mediates the attachment of the osteoclast podosomes to the bone surface .

Ultrastructurally, the ruffled border is a membrane domain formed by microvilli, which is isolated from the surrounded tissue by the clear zone, also known as sealing zone. The clear zone is an area devoid of organelles located in the periphery of the osteoclast adjacent to the bone matrix. This sealing zone is formed by an actin ring and several other proteins, including actin, talin, vinculin, paxillin, tensin,and actin-associated proteins such as α-actinin, fimbrin, gelsolin, and dynamin [95]. The $\alpha v\beta 3$-integrin binds to noncollagenous bone matrix containing-RGD sequence such as bone sialoprotein, osteopontin, and vitronectin, establishing a peripheric sealing that delimits the central region,where the ruffled border is located (Figures 4(b)–4(d)). The maintenance of the ruffled border is also essential for osteoclast activity; this structure is formed due to intense trafficking of lysosomal and endosomal components.

In the ruffled border, there is a vacuolar-type H+-ATPase (V-ATPase), which helps to acidify the resorption lacuna and hence to enable dissolution of hydroxyapatite crystals. In this region, protons and enzymes, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K, and matrix metalloproteinase-9 (MMP-9) are transported into a compartment called Howship lacuna leading to bone degradation (Figure 5). The products of this degradation are then endocytosed across the ruffled border and transcytosed to the functional secretory domain at the plasma membrane.

Abnormal increase in osteoclast formation and activity leads to some bone diseases such as osteoporosis, where resorption exceeds formation causing decreased bone density and increased bone fractures. In some pathologic conditions including bone metastases and inflammatory arthritis, abnormal osteoclast activation results in periarticular erosions andpainful osteolytic lesions, respectively.

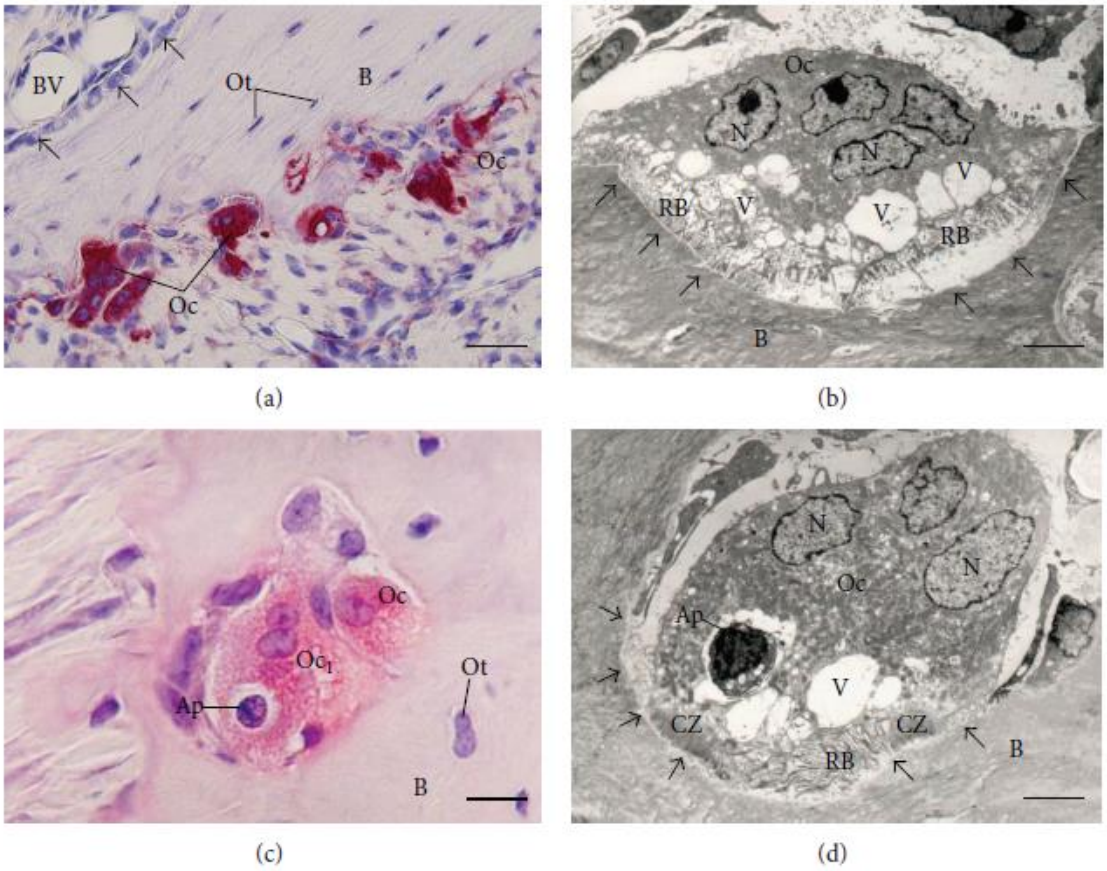


Figure 4: Light (a and c) and electron micrographs (b and d) of portions of alveolar bone of rats. In (a) tartrate-resistant acid phosphatase (TRAP) activity (in red color) is observed in the cytoplasm of osteoclasts (OC) adjacent to the alveolar bone (B) surface. Note that in the opposite side of the bony trabecula B is covered by large and polarized osteoblasts (Ob). Ot, osteocytes (Ot); BV: blood vessel. Bar: 40 μm. (b) Multinucleated osteoclast (OC) shows evident ruffled border (RB) adjacent to the excavated bone surface (arrows). Several vacuoles (V) are observed in the cytoplasm adjacent to ruffled border (RB). N: nucleus. Bar: 4 μm. (c) Portions of TRAP-positive osteoclasts (Oc andOc1) are observed in a resorbing bone lacuna. A round cell (Ap) with condensed irregular blocks of chromatin, typical apoptotic cell, is observed inside a large vacuole of theOc1. B: bone matrix;Ot: osteocyte. Bar: 15 μm. (d) An osteoclast (Oc) showing ruffled border (RB) and clear zone (CZ) is in close juxtaposition to the excavation of the bone surface (arrows), that is, Howship lacuna. Vacuoles (V) with varied size are present next to the ruffled border (RB); one of them contains a round cell with masses of condensed chromatin (Ap), typical of cell undergoing apoptosis.
B: bone matrix; N: nucleus. Bar: 3 μm.

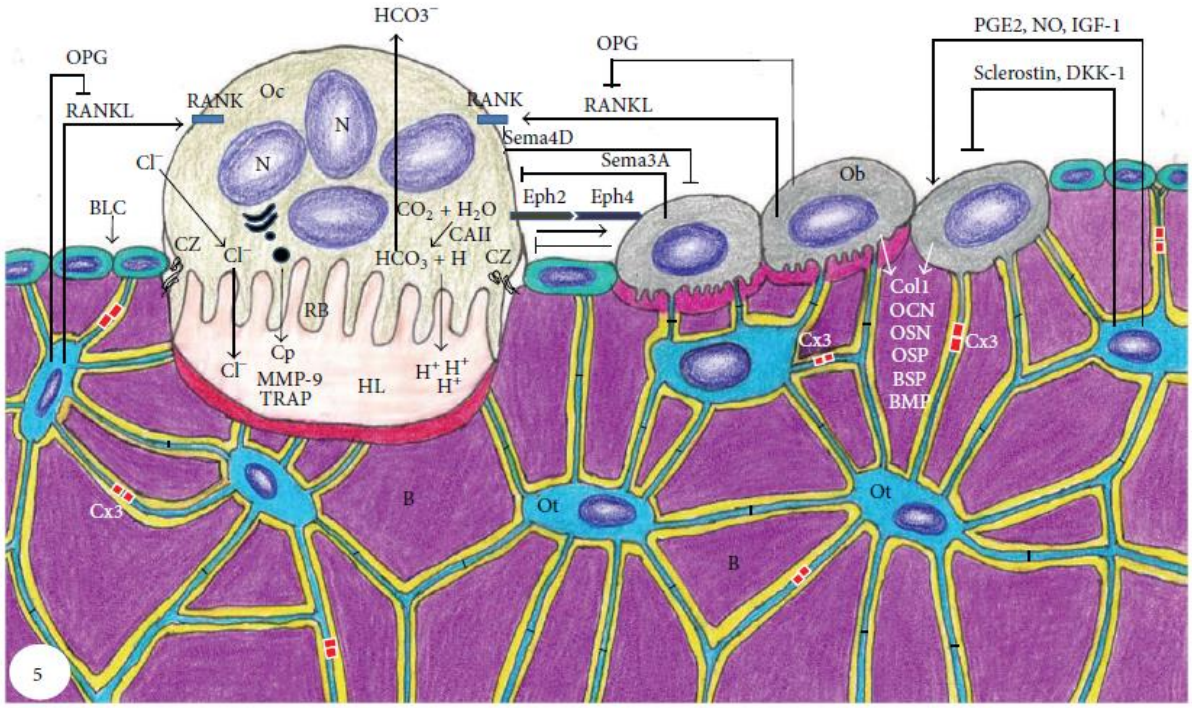


Figure 5: Schematic summary of bone tissue showing bone cells and the relationships among them and with bone matrix (B). Osteoclast (Oc) activation occurs after binding of RANKL to its receptor RANK, present in the membrane of osteoclast precursors. Then, osteoclast becomes polarized through its cytoskeleton reorganization; the ruffled border (RB) and clear zone (CZ) are membrane specializations observed in the portion of the osteoclast juxtaposed to the bone resorption surface, Howship lacuna (HL). Dissolution of hydroxyapatite occurs in the bone surface adjacent to the ruffled border (RF) upon its acidification due to pumping of hydrogen ions (H+) to the HL. H+ and ions bicarbonate (HCO3 −) originate from the cleavage of carbonic acid (H2CO3) under the action of carbonic anhydrase II (CAII). After dissolution of mineral phase, osteoclast (Oc) releases cathepsin (Cp), matrix metalloproteinase-9 (MMP-9), and tartrate-resistant acid phosphatase (TRAP) that degrade the organic matrix. EphrinB2 (Eph2) present in osteoclast membrane binds to ephrinB4 (Eph4) in osteoblast (Ob) membrane, promoting its differentiation, whereas the reverse signaling (ephrinB4/ephrinB2) inhibits osteoclastogenesis. Sema4Dproduced by osteoclasts inhibits osteoblasts,while Sema3Asecreted by osteoblasts inhibits osteoclasts.Osteoblasts (Ob) also produce receptor activator of nuclear factor KB (RANKL) and osteoprotegerin (OPG), which increase and decrease osteoclastogenesis, respectively. Osteoblasts (Ob) secrete collagenous (Col1) and noncollagenous proteins such as osteocalcin (OCN), osteopontin (OSP), osteonectin (OSN), bone sialoprotein (BSP), and bone morphogenetic proteins (BMP). Osteocytes (Ot) are located within lacunae surrounded by mineralized bone matrix (B). Its cytoplasmic processes cross canaliculi to make connection with other neighboring osteocytes processes by gap junctions, mainly composed by connexin 43 (Cx3), as well as to cytoplasmic processes of osteoblasts (Ob) and bone lining cells (BLC) on bone surface. RANKL secreted by osteocytes stimulates osteoclastogenesis, while prostaglandin E2 (PGE2), nitric oxide (NO), and insulin-like growth factor (IGF) stimulate osteoblast activity. Conversely, osteocytes produce OPG that inhibits osteoclastogenesis;moreover, osteocytes produce sclerostin and dickkopfWNT signaling pathway inhibitor (DKK-1) that decrease osteoblast activity.

Discovery of osteoprotegerin, RANKL, and RANK

Between 1981 and the mid 1990s, the Rodan–Martin hypothesis was supported by many studies, but the factor(s) expressed by osteoblast/stromal or other cells remained undetermined until they were discovered independently by four groups using different approaches. Boyle and coworker at Amgen Inc. (Thousand Oaks, CA, USA) discovered OPG unexpectedly in studies to identify tumor necrosis factor (TNF) receptor related molecules with possible therapeutic utility by generating transgenic mice that over-express various TNF receptor related cDNAs. Mice overexpressing one particular cDNA developed marked osteopetrosis because they did not have any osteoclasts in their bones. The protein encoded by the gene was named osteoprotegerin (the bone protector) , because it appeared to protect the skeleton from excessive bone resorption by limiting osteoclastic bone resorption.

Independently, researchers at the Snow Brand Milk Products Co. (Sapporo, Hokkaido, Japan) reported their discovery of an identical molecule using the standard approach to test the Rodan–Martin hypothesis of purifying a factor from human embryonic fibroblasts that inhibited osteoclastogenesis. They obtained a partial protein sequence and subsequently cloned the cDNA for OPG. Using expression cloning and OPG as a probe, both groups quickly identified its ligand, which they called OPG ligand and osteoclast differentiation factor, respectively. This ligand turned out to be identical to a member of the TNF ligand family, which had been identified in the preceding year as RANKL and TNF-related activation induced cytokine. Soon after OPG ligand/osteoclast differentiation factor was identified as a ligand for OPG, the cellular receptor was identified as being identical to the previously identified RANK, which Anderson and coworkers at Immunex (Seattle, WA, USA) had discovered while they were sequencing cDNAs from a human bone marrow derived myeloid dendritic cell cDNA library.

They found that RANK had partial homology to a portion of the extracellular domain of human CD40, a member of the TNF receptor superfamily, and that it was involved in the activation of T cells in the immune system. They then isolated RANKL by direct expression screening and found, like Wong and coworkers did, that it increased dendritic cell stimulated naïve T cell proliferation and survival of RANK-expressing T cells. These discoveries that RANKL is involved in osteoclastogenesis and T cell activation have spawned the now growing field of osteoimmunology. Transcription factor activation by RANKL/RANK in osteoclasts With the

knowledge that RANKL/RANK signaling is essential for osteoclast formation, major efforts have been made to identify the signaling pathways that are activated downstream and to determine the full extent of the involvement of RANKL in osteoclast biology and common bone diseases. After RANKL binds to RANK, a key preliminary step in downstream signaling is binding of TRAFs to specific sites within the cytoplasmic domain of RANK, which is a transmembrane protein that - like the TNF receptors - has no intrinsic ability to activate protein kinases to mediate signaling. TRAF2, -5, and -6 all bind to RANK, but of these only TRAF6 appears to be essential in osteoclasts, because only TRAF6 knockout mice develop osteopetrosis. Interestingly, although two independently produced mutant TRAF6 mice have osteopetrosis, surprisingly one has normal numbers of osteoclasts (but they are inactive) and the other has no osteoclasts . At least seven signaling pathways are activated by RANK-mediated protein kinase signaling; four of them directly mediate osteoclastogenesis (inhibitor of NF-κB kinase/NF-κB, c-Jun amino-terminal kinase/activator protein-1, c-myc, and calcineurin/nuclear factor of activated T cells [NFAT]c1) and three mediate osteoclast activation (src and MKK6/p38/ MITF) and survival (src and extracellular signal-regulated kinase).

It remains unexplained how inactivation of TRAF6 resulted in two different osteoclast phenotypes, however. Several adapter molecules bind to RANK along with TRAFs to mediate signaling. Among these is Grb-2 associated binder protein 2, a member of a family of adapter molecules that are phosphorylated at tyrosine residues and recruit a variety of signaling molecules that contain Src homology 2 domains. Loss of Grb-2 associated binder protein 2 results in reduced RANKL/RANK-induced osteoclast differentiation, decreased bone resorption, and mild osteopetrosis. This indicates that it plays a significant role in RANKL-induced osteoclastogenesis. The essential role played by NF-κB/activator protein-1/ NFATc1 signaling for osteoclast formation was discovered

RANKL

RANKL is a type II homotrimeric transmembrane protein that is expressed as a membrane-bound and a secreted protein, which is derived from the membrane form as a result of either proteolytic cleavage or alternative splicing. The proteolytic cleavage of RANKL requires ADAM (a disintegrin and metalloprotease domain) and matrix metalloproteases. RANKL expression is stimulated in osteoblast/stromal cells by most of the factors that are known to stimulate

osteoclast formation and activity. It is highly expressed in lymph nodes, thymus and lung, and at low levels in a variety of other tissues including spleen and bone marrow. In inflamed joints it is expressed by synovial cells and secreted by activated T cells. These sources of RANKL appear to be responsible, at least in part, for mediating the joint destruction in patients with rheumatoid arthritis. TNF also mediates joint destruction in rheumatoid arthritis by systemically increasing the number of circulating OCPs, and by promoting their egress from the bone marrow into the peripheral blood and then to the inflamed joints, where it promotes fusion of these cells to osteoclasts along with RANKL and interleukin-1. RANKL, like TNF, stimulates the release of immature progenitors into the circulation. However, RANKL does not induce OCP mobilization in protein tyrosine phosphatase-ε knockout mice with osteoclasts that are defective in terms of bone adhesion and resorption. Thus, RANKL-induced osteoclast activation may regulate progenitor recruitment as part of homeostasis and host defense, linking bone remodeling with regulation of hematopoiesis.

Preclinical studies in mice have shown that RANKL is also expressed in mammary epithelial cells during pregnancy and is required for lactational hyperplasia of mammary epithelial cells and milk production. It is also expressed by some malignant tumor cells that also express RANK, and thus it may play a role in inducing tumor cell proliferation by an autocrine mechanism or in a paracrine manner if it is produced by accessory cells, such as activated T cells. However, production by T cells of RANKL also induces expression of interferon-β by activated osteoclasts through c-Fos to negatively regulate their formation. This mechanism can be enhanced by T-cell produced interferon-γ, which degrades TNF receptor associated factor (TRAF)6, an essential adapter protein that is recruited to RANK to mediate RANK signaling (see below).

RANK

RANK is a type I homotrimeric transmembrane protein whose expression was initially detected only on OCPs, mature osteoclasts, and dendritic cells. Like RANKL, however, it is expressed widely [17]. RANK protein expression has been reported in mammary gland and some cancer cells, including breast and prostate cancers, two types of tumors with high bone metastasis potential. Although no humans have been identified to date with inactivating mutations or deletions of RANK, a deletion mutation occurred spontaneously in a line of transgenic mice, which consequently had all of the features of mice with targeted deletion of RANK, confirming

the importance of RANK for osteoclast formation. Activating mutations in exon 1 of RANK that cause an increase in RANK-mediated nuclear factor-κB (NF-κB) signaling and a resultant increase in osteoclast formation and activity account for the increased osteolysis seen in some patients with familial Paget's disease and have confirmed the importance of this system in humans . A potential role for RANK in tumor cell proliferation is being investigated and, if proven, could be a future target for anti-tumor therapy.

OPG (the Decoy Receptor, Belonging to the TNF Receptor Family)

OPG is a new member of the tumor necrosis factor (TNF) receptor family which plays a key role in the physiological regulation of osteoclastic bone resorption. The protein, which is produced by osteoblasts and marrow stromal cells, lacks a transmembrane domain and acts as a secreted decoy receptor which has no direct signaling capacity. OPG acts by binding to its natural ligand OPGL, which is also known as RANKL (receptor activator of NF-kappaB ligand). This binding prevents OPGL from activating its cognate receptor RANK, which is the osteoclast receptor vital for osteoclast differentiation, activation and survival (fig 6 & 7). Overexpression of OPG in transgenic mice leads to profound osteopetrosis secondary to a near total lack of osteoclasts. Conversely, ablation of the OPG gene causes severe osteoporosis in mice. Ablation of OPGL or RANK also produces profound osteopetrosis, indicating the important physiological role of these proteins in regulating bone resorption.

The secretion of OPG and OPGL from osteoblasts and stromal cells is regulated by numerous hormones and cytokines, often in a reciprocal manner. The relative levels of OPG and OPGL production are thought to ultimately dictate the extent of bone resorption. Excess OPGL increases bone resorption, whereas excess OPG inhibits resorption. Recombinant OPG blocks the effects of virtually all factors which stimulate osteoclasts, in vitro and in vivo. OPG also inhibits bone resorption in a variety of animal disease models, including ovariectomy-induced osteoporosis, humoral hypercalcemia of malignancy, and experimental bone metastasis. OPG might represent an effective therapeutic option for diseases associated with excessive osteoclast activity

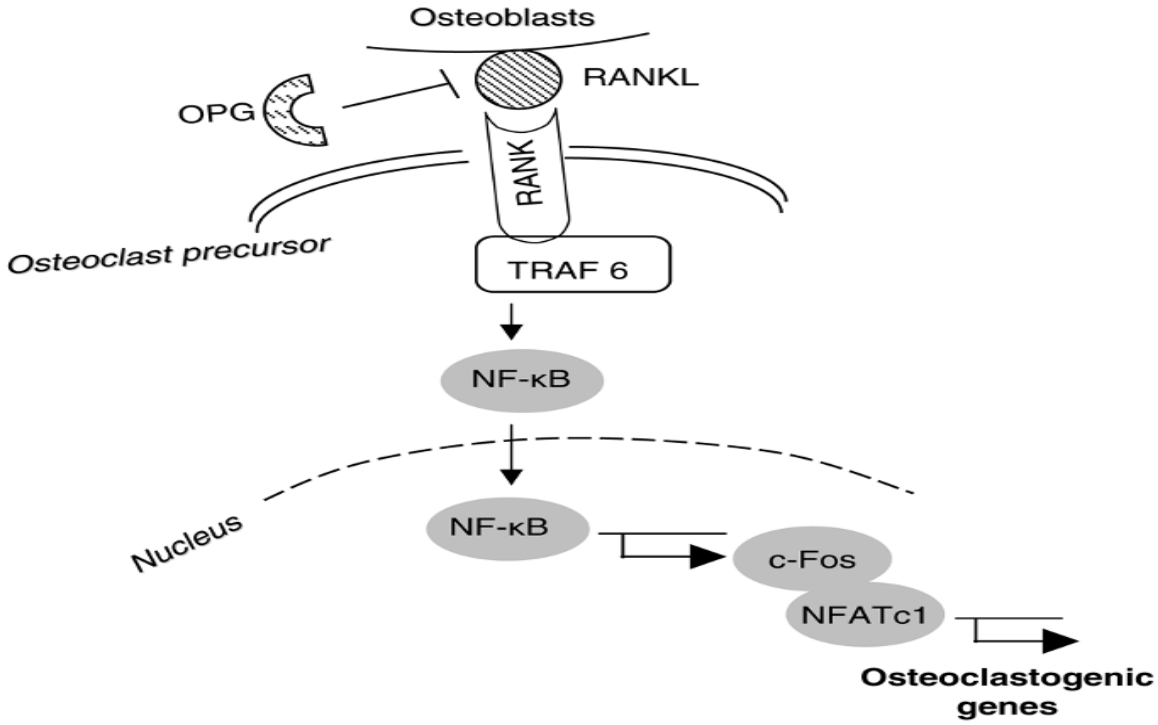


Fig 6 The essential signaling pathway for normal osteoclastogenesis. Under physiologic conditions, RANKL produced by osteoblasts binds to RANK on the surface of osteoclast precursors and recruits the adaptor protein TRAF6, leading to NF-κB activation and translocation to the nucleus. NF-κB increases c-Fos expression and c-Fos interacts with NFATc1 to trigger the transcription of osteoclastogenic genes. OPG inhibits the initiation of the process by binding to RANKL. NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor-κB; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-κB ligand; TRAF, tumor necrosis factor receptor associated factor.

Regulation of Osteoclast Formation: The RANKL–RANK–OPG Pathway

The RANKL/RANK/NF-κB signaling system and osteoprotegerin (OPG), which positively and negatively regulate the mid-to-later stages of osteoclastogenesis were discovered >20 years ago. These seminal studies revealed that $RANKL^{-/-}$ and transgenic mice overexpressing OPG had marked osteopetrosis because they lacked OCs, while $OPG^{-/-}$ mice had severe osteoporosis due the unopposed OC-inducing effects of RANKL. Mice deficient in NF-κB1 and 2, two major components of the NF-κB signaling pathways that regulate immune responses, were reported earlier to have marked osteopetrosis due to a lack of OCs. Later, it became clear that NF-κB signals downstream from RANK, and is required for RANKL-induced OC formation.

RANKL is a member of the TNF superfamily of proteins and is typically expressed on the surface of osteoblastic cells in response to most of the factors known to stimulate bone resorption. However, like M-CSF, it is also secreted by osteoblastic cells and by numerous other cell types, including activated T cells, B cells, and synovial cells in joints of patients with rheumatoid arthritis (RA) where, along with other cytokines, it contributes to joint destruction, and where synoviocytes appear to be the major source of RANKL.

Chondrocytes in growth plates express RANKL, RANK, and OPG. 1,25-dihydroxyvitamin D, bone morphogenetic protein 2 (BMP2) and Wnt/β-catenin signaling regulate chondrocyte expression of RANKL to attract OCPs to growth plates for removal of newly formed bone.[2] $RANKL^{-/-}$ and $RANK^{-/-}$ mice, like NF-κB1/2 dKO mice, also have impaired B-cell development, and failure of lymph node formation[2] and mammary lobular hyperplasia during pregnancy leaving them unable to feed their offspring. RANKL centrally controls fever and body temperature in females and promotes mammary cell proliferation, carcinogenesis, and breast cancer metastasis to bone. It also has antiinflammatory and neuroprotective effects in a mouse model of ischemic stroke in which it limits infarct volume. Mutations in RANKL occur rarely in humans associated with osteopetrosis, but affected individuals do not appear to have obvious immunological defects.

RANK, a TNF receptor superfamily member, is expressed by OCPs in response to PU.1, microphthalmia-induced transcription factor (MITF), M-CSF, TNF[2], Il-34, and Wnt5a, thus priming them for terminal differentiation when they encounter RANKL. RANK is also expressed by dendritic cells, normal breast epithelial cells,and in some cancers, including breast and prostate. Inactivating mutations of *rank* are rare in humans and are associated with OC-poor osteopetrosis. Activating *rank* mutations are more common[13] and account for the increased OC formation, activity, and osteolysis seen in some patients with early-onset (juvenile) Paget's disease, expansile skeletal hyperphosphatasia, and familial expansile osteolysis. OPG binds to RANKL and prevents its interaction with RANK and downstream signaling through TNF receptor-associated factor 6 (TRAF6) (Fig.6& 7).

It is secreted by OBs in response to most of the factors that also upregulate RANKL expression and in this way it limits OC formation, activity and survival.[12] Cells in numerous other organs express OPG, including the liver, heart, spleen, and kidney, where it has other regulatory functions. Some patients with juvenile Paget's disease, an autosomal recessive disorder,[17] have

homozygous partial deletions of *opg* resulting in osteopenia and fractures. Some children with idiopathic hyperphosphatasia, an autosomal recessive disease associated with increased bone turnover and deformities of long bones, acetabular protrusion, and kyphosis, have an inactivating deletion in exon 3 of *opg*.

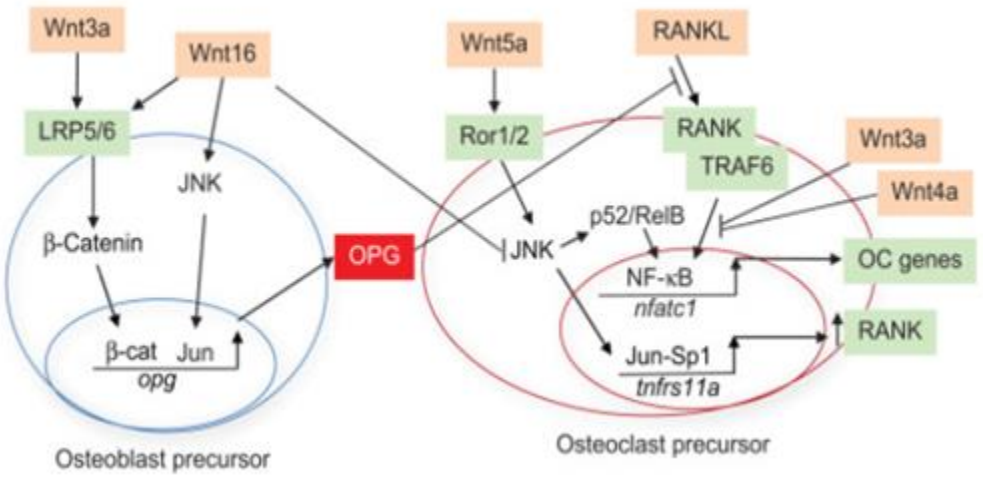


Figure 7:Wnt regulation of ostoclastogenesis through diret and indirect mechanisms. Wnt3a and Wnt16 inhibit Ostoclast (OC) formation by upregulat OPG expression in Ostoblastic cells via β catenin in the Wnt canonical pathway. Wnt 16 also upregulates OPG expression through *Jun* kinase *Jun* signaling in the none canonical pathway; in addition,Wnt16 expressed by ostoblastic cell also limits Oc formation and activation directly by inhibiting RANKL-induced JNK/NF-kB-mediated *nfatc1* expression.In contrast,Wnt5a, also expressed by ostoblastic cells,increases *tnfrs11a* (rank) expression via Ror2/JNK signaling in the noncanonical pathway,leading to inhanced OC formation .Wnt4 inhibits RANK/TRAF6-induced expression of NF-kB-mediated nfatc1,which Wnt3a also inhibits.

Major pathways that regulate OB formation and differentiation of MSCs to OBs also affect RANKL/RANK and/or OPG expression and OC formation. For example, Wnt3a- and Wnt16-induced canonical signaling through β-catenin upregulates OPG expression in OBs (Fig.7). Wnt16, Wnt3a, and Wnt4a expressed by osteoblastic cells also limit OC formation noncanonically by inhibiting RANKL-mediated noncanonical NF-κB-induced NFATc1 expression (Fig. 7). Wnt5a, also expressed by osteoblastic cells, positively regulates OC formation noncanonically through receptor tyrosine kinase (TK)-like orphan receptor (Ror) proteins expressed on OCPs that leads c-Jun N-terminal kinase (JNK)-mediated increased expression of RANK[15] (Fig. 7). Importantly, a soluble form of Ror2 acted as a decoy receptor of Wnt5a and abrogated bone destruction in mouse models of arthritis.Activation of β-catenin signaling in early OCPs promotes their differentiation into OCs, but activation of this pathway in

more differentiated precursors inhibits OC formation Thus, Wnts can have positive and negative regulatory roles in osteoclastogenesis.

In addition, Jagged1/Notch1 signaling, which also regulates MSC numbers and OB differentiation, negatively regulates OC formation indirectly by affecting the OPG/RANKL ratio in stromal cells. However, the effects of Notch ligands and receptor signaling on osteoclastogenesis are complex and may be time- and context-dependent. $OPG^{-/-}$ mice also have aortic and renal artery medial calcification, which complicates atherosclerotic plaques on the intimal surfaces of arteries, particularly in patients with diabetes mellitus and/or chronic renal failure. OPG has been implicated in calcification because OPG/apoE dKO mice have more accelerated calcific atherosclerosis than $apoE^{-/-}$ mice. Aberrant OPG and RANKL expression have been implicated in cardiovascular disease, diabetes, hypertension, and numerous other diseases, suggesting that OPG or RANKL inhibitors could be used to limit calcification of arteries in these common diseases.

Ossification

There are two types of bone ossification; intramembranous ossification and endochondral ossification

Intramembranous ossification is defined as the direct mineralisation of a highly vascular connective tissue. This process occurs in the flat bones of the skeleton, such as the facio maxillary bones. In summary, intramembranous ossification can be considered a four step process

1. An ossification centre forms.
2. Osteoblasts secrete osteoid elements and the subsequent mineralisation of the osteoid matrix begins working outward from the ossification centre.
3. The early trabeculae form.
4. The periosteum develops. This is the process that creates woven bone.

Endochondral ossification is different from intramembranous ossification in that a cartilage precursor (template) to the bone is initially laid down, then the bone begins to take shape in this template. In summary, endochondral ossification can be considered a ten step process:

1. The hyaline cartilage model or template grows and develops. This occurs when chondroblasts (the cartilage equivalent of the osteoblast) begin to secrete collagen and other proteins forming

the cartilage matrix. Chondroblasts that become caught in the matrix are subsequently known as chondrocytes. A membrane (perichondrium) surrounds the cartilage model.

2. Blood vessels form in and around the cartilage matrix to bring nutrients and allow the removal of waste.

3. A bone collar and the periosteum form around the diaphysis of the cartilage precursor when the perichondrium develops osteogenic cells.

4. Hypertrophic chondrocytes located at the edges of the primary ossification centres enlarge. The cells eventually degenerate, the matrix becomes compressed and subsequently mineralises. This area is known as the proliferative zone. A representation of the growth plate and adjacent metaphysis during growth is illustrated in Figure 8.

5. The original cartilage model is then gradually replaced by woven bone in a complex system of bone apposition, resorption and elongation. This process occurs in the zone of cartilage transformation and the resulting trabeculae form the primary spongiosa.

6. Osteoclasts begin to resorb the woven bone and cartilage remnants, osteoblasts begin to deposit bone matrix and the crystals produced inside the matrix vesicles become organised into the matrix, producing lamellar bone. This process occurs in the zone of ossification and the resulting mature trabeculae form the secondary spongiosa.

7. More blood vessels form during growth because of the constant need to bring nutrients to the newly developing bone.

8. Secondary ossification centres appear at the epiphyses of long bones and more blood vessels form to bring nutrients to these areas. In bones such as the carpals and tarsals, there is a variation in the number and location of secondary ossification centres.

9. The development of a medullary cavity occurs through osteoclastic resorption and the endosteum begins to form. Then bone marrow begins to develop in the newly created cavity. The epiphyses do not have separate medullary cavities. The smaller bones do not develop medullary cavities but all bones have some degree of marrow within their trabecular structure.

10. The articular cartilage forms and remains to form the joint margins.

When the epiphysis and diaphysis of a growing long bone become adjacent to one another, the diaphysis will gradually fuse with the epiphysis via the creation of mineralised bridges until the hyaline-cartilaginous growth plate has been completely replaced by bone and bone marrow,

making a complete bone. In same study has separated this union process into a nine stage process. The articular cartilage remains at joint surfaces to form the joints and prevent attrition

Long bones must also have a mechanism for increasing their diaphyseal circumference and their length. They do this by subperiosteal addition of bone combined with endosteal resorption. This mechanism ensures that the cortical bone layer does not become too thick, which would therefore make the bone much heavier and mechanically disadvantaged. The size, shape and density of the cortical and trabecular bone will generally develop to a stage at which they are sufficiently strong and light to accommodate loading and activity to which the skeleton is normally exposed.

Mineralisation

Once the matrix vesicles are produced by osteoblasts and/or chondroblasts, normal mineralisation requires the enzyme alkaline phosphatase, normal pH, calcium and phosphate ions and the active metabolite of vitamin D 1,25(OH)2D to enable mineralisation of osteoid and crystal formation. The formation of matrix vesicles is aided by vitamin D and its metabolites and alkaline phosphatase. As the crystals grow and proliferate, the matrix vesicles break down and the crystals are exposed to the matrix. During that process, collagen fibrils and other non-collagenous proteins such as osteopontin and osteonectin help to determine the organisation and orientation of the crystal.

Mineralisation can also occur as the result of crystals forming at sites on collagen fibrils. Although the mechanism for the formation of these crystals outside the vesicle microenvironment is not completely understood, there may be an association of mineral in the collagen matrix with the mineral in vesicles, such as minerals in vesicles serving as a calcium and phosphate ion source to assist early collagen-based mineralisation. However, one feature the two mineralisation processes have in common is the need for calcium and phosphate ions.

Vitamin D deficiency reduces the mineral-osteoid ratio and particularly affects endochondral ossification. If the vitamin D deficiency is sufficiently severe it is referred to as rickets in childhood and osteomalacia in adulthood. It results in growth plate expansion and non-mineralised bone matrix, leading to bone deformities such as abnormal curvature of the spine or bowed legs. Further information regarding vitamin D deficiency and the effects of vitamin D on the growth plate are discussed in letter.

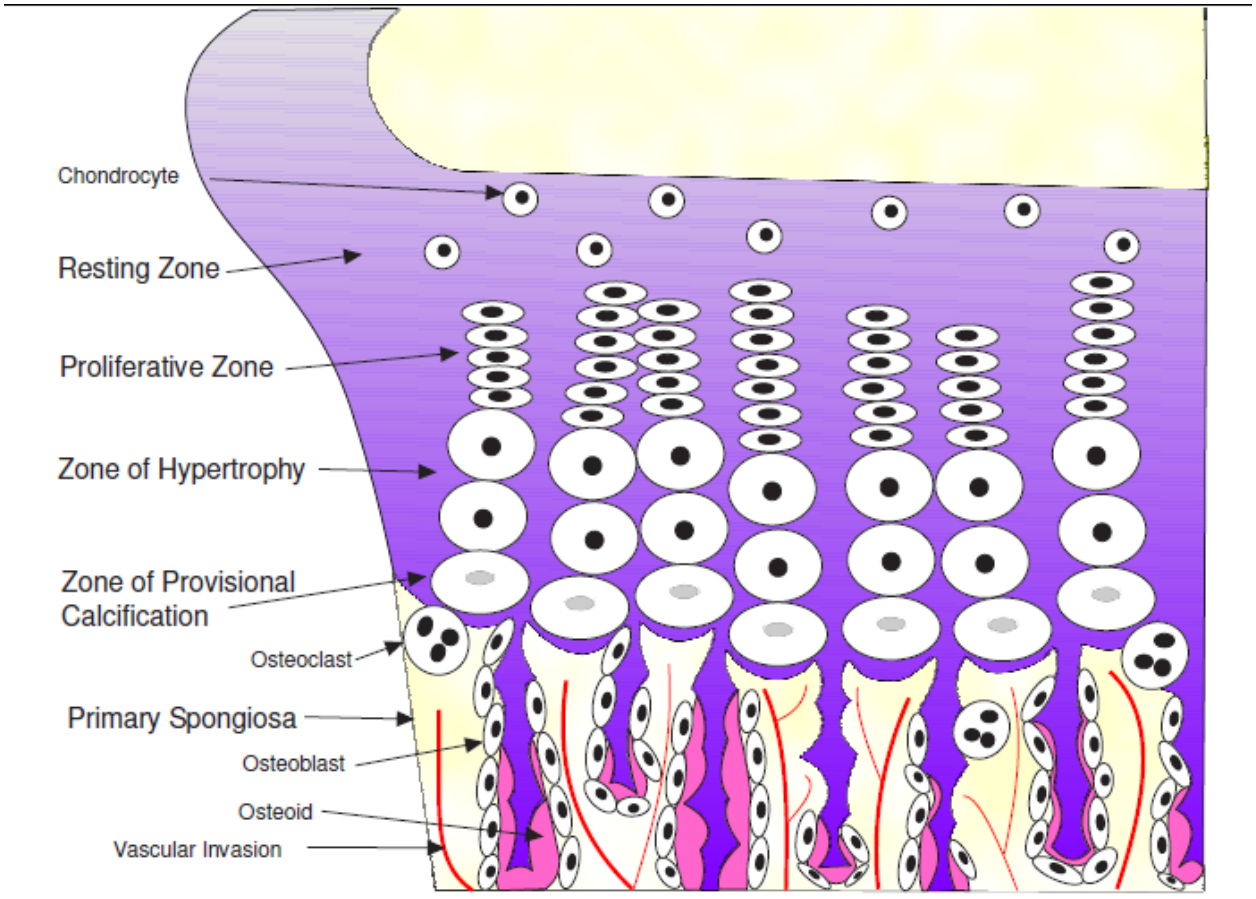


Figure 8: Diagram of the physeal growth plat, showing the resting, proliferatve, hypertropic and provisional calcification zones, and primary spongiosa

Remodeling of Bone

Bone undergoes remodeling throughout life. This involves the coupling of resorption of existing bone and the formation of new bone. Thus the entire bony skeleton is "renewed" on a continuous basis. This mechanism is important, because of the cyclical loading and torsional stresses that the skeleton undergoes. In the absence of renewal, bone would exceed its tolerance limits within a short period of time. Bone turnover or remodeling is thought to occur in discrete foci or packets scattered throughout the skeleton. Each packet takes 3-4 months to complete. Such foci have been termed bone remodeling units or BRUs by Frost, who described the process in 1964. About 25 percent of the metabolically active trabecular bone and about 3 percent of the cortical bone completely renews itself each year. The amount of bone added in each remodeling cycle, however, reduces slightly with age. This is probably due to a decreased number of osteoblasts.

This has been suggested as a possible mechanism for age related (but not post-menopausal) osteoporosis.

In the adult bone, remodeling involves activation, resorption and formation at endosteal and periosteal surfaces and within Haversian systems. Remodeling at the endosteal and periosteal surfaces would result in alterations in the thickness and width of tubular bones. Conditions such as acromegaly, osteopetrosis and hypo- or hyperthyroidism alter trabecular and cortical bone mass. Bone formation during a remodeling process requires a prior resorption. Resorption takes approximately 10 days. The resorption is carried out by a "cutting cone" of osteoclasts.

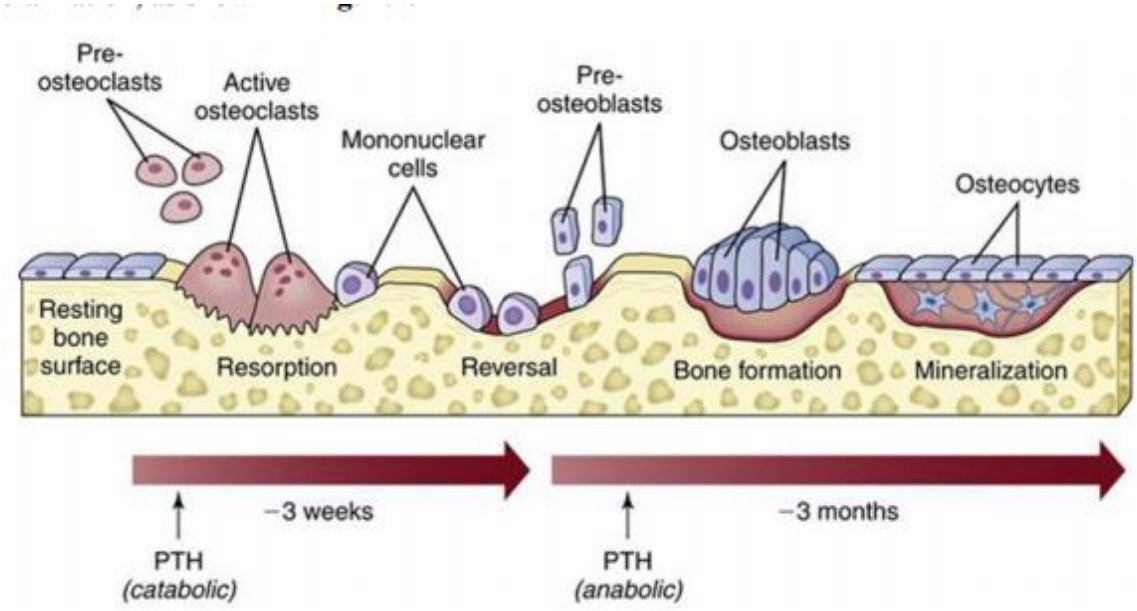


Figure 8. Schematic representation of the bone remodeling cycle.

The trigger for resorption includes the stimulatory cytokines IL-1 and IL-6 produced by osteoblasts, as well as the modulation of the integrin-RGD sequence interaction and other factors such as transcription factors and membrane proteins. The defect created after resorption, is filled in by fibrovascular tissue. The vessel component is especially important. The formation of the Haversian and the Volkmann systems are thought to be created by these mechanisms. In addition, the fibrovascular core contains pericytes, loose connective tissue, macrophages, mesenchymal stem cells and undifferentiated

osteoprogenitor cells. The outer edge of the osteon (where resorption ends and bone formation first starts) is marked by an intensely basophilic line - the "cement" or the "reversal" line. This

area is poor in collagen and mineral, and has a high content of sulfur. Bone formation is carried out by osteoblasts. The process takes approximately 3 months. As the remaining bone and its osteocytes (old, partially resorbed osteons) gets cut off by newly forming osteons, they remain as "interstitial" lamellae. Osteoblastogenesis has identifiable processes of chemotaxis, proliferation and differentiation of osteoblasts. This is then followed by mineralization and the cessation of osteoblast activity.

Mediators of osteoblastic activity and bone formation include transforming growth factor-beta (TGF-β, bone Gla protein fragments, platelet derived growth factors A and B (PDGF A and B), all of which are chemotactic for osteoblasts. The second event, proliferation of osteoblasts, is thought to be mediated by TGF-, β PDGF, IGF I and II, and fibroblast growth factors (FGFs). Cytokines that may play a role in the differentiation of osteoblasts and the production of alkaline phosphatase activity within these cells include IGF-I and bone morphogenetic protein-2 (BMP-2).

The linking or "coupling" of bone resorption and bone formation is complex and difficult to explain. There is emerging evidence however to suggest that "osteoclastogenic" cytokines such as IL-6, IL-1 and IL-11 as well as "osteoblastogenic" cytokines such as leukemia inhibitory factor may be stimulated together by the same signal transduction pathway.

Glycoprotein 130 is a molecule present in this pathway, and is involved in the transduction of the signal delivered by each of these cytokines. Sex steroids inhibit, whereas parathyroid hormone and vitamin D increase glycoprotein 130 in experimental models. This type of model would conveniently explain bone formation-resorption coupling as well as the various acknowledged effects of these hormones on bone turnover.

Another mechanism that may help explain coupling, is the release of osteoblast stimulating factors such as IGF I and II and TGF- β during the osteoclastic process. Another possible mechanism to explain coupling is the RANK/ RANKL interaction in which osteoblasts regulate the development and function of osteoclasts (please refer to regulation of osteoclastogenesis). Coupling is the rationale for the counterintuitive, but clinically validated method of treating osteoporosis by giving intermittent parathyroid hormone therapy.

Several diseases of bone are superimposed on this normal cellular remodeling sequence. In diseases such as primary hyperparathyroidism, hyperthyroidism and Paget's disease, there is osteoclast activation. However, there is also a compensatory and relatively balanced increase in

bone formation, due to the coupling of these events. Other diseases of bone are the result of abnormal coupling. One example is the decreased bone formation after extensive resorption in the osteolytic lesions of myeloma, where there may be a defect in osteoblast maturation. In solid tumors and in elderly patients with age-related osteoporosis there may be similar mechanisms operating, increased bone resorption and decreased bone formation. Osteoblastic activity in the absence of prior osteoclastic activation is thought to occur in some special situations such as osteoblastic metastases and in the response of bone to fluoride therapy.

Organic Matrix of Bone

The organic matrix of bone is 90 to 95 percent *collagen fibers,* and the remainder is a homogeneous gelatinous medium called *ground substance*

Collagen

Classification of Collagens

Connective tissues contain varying amounts of collagen, elastin (a related fibrous protein), glycosaminoglycans and proteoglycans. Of these, collagen is the most abundant. The details of collagen synthesis and function have been extensively reviewed in several specialized texts, and only some of the relevant aspects will be discussed. Collagens are a class of proteins with common features such as a unique triple helix composed of three component polypeptide alpha chains. However, there are several subtypes (types I to XIII) . Each of these are a product of a different gene and differ from each other in their biochemical structure. Several different types of *fibrillar, basement membrane-associated, fiber-associated, and short chain* collagens are recognized. Type I collagen is the most abundant type of collagen in most connective tissues.

Type I Collagen,

The Primary Component of Bone

Type I collagen is a fibrillar type collagen and is found in bone, skin, meniscus, tendon, ligaments, annulus fibrosis and joint capsules. About 90% of bone matrix is composed of type I collagen. There are several subtypes of type I collagens. The bone type I collagen appears to have predominantly galactosyl-hydroxylysine as opposed to glucosyl-galactosylhydroxylysine, the predominant amino-acid configuration found in the skin. Hydroxylation and glycosylation are posttranslational modifications of collagen and are specific to bone. These modifications, partially explain why mineralization only occurs in bone and not in other sites. The basic structure of type I collagen is composed of a repeating tripeptide sequence that form a left

handed helix. Type I collagen is a hetero-trimer of two pro-αI and one pro- α2 chains. The helix (the α chain) is highly coiled. The α chain corresponds to the basic chemical structure of Gly-X-Y where X and Y represent various amino acids; in practice however, X and Y are rich in proline and hydroxyproline and to a lesser extent, lysine and hydroxylysine. The helix is supertwisted, which provides enormous strength. Collagen fibers can support 10,000 times their own weight and are said to have greater tensile strength than steel wire of equivalent cross section.

Other Collagen Types

Types II, III, V and XI are also fibrillar type collagens. Type II collagen is located mainly in articular cartilage, fibrocartilage, the vitreous humor of the eye and the nucleus pulposus of the intervertebral disk. The other fibrillar collagens are the "minor" collagens. Type III is present in large blood vessels (30%), and in other tissues in association with Type I. Type III collagen is also present in tissues undergoing repair. Type V collagen is present in large blood vessels (5%), cornea, bone, and a few other connective tisues, while Type XI is present only in articular cartilage, comprising 5-20% of articular cartilage. Basement membrane-associated and fiber-associated type collagens include Types IV, VII, IX and XII. Type IV collagen is the prototype and major component of the *basement membrane* (95%). Type VII forms the anchoring filament in epithelial basement membranes, while Type IX comprises 5-20% of cartilage. Small amounts of Type XII collagen are associated with Type I. The least understood class of collagens is the *short chain* collagens and are comprised of the Types VI, VIII and X. Short chain collagens may function in association with other collagens and have a role in cartilage physiology.

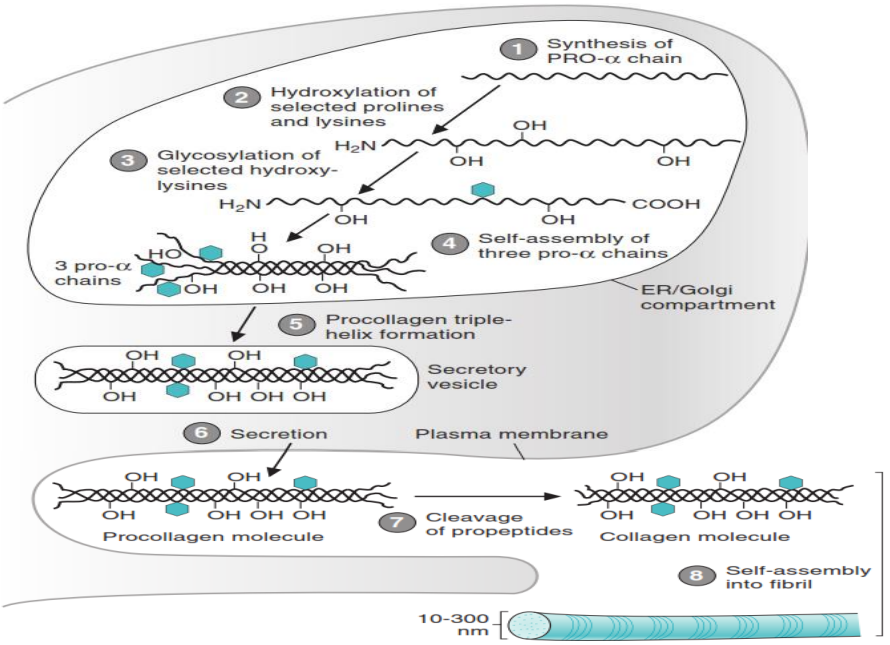
Collagen Synthesis and Cross-linking

Collagen synthesis is under the control of over 20 genes. The biosynthesis of collagen, its secretion and aggregation is a complicated process, and has been the subject of several reviews**.** Aspects directly applicable to musculoskeletal pathology will be mentioned in other chapters of this book. The promoters for synthesis of many of the collagen chains have been identified. Many growth factors and hormones also exert their effect on collagen synthesis at the transcriptional level. Collagen mRNAs usually contain a large number of introns. Once the precursor mRNA is transcribed, the introns are removed and the mRNA is transported from the nucleus to the cytoplasm for translation. At this point, additional translational control can be exercised. The N and C propeptidases (see later) are thought to act at this level. Like many other proteins, a precursor form (procollagen) is first synthesized, with peptide extensions at each end.

It is at this point that the α chain of collagen is formed and is transfered into the endoplasmic reticulum. At this stage of synthesis, several amino acids are modified posttranslationally (such as hydroxylation of proline residues and lysine residues, forming hydroxyproline and hydroxylysine, respectively), the addition of sugars (such as glucose and galactose to the hydroxylysines), and the formation of hydroxylysine and lysine aldehydes.

Co-factors for these processes include atmospheric oxygen, ascorbic acid, ferrous ion and several required enzymes. Once the translation is complete, the triple helix forms, from the C terminal end, and intra- and inter- molecular disulfide bonds are formed. The completed procollagen is then secreted via vesicles into the extracellular space. Glycosylation may be important to facilitate this final step. As stated earlier, the fundamental units of collagen fibrils are three polypetide chains arranged in a helical fashion. The polypeptide chains aggregate in units of threes to form *tropocollagen*. The tropocollagen, in turn, aggregates in a staggered fashion in a collagen microfibril. Collagen fibers are made up of several of these microfibrils. The process of collagen fibril formation is not fully understood. Removal of the N and C propeptides may be important. After their removal, the molecules aggregate in a head-to-tail fashion with a characteristic stagger, resulting in a 64 nm banding pattern seen under the electron microscope. The ultrastructurally dark area between two tropocollagen molecules is termed a “hole”. It measures about 41 nm and is the site where mineralization is thought to first occur.

The collagenous scaffolding is stabilized by cross linking and perhaps by interaction with proteoglycans. The process of cross-linking is important for stabilization and structural integrity. The first step is the enzymatic production of aldehydes by the removal of terminal amino groups of lysyl or hydroxylysyl groups of tropocollagen . These then can either condense with a lysyl or hydroxyl group to form a cross link and produce a Schiff base, or condense with a similar aldehyde in an aldol reaction (a stronger bond). The amino-oxidase enzyme that catalyzes the aldehyde formation is susceptible to blockage by nitriles. Nitriles are alkyl cyanide substances involved in the disorder called Lathyrism. Lathyrism is characterized by spinal deformities, demineralized bone, dislocations, aortic aneurysm, and various nervous system manifestations.



Steps in the formation of mature collagen fibrils 42

figure 9:

Cross-linking of collagen occurs in the extra-cellular space. Collagen molecules are cleaved in this space at both the N- and the C- terminal ends by specific peptidases. Crosslinking then occurs and the collagens are packed into a one-quarter stagger array. Specific interactions also occur among collagen and other extracellular macromolecules such as fibronectin, osteonectin and the proteoglycans. Extensive “cross-linking” between α component chains results in a rigid, brittle character to the connective tissue.

This type of cross-linking is found in aging individuals. Defects in the process of forming cross links can render the collagen susceptible to collagenases (discussed more below).

Penicillamine prevents collagen cross-linking; and is administered to patients with scleroderma, a disorder of excessive collagen deposition. Genetic defects in collagen can also result in several lethal and non-lethal conditions. Examples include Ehlers-Danlos syndrome (loose joints, characterized by a Gly to Serine change) or Osteogenesis imperfect (brittle bones, characterized by a Gly to Cystine change). Osteogenesis imperfecta (see section on metabolic diseases) is a heritable disorder of Type I collagen. It is due to a varietyof point mutations in either the pro-α1 or pro-α2 collagen chains. Over 100 point mutations have been found in probands with osteogenesis imperfecta. In a few cases, the mutations cause a decrease in the synthesis of pro-α1 and pro-α2 chains. In the majority of cases, however, there is a production of structurally

abnormal collagen chains. A mouse model has also been developed which demonstrates the relationship between abnormal collagen genes and osteoporosis in heterozygous animals. In homozygous animals, osteogenesis imperfecta develops.

Collagenases are enzymes that catalyze the hydrolysis of collagen. Several collagenases have been isolated, purified and synthesized. Collagenase levels are increased in rheumatoid arthritis nodules and in synovial fluid from patients with rheumatoid and septic arthritis. Colchicine and heparin increase collagenase synthesis. Collagenases and several other enzymes, such as cathepsin B, are capable of degrading collagen and have been implicated in the pathogenesis of collagen-vascular diseases. Urinary excretion of hydroxyproline (found exclusively in collagen) and other products of collagen degradation (cross-linked products such as pyridinoline and deoxypyridinoline), act as markers of collagen breakdown. The level of collagen degradation byproducts in urine or serum reflect the amounts of bone turnover (see section on the laboratory in orthopaedic practice).

Non Collagenous Matrix Proteins *Calcium Binding Proteins*

(The Glyco- and Phosphoproteins)

This is not an easily classified group, since some glycoproteins are also phosphorylated. In the former class are three "sialoproteins" (bone sialoprotein or BSP), including BSP I or osteopontin, BSP II, and bone acidic glycoprotein-75 or BAG-75. There is also a dentin sialoprotein which is found in the jaw. These compounds play a role in the control of extracellular calcium, regulation of crystal growth and shape, and cell adhesion to bone surfaces. Another important phosphorylated glycoprotein is osteonectin.

Osteopontin

The amino acid sequence has been determined, and its gene localized to chromosome 4. Osteopontin is a sialated and highly phosphorylated phosphoprotein, which exists in multiple forms, due to both alternate splicing as well as posttranslational variations in the degree of phosphorylation. This protein is transcriptionally regulated by substances such as 1,25 dihydroxyvitamin D, TGF-β, dexamethasone and parathyroid hormone, at least in experimental models. Osteopontin contains a GRGDS cell attachment sequence similar to binding proteins such as fibronectin (see below). Osteopontin in particular is important in that it has been shown to bind to the integrin receptor on osteoclasts. This binding leads to the activation of the phospholipase C pathway in osteoclasts, and a resultant increase in intracellular calcium. This

process may involve the src tyrosine kinase. Immunolocalization of osteopontin reveals high amounts in the extracellular matrix of developing intramembranous and endochondral bones. Its localization within cells reveals a broad pattern, including osteoblasts, osteocytes, osteoclasts, precursor cells, chondrocytes and fibroblasts. In situ hybridization studies have also shown the presence of mRNA in mononuclear marrow cells, proximal convoluted tubules of the kidney, neuronal cells within the brain and inner ear as
well as (murine) placenta.

Bone Sialoprotein-II (BSP II)

The gene for this sialoprotein has been localized to chromosome 4. Northern blot studies have suggested that BSP II is fairly bone specific. Fetal bone studies have indicated that the initial translation product may differ significantly from the mature form. Like BSP I, this protein has cell attachment properties due to its RGD sequence. However, osteopontin is more active than BSP II in this regard, and maintains cell attachment for more prolonged periods.

Bone Acidic Glycoprotein (BAG -75)

This protein binds to the small bone proteoglycans. There is cross-reaction of antibodies with osteopontin in some species; however there are significant differences between this protein and BSP I at the N-terminal end. Complete characterization of this protein is not clearly understood.

Phosphoproteins (Example: Osteonectin, SPARC or BM-40)

These proteins have a role in regulating the extracellular calcium hydroxyapatite formation and mineralization. Examples include phosphorylated glycoproteins like *osteonectin*. This protein also called secreted protein, acidic, rich in cysteine (SPARC), culture shock protein or basement membrane-40 (BM-40). Osteonectin binds to Ca2+, collagen type I, hydroxyapatite and thrombospondin. It promotes and initiates crystal growth. The gene for osteonectin has been localized to chromosome 5. Several tissues express osteonectin, however, its concentration is extremely high in bone (up to 10,000 times that of other connective tissues). In fact, in bone it may be the most abundant non-collagenous protein. The concentration of osteonectin in bone increases with maturity. Other tissues /cells having osteonectin include skin fibroblasts, tendon cells (but not tendon matrix) and odontoblasts. Interestingly, when osteonectin was activated by the use of blocking antibodies during tadpole development, there was a disruption of somite formation and malformation in the head and trunk.

Mice lacking osteonectin develop severe cataracts and low turnover osteopenia. *In vitro* studies of osteonectin-null osteoblastic cells showed that osteonectin supports osteoblast formation, maturation and survival. Osteonectin also plays role in cell attachment, migration, proliferation and differentiation.

Mineralization Proteins: Gammacarboxyglutamic acid proteins ("Gla" proteins)

Osteocalcin (also called bone Gla protein) is an example of this group. Osteocalcin contains three γ-carboxyglutamic acid (Gla) residues. It comprises about 20% of the non collagenous proteins in human bone. There is also a matrix Gla protein found in bone, cartilage, lung, heart and kidney. Osteocalcin is made by osteoblasts and odontoblasts in response to 1,25 dihydroxyvitamin D3. It is secreted into the osteoid after the initiation of mineralization. The bone localization of osteocalcin has been confirmed by several different methods including Northern blotting, immunohistochemistry and electron microscopy. It therefore serves as marker for mineralized tissue. In fact, both osteocalcin and alkaline phosphatase are valuable markers in the repertoire of the surgical and clinical pathologist. Osteocalcin serves as a marker of increased bone turnover, in particular of enhanced

osteoblastic activity. Serum osteocalcin levels do not always correspond well with the levels of serum alkaline phosphatase, suggesting that these two markers may be synthesized by osteoblasts at different stages of development. These substances can be used for following the progress of patients with osteosarcoma and may be can be used as a marker for recurrences or metastases in this situation. The role of osteocalcin in the body is unclear, but it may function in regulating mineralization and remodeling. It may also act as a chemoattractant for osteoclast progenitors (also see section on mineralization). Its secretion is under the control of many factors including Vitamin D, TGF-β, PTH and others. Serum levels reflect bone turnover. Osteocalcin has an affinity for Ca2+ that is dependent on the presence of Gla residues and an intact disulfide bond. It therefore may have a role in the regulation of crystal growth and recruitment of osteoclasts. Developmentally, low levels are found in the early stages of bone development while maximal levels are reached at maturity. The entire primary structure of osteocalcin has been determined (amino acid sequencing, cDNA clone sequencing, etc.) and the gene is localized to chromosome 1 in humans. The promoter region has a TATA box and a CCAAT sequence. There is a NF1 site, and a binding site for two other nuclear factors AP1 and AP2. There is a cAMP responsive region as well as a 1,25 dihydroxyvitamin D3 enhancer element. Genetic studies

showed that osteocalcin acts as an inhibitor of osteoblast function. Osteocalcin knockout mice were reported to have increased bone mineral density compared to normal controls, but the changes in mineral properties that occur with age were not observed in osteocalcin deficient mice compared to age-matched normal control mice. Collectively, the published literature provides evidence that osteocalcin is required to stimulate bone mineral maturation.

Adhesion Proteins (Osteopontin, Fibronectin, Sialoproteins and Thrombospondin)

These proteins contain an arginine-glycine-aspartic acid (RGD) amino acid sequence in their composition. This sequence mediates the attachment to certain integral membrane proteins or integrins, which are located on cell surfaces.

Osteopontin: Discussed earlier (see section on Calcium binding proteins) Fibronectin (FN)

FN is a multifunctional glycoprotein present in the extracellular matrix as an insoluble component or in circulating plasma as a soluble protein. FN mediates the adhesion, migration, differentiation, and proliferation of cells and has been implicated in wound healing and embryonic development FN is one of the most prevalent and versatile of the extracellular matrix proteins. Disruption of the FN gene in mice results in an embryonic lethality, confirming the importance of FN in embryonic development. The molecule is a dimer, its subunits being held together by two disulfide bonds. The subunits contain binding domains for fibrin, heparin, bacteria, gelatin, collagen, other extacellular matrix proteins, DNA and cell surfaces. The primary sequence of fibronectin has been determined, and the gene localized to chromosome 7. Fibronectin is characterized by several repeat sequences, for fibrin, collagen and integrin receptor binding. The latter is composed of the Gly-Arg- Gly-Asp-Ser cell attachment consensus sequence known as the GRGDS sequence.

There is heterogeneity associated with fibronectin mRNA, both dependent on origin (plasma versus tissue) and on stage of development (fetal versus adult). This results from alternative splicing of the primary transcript. This may allow the cell to utilize the form more suited to its needs. FNs exhibit molecular heterogeneity arising from alternative splicing of the primary transcript at three distinct regions termed EDA, EDB, and IIICS. Alternative splicing at the EDA and EDB regions is regulated in a tissue specific and developmental stage-dependent manner. Despite accumulating evidence for the regulated expression of EDA- and/or EDBcontaining FNs *in vivo*, the biological functions of these isoforms are poorly understood. Recent studies have shown that the EDA segment regulates the binding affinity of FNs for integrin α5β1 and thereby

stimulates integrin-mediated signal transduction and subsequent cell cycle progression. Unlike the EDA segment, the EDB segment does not enhance FN binding to integrin α5β1. Fibronectin is synthesized during bone development. During embryonic development, fibronectin is present at high levels during mesenchymal condensations and plays a crucial role in the overt differentiation of these cells into chondrocytes. It is also present around osteoblasts during osteogenesis. Osteoblasts can utilize fibronectin as a cell attachment protein. The synthesis of fibronectin from osteoblasts is probably under the control of TGF-β. Mice deficient for the EDB domain of FN were apparently normal and fertile, although the fibroblasts obtained from the homozygous mice exhibited reduced potential for cell growth and FN matrix assembly *in vitro*. Skeletal characterization of EDB null mice revealed no changes in any cartilage elements of skeletal development when compared to the wild type mice.

Thrombospondin (TSP)

This is a 450 kilo Dalton trimeric glycoprotein. It is composed of identical subunits that are disulfide-bonded to each other. It is the predominant protein of the α granules of platelets, but is synthesized in several connective tissues. Like fibronectin, there are "domains" for binding to a host of connective tissues and serum proteins. The molecule also binds Ca2+ to hydroxyapatite and to osteonectin. Thrombospondin and osteonectin co-localize in the α granules of platelets, where they bind to one another. The structure of thrombospondin reveals a homology to fibrinogen with binding sites to collagen, thrombin, fibrinogen, laminin, plasminogen activator and plasminogen. There are areas with homology to α (1) chains of types I and III collagen, von Willebrand factor and epidermal growth factor.

There is a region for activating platelet aggregation, as well as sequences with homology to calmodulin and paralbumin. In addition there is an RGD sequence in the middle of a Ca2+ binding region. Thrombospondin is distributed in a variety of tissues, including the dermo-epidermal junction of skin, in small blood vessels, surrounding skeletal muscle and beneath glandular epithelium. Temporally, there is an orderly increase in amounts during organogenesis, followed by a reduction as differentiation proceeds. There is evidence to suggest that TGF-β may be involved in the modulation of thrombospondin biosynthesis.

The proposed functions of this molecule include mediation of platelet aggregation, organization of the extracellular matrix (by its multiple binding sites) and action as an autocrine growth factor. TSP is expressed by bone cells such as osteoblasts as well as chondrocytes and this protein is

usually deposited into the matrix and regulates other extracellular matrix proteins. There are different types of TSP including TSP1, 2, 3 and 4, some of which have common physiological roles while others do not.

Genetically targeted mouse models have been used to define the physiological role of TSPs in bone and other tissues. Mice lacking TSP1 exhibit curvature of the spine and minor abnormalities in trabecular bone. TSP2-null mice display increased endocortical, but not periosteal, bone formation rates, compared to wild-type, normal mice, as a result of a larger pool of marrow osteoprogenitor cells. From the above information it is evident that the role of TSPs in bone is varied and is largely context-dependent.

Other Proteins, Cytokines and Growth Factors

Osteoblast cell culture studies have revealed the presence of several bio-products. Plasminogen activator and its inhibitor have been identified. Collagenase and tissue inhibitor of metalloproteinase (TIMP) have been isolated from such experiments. The extrapolation of these results across species and to *in vivo* situations should be treated with caution. Several plasma products including albumin and α 2 HS-glycoprotein can bind to bone. There is evidence in the literature suggesting a role for plasminogen activators in bone remodeling. Plasminogen activators tPA and uPA are involved in tissue remodeling and bone metabolism. Mice lacking tPA and uPA show increased bone formation and bone mass associated with increase osteoblast function and delay in extracellular matrix degradation.

Connective Tissue Growth Factor

Connective Tissue Growth Factor (CTGF) is a cysteine-rich protein first discovered by Bradham and colleagues while screening a human umbilical vein endothelial cell cDNA expression library using a polyclonal anti-PDGF antibody. At about the same time, two independent groups isolated mouse CTGF (Fisp 12/βIG-M2) from serum-stimulated NIH-3T3 cells and TGF-β -stimulated mouse AKR-2B cells using differential cloning techniques. Since that time CTGF has been isolated, cloned and sequenced in other species including the cow, pig, frog, and most recently in the rat. The CTGF gene belongs to a larger CCN gene family that also includes Cyr61/CEF10 and *nov*. Cyr61 and CEF10 were isolated by differential cloning techniques from mouse and avian fibroblasts, respectively, and *nov* was identified from myeloblastosis associated virusinduced avian nephroblastomas. More recent additions to this protein family include ELM-1 (WISP-1), WISP-3 (161) and COP-1 (WISP-2), bringing the total to six distinct members. With

the exception of *nov*, CTGF family members are immediate early growth-responsive genes that regulate the proliferation and differentiation of various connective tissue cell types. Most of the functional information on CCN proteins has emerged within the last 5 years, including the identification of receptors (i.e. integrins) and the elucidation of potential mechanisms of action, the field is poised for major advances in understanding the activities and functions of these proteins. All members of the CTGF gene family share 30-50% amino acid sequence identity overall, possess a secretory signal peptide at the N terminus, and contain 38 cysteine residues that are largely conserved. The CCN proteins are organized into four discrete and conserved structural domains, each encoded by a separate exon.

Domain I shares significant sequence homology with the N-terminal region of the insulin-like growth factor binding proteins (IGFBPs), although only low levels of IGF binding activity have been demonstrated for CTGF. Since the affinity of CTGF for IGF is much lower than that of the IGFBPs, the physiological significance of this binding is unclear. Domain II includes a von Willebrand factor type C repeat followed by a variable region that is highly charged and devoid of cysteine residues. This variable region may serve as a hinge connecting the N- and C-terminal halves of the protein. The central hinge region located between domains II and III of CTGF and other CCN family members is highly susceptible to enzymatic cleavage with additional sites of proteolysis between other domains. Domain III contains a region that is homologous to the thrombospondin type I repeat and may be involved in binding to the extracellular matrix via sulfated glycoconjugates. Domain IV is the C-terminal (CT) module resembling the CT domains of several other extracellular proteins believed to mediate protein-protein interaction or dimerization . Within this domain are six cysteines forming a motif called a cysteine knot. Cysteine knots are also found in other growth factors (TGF-β, PDGF and NGF) and are involved in their dimerization

Regulation of Bone by Endocrine and Paracrine Factors

Endocrine Control

Endocrine control of the bony skeleton is multifarious and includes the need to maintain a balance between bone formation and loss, maintenance of homeostasis in calcium and phosphate levels in the body, and maintenance of a reservoir of phosphate required for generating energy. The major players in the endocrine system that participate in this regulation include parathyroid

hormone, PTH-related peptide, calcitonin, vitamins A and D, estrogens, androgens and growth hormone.

Parathyroid Hormone

Parathyroid hormone (PTH) viewed as catabolic for bone is synthesized in the parathyroid gland from a biosynthetic precursor pro-PTH. PTH, a single chain polypeptide (84 amino acids referred to as PTH 1-84) impacts bone, intestine and kidney function. PTH mediates bone loss in older animals in its role to maintain calcium homeostasis and is required in fetal and neonatal animals for normal trabecular bone formation. In response to a decrease in serum calcium, PTH is released from the parathyroid gland. It targets the kidney to reduce calcium excretion, inhibits phosphate resorption and stimulates 1, 25 - dihydroxy vitamin D production which in turn targets the gastrointestinal tract to increase dietary absorption of calcium resulting in suppression of PTH. In addition, both PTH and 1, 25 (OH)2-vitamin D are able to bind to osteoblasts and through RANK and RANKL increase osteoclastic activity which results in calcium and phosphate release from the bony skeleton returning serum calcium levels to normal by an increase in bone resorption.

Receptors for PTH are found on pre-osteoblasts, osteoblasts and chondrocytes. They are not, however present on osteoclasts supporting the notion that the action of PTH on osteoclasts is osteoblast-dependent and mediated via substances such as IL-1, IL-6 and prostaglandins of the E series. The net result is osteoclast activation and initiation of bone resorption leading to calcium release from bone. Evidence suggests that in certain situations PTH stimulates bone formation. When administered continuously, it increases osteoclastic resorption and suppresses bone formation. When administered in low doses, intermittently, it stimulates bone formation without resorption. This anabolic effect, like the resorptive effect is probably indirect, and mediated via IGF-1, TGFβ, etc. High serum PTH levels, maintained for even a few hours, initiates osteoclast formation resulting in bone resorption that overrides the effects of activating genes that direct bone formation. Identification of PTH-related protein (PTHrP) expression early in the osteoblast progenitor cells, its action through the PTH 1 receptor

(PTH1R) on mature osteoblasts, and the observation that PTHrP+/- mice are osteoporotic, raise the possibility that PTHrP is a crucial paracrine regulator of bone formation.

Calcitonin

Calcitonin is a peptide hormone synthesized and secreted by thyroid parafollicular C cells, is regulated by extracellular calcium levels, and gastrointestinal hormones such as gastrin. It is encoded by a gene that undergoes alternate splicing to generate several other peptides including calcitonin gene related peptide. Calcitonin receptors are present on osteoclasts,
preosteoclasts, monocytes and certain tumor cells and increased levels result in a short lived fall in plasma calcium. In bone, calcitonin blocks bone resorption probably via mature osteoclasts, by enhancement of adenylate cyclase and cAMP or as a mitogen acting on bone cells. It promotes renal calcium excretion possibly to maintain normocalcemia after
a large calcium containing meal.

The physiological role of calcitonin remains controversial. Calcitonin and alpha-calcitonin gene-related peptide (alphaCGRP)-deficient mice exhibit high bone mass mediated by increased bone formation with normal bone resorption. The absence of significant changes in bone mineral density caused by a decline or overproduction of calcitonin in humans questions the physiological relevance of calcitonin as an inhibitor of bone resorption. A recent study on the agedependent bone phenotype in two mouse models, one lacking calcitonin and alphaCGRP (Calca-/-), the other lacking alphaCGRP (alphaCGRP-/-) reported osteopenia at all ages in AlphaCGRP-/- mice. However, the Calca-/- -mice displayed increased bone turnover with age and at 12 months of age a significant increase in bone formation and resorption. These data suggest that calcitonin may have dual actions, in bone formation and resorption, which may explain, at least in part, why alterations of calcitonin serum levels in humans do not result in major changes in bone mineral density. In addition, calcitonin has a role in the therapy of hypercalcemia of malignancy, in Paget's disease and in osteoporosis. Osteoclasts from Paget's patients are hyper-responsive to
calcitonin, for longer periods of time than control cells although the molecular mechanism(s) for this hyperresponsivity is unknown.

Vitamin D

Ergosterol and 7-dehydrocholesterol are the precursors for vitamin D, best labeled as a hormone and vitamin. These compounds are stored in the skin, transported in the body via an alpha-globulin binding protein/vitamin D binding protein (DBP) and become activated by ultraviolet light. Findings procured from gene targeting experiments in mice suggest that DBP possibly

maintains stable serum stores of vitamin D metabolites and modulates the rate of its bioavailability, activation, and end-organ responsiveness. These properties may have evolved to stabilize and maintain serum levels of vitamin D in environments with variable vitamin D availability. Activation of ergosterol and 7-dehydrocholesterol in turn generates calciferol and cholecalciferol. These substances are hydroxylated in the liver to yield 25-hydroxyvitamin D in the presence of magnesium, and then are converted in the proximal tubule of the kidney to generate metabolites of 25-hydroxy-vitamin D. The most active form of vitamin D is 1,25-dihydroxyvitamin D. This hormone is key to the control of calcium metabolism in the gut, proximal tubule in the kidney and bone. 1,25-dihydroxyvitamin

D production is regulated by calcium and PTH. It stimulates calcium binding protein, affects osteocalcin production, osteoclastic resorption, monocytic maturation, myelocytic differentiation, skin growth and insulin secretion. Lack of vitamin D results in impaired mineralization of newly formed bone which results in rickets in children, and osteomalacia in adults. These conditions are typified by an increase in proteinaceous bone matrix which does not mineralize. An excess of vitamin D leads to an increase in bone resorption and hypercalcemia. Vitamin D acts via vitamin D receptors, and receptor sites of 1,25-dihydroxyvitamin D have been identified on several cell types. The vitamin D receptor is a transcription factor which forms homo- or heterodimers with members of the steroid hormone receptor superfamily (most notably the retinoic acid receptor RXR). Errors in genes that code for these nuclear receptors are reported in several forms of rickets. It is also suggested that postmenopausal osteoporosis may be genetically predetermined by polymorphisms present

on the vitamin D receptor gene.

The vitamin D receptor type II (VDR-II) null mouse suggests a role for vitamin D in bone metabolism. These mice are phenotypically normal at birth, survive to 6 months of age, develop hypocalcemia at 21 days of age, at which time their parathyroid hormone (PTH) levels begin to rise. They also develop hyperparathyroidism accompanied by an increase in the size of the parathyroid gland with a concomitant increase in PTH mRNA levels. This phenotype is also associated with rickets and osteomalacia as early as day 15, and there is an expansion in the zone of hypertrophic chondrocytes in the growth plate. Interestingly the VDR-II knockout mouse also develops progressive alopecia at 4 weeks of age.

Studies using primary calvarial cultures revealed that ablation of VDR-enhanced osteoblast differentiation was associated with an increase in alkaline phosphatase activity, as well as an early sustained increase in formation of mineralized matrix. The expression of bone sialoprotein, a marker for mineralization, was also increased in VDR null osteoblasts. These studies demonstrate that VDR attenuates osteoblast differentiation in vitro, and that other endocrine and paracrine factors may modulate the effect of VDR on osteoblast differentiation in vivo.Evidence suggests that marrow mononuclear cells and monocytes fuse to form osteoclasts on exposure to vitamin D. Vitamin D receptors are not present on mature osteoclasts, thus osteoblasts are needed to mediate the effects of vitamin D to induce bone resorption and PTH may act synergistically with vitamin D to mediate this activity. In addition, it is likely that IL-1 and IL-2 play an intermediary role in bone resorption mediated by vitamin D.

Calcitriol and Osteogenesis

Calcitriol (1α, 25(OH)2 D3), the active form of vitamin D3, is synthesized from 25 hydroxyvitamin D3 by the action of 1 α hydroxylase which is present predominantly in the kidne. Mutations in the human1 α hydroxylase gene cause pseudo-vitamin D deficiency rickets. Targeted ablation of the 1 α hydroxylase gene in a mouse model leads to development of retarded growth, and skeletal abnormalities characteristic of rickets. Calcitriol resorbs bone by stimulating the formation of osteoclasts. Receptors for 1 α, 25(OH)2 D3 are found on osteoblasts and osteoprogenitor cells but not osteoclasts. Stimulation of osteoclast formation requires cell-cell contact between osteoblasts and osteoclast precursor cells, and involves the upregulation of the osteoclast-differentiating factor, RANK ligand in osteoblasts, and downregulatation of OPG expression, an osteoclastogenesis inhibitory factor that works as a decoy receptor for RANK. Through stimulation of osteoclast formation, 1, α 25(OH)2 D3 is believed to mediate bone resorption and remodeling. In addition, 1 α, 25(OH)2 D3 has been shown to inhibit osteoblast proliferation and stimulate apoptosis through induction of tumor necrosis factor alpha.

I*n vitro* studies demonstrate that vitamin D3 stimulates osteoblast differentiation through induction of osteocalcin and alkaline phosphatase expression (both markers of mature osteoblasts). These findings are supported by studies showing that the Ca+2 binding proteins osteocalcin and osteopontin secreted by osteoblasts during differentiation, are upregulated by 1α, 25(OH)2 D3 through its response element on the osteocalcin and osteopontin promoter. Moreover, 1 α, 25(OH)2 D3 stimulates osteoblast differentiation by the release of alkaline

phosphatase (ALP) through the ERKMAPK signaling pathway. Treatment of primary osteoblast cultures with an ERK inhibitor resulted in reduced 1 α, 25(OH)2 D3 induction of ALP, which confirms that 1α, 25(OH)2 D3 stimulates ERK expression in primary human osteoblasts.

Vitamin A

Retinoids, in excess, decrease the formation of bone and cartilage matrix, whereas a deficiency has the opposite effect. Several years ago, it was discovered that an imbalance of vitamin A during embryonic development had dramatic teratogenic effects. These effects have since been attributed to vitamin A's most active metabolite, retinoic acid (RA), which itself profoundly influences the development of multiple organs including the skeleton. After decades of study, researchers are still uncovering the molecular basis whereby

retinoids regulate skeletal development. Retinoid signaling involves several components, from the enzymes that control the synthesis and degradation of RA, to the cytoplasmic RA-binding proteins, and the nuclear receptors that modulate gene transcription. As new functions for each component continue to be discovered, their developmental roles appear increasingly complex and each has been implicated in skeletal development. Moreover, retinoid signaling comes into play at distinct stages throughout the developmental sequence of skeletogenesis, highlighting a fundamental role for this pathway in forming the adult skeleton. Consistent with these roles, manipulation of the retinoid signaling pathway significantly affects the expression of the skeletogenic master regulatory factors, Sox9 and Cbfa1. In addition to the fact that we now have a greater understanding of the retinoid signaling pathway on a molecular level, we are able to place retinoid signaling within the context of other factors that regulate skeletogenesis. Here we review these recent advances and describe our current understanding of how retinoid signaling functions to coordinate skeletal development.

We also discuss future directions and clinical implications in this field. Retinoic acid (RA) is an endogenous metabolite of vitamin A that acts as potent regulator of osteoblast growth and differentiation of. The actions of RA are mediated by nuclear receptors that belong to the steroid hormone receptor superfamily. Changes in levels of RA during skeletal development result in severe abnormalities in the appendicular and craniofacial skeleton. Several studies have investigated the effects of RA on osteoblasts *in vitro*. Low doses of RA (0.01 μM) resulted in a increased levels of osteopontin and osteocalcin mRNA in fetal rat calvarial osteoblasts. Similarly, treatment of clonal pre-osteoblasts with pharmacologic doses (1 μM) of RA have

shown an increase in osteopontin transcript levels and enhancement in nuclear processing of primary mRNA transcripts.

While these reports suggest a direct relationship between RA level and osteoblast differentiation, other studies have demonstrated a decrease in alkaline phosphatase activity with both low and high-doses of RA, and decreases in osteocalcin transcription at higher doses. Thus, the actual effect that RA has on osteoblast differentiation and matrix mineralization remains to be determined. An extensive literature on the role of steroid hormones (estrogens and androgens), and growth hormone reviews their impact on musculoskeletal development and disease and is not covered in the introductory chapter. In brief, in experimental situations, reduced estrogen leads to bone loss. This may be a direct effect on osteoblasts and possibly osteoclasts, and may be mediated via PTH and calcitonin. Androgens are reported to maintain bone mass via receptors on osteoblasts and the effect of growth hormone on bone is primarily mediated via insulin-like-growth factor (IGF). There may however also be a direct effect through growth hormone receptors on osteoblasts and chondrocytes.

Growth Factors

Transforming Growth Factor-Beta

Initially isolated from "transformed" neoplastic cells in tissue culture studies. Two "factors" were isolated and named TGFα and-β. TGFα is not found in bone and is now called epidermal growth factor. A number of similar compounds also exist (the TGF β supergene family) including bone morphogenetic proteins (BMP). There are four known receptors for TGF β. Additionally, there are cross effects from the stimulation of similar receptors. The net effect is to increase DNA at low concentrations, enhance the synthesis of type I collagen and non collagenous proteins (fibronectin, proteoglycans etc.), and reduce the activity of alkaline phosphatase. There is less information on the effect on osteoclasts. There may be stimulation at low and inhibition at high concentrations. The latter effect is in association with the production of prostaglandins. TGF β is said to have a prominent role in soft tissue healing, in a cascade fashion. It is released from the degranulation of platelets as well as from macrophages. It may help in the deactivation of the production of hydrogen peroxide, inhibit proteolytic enzymes and upregulate the integrin receptors for extracellular matrix proteins allowing the production of abundant granulation tissue. The current hypothesis is that TGF β induces bone formation during remodeling. Additionally, high amounts are seen in tissues undergoing endochondral

ossification. Experimental evidence suggests that TGF β plays a positive role in intramembranous and endochondral bone formation as well as fracture and wound healing in experimental animals. The action of TGF β in bone induction may however be only in conjunction with other factors such as the BMPs.

Role of TGFb-1 in Osteoblast Development *in Vitro*

It is well established that the members of the TGF β superfamily play a crucial role in bone development, remodeling, and disease. However, the various TGF β members have contradictory functions that have been documented *in vitro* and *in vivo* models.

For example, knockout of TGF β -2 has been shown to result in bone defects, indicating a positive role for these molecules in bone development. However, transgenic mice over-expressing TGF β -2 under the control of an osteocalcin promoter displayed an osteoporosis-like phenotype. On the other hand, TGF β -1 has been demonstrated to either stimulate or inhibit bone formation *in vivo*, and to differentially modulate distinct osteoblast markers *in vitro*. It has been suggested that TGF β -1 enhances the proliferation and early differentiation of osteoblasts *in vitro*, which is characterized by a high rate of collagen synthesis, but impairs their terminal differentiation based on osteocalcin production (a differentiation marker) and mineralization of culture matrix. The TGF β -1 signaling pathway begins by the binding of TGF β to TGF β specific type I and type II receptors leading to the phosphorylation of Smads 2 and 3, complex formation with Smad 4, translocation of Smad 2/3/4 to the nucleus, and transcriptional activation of specific target genes.

TGF β -1 enhances intracellular Ca+2 transport. This is crucial for osteoblast adhesion and early development in culture, since Ca+2 enhances expression of α5 integrin, which is important in the formation of focal contact adhesions and cytoskeletal reorganization. These early events are necessary for osteoblast adhesion. Thus, they determine the fate of the osteoblast cell and ultimately affect bone function. TGF β -1 abrogates the steady-state levels of mRNA for lysyl hydroxylase in human osteoblast-like cells *in vitro* thus inhibiting the matrix maturation by affecting the degree of lysyl hydroxylation in newly synthesized collagen.

The mRNA for lysyl hydroxylase was reduced by one-third under the influence of TGF β -1. However, the mRNAs for both procollagen I alpha-chains were stimulated by TGF β -1. Thus,

TGF β -1 increases collagen production and decreases its maturation. TGF□-1 also stimulates osteoblast proliferation indirectly through inhibition of p57 cyclin-dependent kinase inhibitory

protein (CKIs), a negative regulator of the cell cycle acting through the ubiquitin-proteasome pathway in newly proliferating osteoblast cells.

Nishimori and his colleagues found when the constitutively active form of the TGF β -1 type I receptor was ectopically expressed in osteoblast cells, the p57 that had been accumulated by serum starvation and causing the cell-cycle arrest was rapidly degraded in a manner analogous to TGF- β 1 stimulation. Moreover, Smad2 or Smad3 binding to Smad4 enhanced the proteolytic pathway of p57. All of the pathways mediated by TGF β -1 growth factor suggest its important role in osteoblast proliferation but not terminal differentiation.

Studies on TGF β -1 null mice have shown that growth plates, alkaline phosphatase (ALP) activity and collagen maturity were reduced in the tibiae at all ages compared to age-matched wild-type (WT) control animals using Fourier transform-infrared imaging (FTIRI) and immunohistochemistry . Also analysis of proximal tibial metaphyses showed significant decreases in the bone mineral content of the TGF β -1 null mice compared to TGF β -1 wild-type (WT) control animals. However, no significant differences were observed in bone mineral density (BMD) between the groups of mice. Histomorphometry revealed that the width of the tibial growth plate and the longitudinal growth rate were significantly decreased in the TGF β -1 null mice, resulting in shorter tibia.

Bone Morphogenetic Proteins (BMP)

At least ten proteins with this property have been extracted from *demineralized bone*, the amino acid sequence has been characterized and synthesized by recombinant DNA technology.

These have been named bone morphogenetic proteins 1-10. BMP 3 is also called osteogenin, BMP 4 is also called BMP 2B, BMP 6 is also Vgr-1 and BMP 7 is known as osteogenic protein-1. This clash of terminology is due to the reclassification after characterization. These BMPs can be thought of as three separate families. One consisting of BMP 2 and 4, the other of BMPs 5, 6 and 7 and the last consisting of BMP 3. These divisions are on basis of homology of structures.

The issue has become complicated by the finding that these proteins (mostly members of the TGF β super gene family) are found in several tissue types other than bone. In fact, the developing embryo has areas such as the apical epidermal ridge, which exhibit this property of bone induction, possibly due to such factors being elaborated. Urist's work had suggested that demineralized bone matrix contains biologic signals to induce endochondral
bone formation when implanted in soft tissues (osteoinduction).

The relative osteoinductive contribution of bone cells as opposed to matrix in demineralized bone is debated. In the 1970's Japanese workers identified bone inducing activity in certain osteosarcoma cell lines. The molecule involved in this bone induction was later characterized as BMP 4. Certain human osteosarcoma cell lines such as the Saos-2 have also shown to produce several BMPs and TGF β. Current recombinant technology however, has allowed the synthesis of these proteins from cDNA, obviating the need of large amounts of demineralized bone or neoplastic cell lines. Most of the BMPs (except for BMP 1) are basic proteins of 15 kDa, existing as dimers and belonging to the TGF β superfamily. Disulfide bonds link these dimers. BMP-1 has recently been shown to be a protease with procollagen as its substrate. The synthesis of most BMPs has been performed by Wozney et at the Genetics Institute (Cambridge, Massachusetts). Their approach has been to isolate and sequence the cDNA for each BMP using a cDNA library obtained from the U-20S human osteosarcoma line.

Following cloning, functional regions of the BMP sequences were transfected into a second mammalian line (Chinese hamster ovary) for expression and secretion of the mature BMP molecules. These were then isolated and purified by a chromatographic method developed by the Genetics Institute and Genentech (Cambridge, Massachusetts). Osteoinductive activity was tested using a bioassay employing rats. Purified BMPs have been used to promote bone repair.

Several trials have shown their efficacy in experimental models. Mixtures of BMPs have also been used, and shown to be more effective than comparable doses of single homodimeric BMP.

Bone Morphogenetic Proteins and Osteogenesis

Bone morphogenetic proteins (BMPs) are osteotrophic factors as well as members of the TGF β superfamily. The activity of BMPs was first identified in the 1960s, but the proteins responsible for bone induction remained unknown until the purification and sequence of bovine BMP-3 (osteogenin) and cloning of human BMP-2 and BMP-4 in the late 1980s BMP-2 induces gene expression and synthesis of osteoblast differentiation markers, alkaline phosphatase and osteocalcin, in pluripotent and preosteoblast cells. BMP-2 exposure for a short duration is sufficient to induce cell differentiation. Functions of bone morphogenetic proteins, such as BMP-2, are initiated by signaling through specific type I and type II serine/ threonine kinase receptors. It was previously reported that BMP receptor type IB (BMPR-IB) plays an essential and specific role in osteoblast commitment and differentiation. Smad1, 5, and 8 are substrates for BMP

receptor I (BMPR-I) and mediators of the BMP signals that inhibit myogenic differentiation and induce osteoblast differentiation, in the mesenchymal C2C12 cell line.

Studies from transgenic and knockout mice and from animals and humans with naturally occurring mutations in BMPs and related genes have shown that BMP signaling plays critical roles in heart, neural and cartilage development. BMPs also play an important role in postnatal bone formation. BMP-2 is known to induce osteoblast differentiation by inducing Runx2. a global regulator for osteogenesis. Runx2 co-operates with BMP-2-induced Smad proteins to stimulate osteoblast differentiation. BMP-2 receptor activated Smad proteins induce Runx2; however, Smad does not directly induce Runx2 expression. The mitogen-activated protein kinase/p38 (MAPK/p38) cascade is also involved in the induction of Runx2 by BMP-2 .

In addition, BMP-2 induces osteoblast differentiation through activation of an endogenous β -catenin signaling pathway thus implicating β -catenin in early steps of BMP-2 mediated osteoblast differentiation. In support, ectopic expression of stabilized β -catenin in the murine embryonic mesenchymal C3H10T1/2 cell line or activation of endogenous β –catenin signaling with lithium chloride induced expression of alkaline phosphatase mRNA and protein (an early osteoblast differentiation marker). However, unlike BMP-2 protein, stabilized β -catenin does not induce osteocalcin gene expression (a late osteoblast differentiation marker).

Insulin like Growth Factors: IGF I and II

Insulin-like growth factors are produced by many cell types including osteoblasts and chondrocytes. They act via receptors to promote proliferation, differentiation and matrix production of bone and cartilage. The action of growth hormone is closely linked with the IGFs. It is thought that growth hormone binding with specific receptors in target tissues stimulates the production of IGF-1. This, in turn, may have endocrine, paracrine and autocrine effects. IGF-1 is transported via carrier proteins, such as IGF-binding proteins and IGFBPs of which, IGFBP-3 is the most important. Deficiency of IGF or IGFBP-3 may be responsible for certain kinds of dwarfism, such as Laron type dwarfism.

Other Growth Factors Growth factors discussed earlier include Epithelial Growth Factor, Acid and Basic Fibroblast Growth Factors and Platelet Derived Growth Factors A and B (PDGFA and PDGFB). PDGFs, in particular, are potent mitogens of osteoblasts in vitro and have a chemotaxic effect on them. PDGFs are thought to be particularly important in bone remodeling.

They are heterodimers of A and B chains, and function via specific receptors. Mutations in fibroblast growth factors are thought to play a role in certain kinds of skeletal deformities, including achondroplasia, Apert's syndrome, Cruzon syndrome, Pfeiffer syndrome, and Jackson-Weiss syndrome.

Cytokines: Prostaglandins and Interleukins

Prostaglandins: Prostaglandins have multiple effects on bone cells, and sometimes opposite effects in different species. Their role is therefore difficult to discern. They are powerful bone resorbers in certain culture studies, yet they are potent anabolic (bone forming) agents when administered in vivo. (Especially true of the E series). Prostaglandins are produced by monocytes under appropriate stimuli. It is possible that some effects of interleukins are mediated by prostaglandins.

Interleukin 6 (IL-6): This cytokine is produced by many cell types, including osteoblasts and bone marrow stromal cells. Bone cells produce IL-6 in response to PTH, Vitamin D3, TGF β, IL-1 and TNFα, to name a few. Human osteoclastoma cells respond to this cytokine; however, it is still unclear whether normal mature osteoclasts respond to IL-6. It is known though that IL-6 has a pathogenetic role in diseases such as multiple myeloma, Paget's disease, rheumatoid arthritis and Gorham's disease (vanishing bone disease). Experimentally, estrogens and androgens inhibit the production of IL-6 by osteoblasts. Additionally, there is evidence to suggest that osteoclastic activity may be inhibited by anti- IL-6 antibodies.

Muscle

Introduction

Muscle is a major determinant of whole-body metabolic activity. The three types of muscle: skeletal, cardiac, and smooth, have both similar and unique biochemical properties related to metabolism and force production. However, the primary function of all muscle is to turn chemical energy into mechanical energy. This is accomplished through the breakdown of ATP. Muscle mass and activity are major determinants of the overall metabolic rate in both the basal and active state. Changes in muscle metabolism occur during prolonged or vigorous physical activity, which affects not only the metabolic rate, but also the relative rate of utilization of glucose and fatty acids as fuels.

Although skeletal muscle is mainly associated with locomotion and heat production, maintenance

of skeletal muscle mass is also essential to provide protein reserves for gluconeogenesis during fasting. Muscle is also the major site of glucose and triglyceride disposal in the body following a meal. Through its GLUT-4 transporter and lipoprotein lipase activity, muscle removes excess fuels from the blood. Loss of muscle mass with age or in wasting diseases, such as AIDS and cancer, leads to glucose intolerance and is associated with increased mortality and morbidity.

ATP is used for muscle contraction

Muscle contraction involves the integration of several biochemical processes including: membrane ion flux, calcium release and re-uptake, and ATP hydrolysis and synthesis. ATP is required for the maintenance of ion gradients, restoration of intracellular calcium levels, and for the actual process of muscle shortening. The functional contractile unit of muscle, the sarcomere, relies on the interaction of two filamentous proteins, actin and myosin, for shortening. The head of the myosin protein has ATPase activity that hydrolyzes ATP, and when calcium is present results in sarcomere shortening. Resting ATP stores do not fluctuate a great deal during muscle contraction. Actively contracting muscle relies on the rapid synthesis of ATP from ADP by the creatine phosphate shuttle, with additional ATP production from both anaerobic and aerobic metabolism. This chapter will describe all three types of muscle, focusing primarily on skeletal muscle and pointing out unique features of biochemistry and regulation in cardiac and smooth muscle. Muscle will be examined from three classic points of view: its structure, the mechanism of mechano-chemical coupling, and its energy metabolism

STRUCTURE OF MUSCLE

Development of Muscle

To understand muscle regulation it is necessary to understand its origin and development. Muscle is derived from proliferating cells that originate from the mesenchyme germ layer in the developing embryo. These cells are 'determined' into the muscle lineage and then become myoblasts. Myoblasts can leave the cell cycle and differentiate into a mature muscle cell phenotype. Differentiation involves the sequential activation of muscle-specific genes including contractile proteins by DNA-binding transcriptional regulator proteins from the family of Myogenic Regulatory Factors (MRFs). Terminally differentiated myoblasts in the heart are called cardiac myocytes; these cells remain

single or bi-nucleated throughout life. To date, no myogenic stem cells have been found in cardiac muscle, which may explain the limited regenerative capacity of the heart after injury.

Smooth muscle myoblasts also differentiate into mature smooth muscle cells (SMC), but unlike heart and skeletal muscle they are not terminally differentiated. SMC phenotype also varies, based on its location and function. SMCs are found throughout the body in the vascular wall, and retain the ability to proliferate, e.g. in response to hypertension or during angiogenesis.

Skeletal muscle cells differ from the other muscle types in that they are multi-nucleated. Proliferating myoblasts fuse and terminally differentiate to form a multinucleated myotube. Innervation by a motor nerve end plate induces the myotube to take on mature muscle fiber characteristics. Satellite cells are undifferentiated muscle precursor cells, found only in skeletal muscle. Since muscle fiber nuclei are post-mitotic, satellite cell proliferation and differentiation are critical events for postnatal muscle growth and regeneration after damage. Alterations in satellite cell differentiation with advancing age or in wasting syndromes are thought to contribute to skeletal muscle loss under these conditions

Muscle contraction: The thick and thin filaments

The sarcomere may shorten by as much as 70% in length during muscle contraction (Fig. 19.2; see also Fig. 19.1). The main sarcomere components producing the shortening are the thick and thin filaments. The thick filament is composed of myosin protein, and the thin filament is mainly made up of actin, with associated tropomyosin and the troponin family proteins. Thick and thin filaments extend in opposite directions from both sides of the M- and Z-lines, respectively, and overlap and slide past one another during the contractile process (Fig. 19.2). The M- and Z-lines are, in effect, base plates for anchoring the filaments. In smooth muscle thick and thin filaments are anchored at structures called dense bodies that are further anchored by intermediate filaments. In striated muscle increased thick-thin filament overlap during contraction causes the H-zone (myosin only) and I-bands (actin only) to decrease. Although all three muscle types contain actin and myosin proteins, each muscle type expresses tissue specific protein types or isoforms; the cardiac actin and troponins differ slightly from those in skeletal muscle.

MUSCLE PROTEINS

Myosin

Myosin comprises two heavy and four light chains and contains two hinge regions

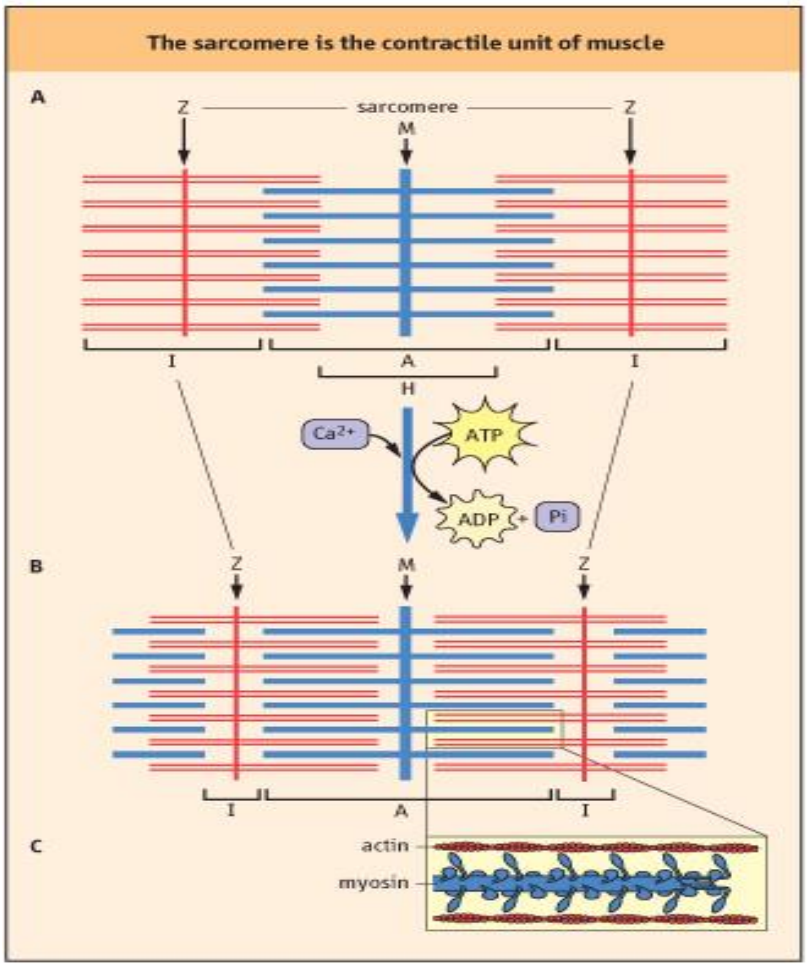


Figure 19.2 **Schematic structure of the sarcomere, indicating the distribution of actin and myosin in the A- and I-bands.** (A) relaxed sarcomere; (B) contracted sarcomere; (C) magnification of contracted sarcomere, illustrating the polarity of the arrays of myosin molecules. Increased overlap of actin and myosin filaments during contraction, accompanied by a decrease in the length of the I-band, illustrates the sliding-filament model of muscle contraction

Table 19-2. Muscle proteins and their functions.

Muscle proteins and their functions	
Protein	**Function**
Myosin	Ca^{2+}-dependent ATPase activity
C-protein	assembly of myosin into thick filaments
M-protein	binding of myosin filaments to M-line
Actin	G-actin polymerizes to filamentous F-actin
tropomyosin	stabilization and propagation of conformational changes of F-actin
troponins-C, I and T	modulation of actin-myosin interactions
α- and β-actinins	stabilization of F-actin and anchoring to Z-line
nebulin	possible role in determining length of F-actin filaments
titin	control of resting tension and length of the sarcomere
desmin	organization of myofibrils in muscle cells
dystrophin	reinforcement of cytoskeleton and muscle cell plasma membrane

Actin and myosin account for over 90% of muscle proteins, but several associated proteins are required for assembly and function of the actomyosin complex.

Myosin is one of the largest proteins in the body, with a molecular mass of approximately 500 kDa, and accounts for more than half of muscle protein (Table 19.2). Under the electron microscope, myosin appears as an elongated protein with two globular heads. Structurally, it consists of two heavy and four light chains. The myosin head has ATPase activity. The heavy chains form an extended α-helical coiled-coil structure, and the light chains are bound to one end of each heavy chain, forming globular domains. Structural analysis by limited proteolysis indicates that there are two flexible hinge regions in the molecule (Fig. 19.3). One is about two-thirds of the way along the helical chain and divides the molecule into light meromyosin (LMM: helical region) and heavy meromyosin (HMM: short helical tail plus globular domains). The other hinge is between the short helical and globular domains of HMM. Thick filaments are formed by self-association of LMM helices, up to 400 myosin molecules per thick filament. The filaments extend outward from the M-line toward the Z-line of each myofibril (compare Figs 19.2 and 19.3). Isoforms of actin and myosin are also found in the cytoskeleton of non-muscle cells, where they have roles in diverse processes, e.g. cell migration, vesicle transport during endocytosis and exocytosis, maintenance or changing of cell shape, and anchorage of intracellular proteins to the plasma membrane.

Myosin light chains have Ca^{2+}-dependent ATPase activity and are involved in reversible interactions with actin

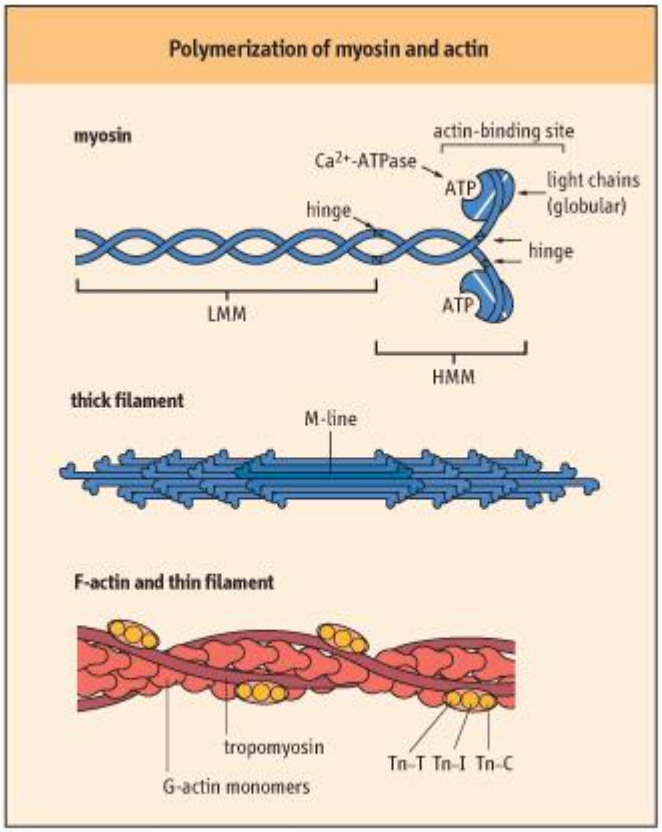


Figure 19.3 **Polymerization of myosin and actin into thick and thin filaments.** Tn-C, calcium-binding troponin; Tn-I, troponin inhibitory subunit; Tn-T, tropomyosin-binding troponin. LMM: light meromyosin, HMM: heavy meromyosin

The myosin light chains in the globular domain are homologous to calmodulin and have Ca^{2+}-dependent ATPase activity. These chains are also involved in reversible interactions with actin. ATP binding to the myosin head groups reduces their affinity for actin. Hydrolysis of the bound ATP to ADP and inorganic phosphate (Pi), catalyzed by Ca^{2+}, results in structural changes that increase by more than a 1000-fold the binding affinity of the myosin head groups for actin. Rigor mortis sets in after death as a result of the inability of muscle to regenerate ATP, which is required to maintain the low calcium concentration in the sarcoplasm. The increase in sarcoplasmic Ca^{2+} and hydrolysis of ATP on myosin after death leads to tight interactions between myosin and actin, forming rigid muscle tissue

Actin

F-actin is a polymer of G-actin subunits; in muscle there are about twice as many actin as myosin chains

Actin is composed of 42 kDa subunits, known as G-actin (globular), but polymerizes spontaneously into a filamentous array (F-actin). The G-actin subunits polymerize in a head-to-tail manner, and two polymer chains coil around one another to form the F-actin myofilament (see Fig. 19.3). The F-actin chains extend in opposite directions from the Z-line, overlapping with the myosin chains extending from the M-line. There are approximately twice as many actin as myosin chains in muscle, yielding an array in which each myosin molecule is associated with six actin molecules and each actin with three myosin molecules (see Fig. 19.1 for a cross-sectional view).

Tropomyosin and troponins

Tropomyosin stabilizes F-actin and coordinates conformational changes among actin subunits during contraction

Tropomyosin is a fibrous protein that extends along the grooves of F-actin, each molecule contacting about seven G-actin subunits. Tropomyosin has a role in stabilizing F-actin and coordinating conformational changes among actin subunits during contraction. In the absence of Ca^{2+}, tropomyosin blocks the myosin binding site on actin. A complex of troponin proteins is bound to tropomyosin: Tn-T (tropomyosin-binding), Tn-C (calcium-binding) and Tn-I (inhibitory subunit). Troponins modulate the interaction between actin and myosin. Calcium binding to Tn-C, a calmodulin-like protein, induces changes in Tn-I, which are then transduced to tropomyosin, moving it out of the myosin-binding site and permitting actin-myosin interactions.

The sliding-filament model of muscle contraction

The general features of the sliding-filament model of muscle contraction are described by a series of chemical and structural changes in the actomyosin complex. The contractile response is powered by reversible 'cross-bridge' interactions between the myosin head and its actin-binding site. A cycle of binding of myosin to actin, conformational changes in the hinge regions of myosin, and release of

myosin occurs, with the conformational change providing the 'power stroke' for muscle contraction. This sequence of reactions is summarized in Figure 19.4. The conformational change in the hinge regions of myosin is induced by hydrolysis of ATP and relaxed by dissociation of ADP and Pi. The latter process, the dissociation of ADP and Pi, rather than the hydrolysis of ATP, is the rate-limiting step in myosin ATPase activity and muscle contraction. The stability of the contracted state is maintained by multiple and continuous actin-myosin interactions, so that slippage is minimized until calcium is removed from the sarcoplasm, allowing the muscle to relax

Excitation-contraction coupling: The calcium trigger

DUCHENNE MUSCULAR DYSTROPHY

A young boy was brought to the clinic because his mother had noticed that he walked with a waddling gait. Physical evaluation confirmed muscle weakness especially in the legs although his calf muscles were large and firm. There was a 20-fold elevation in serum creatine (phospho) kinase (CK) activity, identified as the MM (muscle) isozyme. Histology revealed muscle loss, some necrosis, and increased connective tissue and fat volume in muscle. A tentative diagnosis of Duchenne muscular dystrophy (DMD) was confirmed by immunoelectrophoretic (Western blot) analysis showing the lack of the cytoskeletal protein dystrophin in muscle.

Comment. Dystrophin is a high-molecular-weight cytoskeletal protein that reinforces the plasma membrane of the muscle cell and mediates interactions with the extracellular matrix. In its absence, the plasma membrane of muscle cells is damaged during the contractile process, leading to muscle cell death. The dystrophin gene is located on the X-chromosome and is unusually long, nearly 2.5×10^6 base pairs. Mutations are relatively common, the frequency of DMD being approximately 1 in 3500 male births. DMD is a progressive myodegenerative disease, commonly leading to confinement to a wheelchair by puberty, with death by age 20 years from respiratory or cardiac failure. Dystrophin is completely absent in DMD patients. A variant of the disease, known as Becker muscular dystrophy, has milder symptoms and is characterized by expression of an altered dystrophin protein and survival into the fourth decade. Although there is currently no treatment, the injection of satellite cells (myogenic stem cell) that express dystrophin protein and incorporate into the dystrophic skeletal muscle has shown promise in animal

trials.

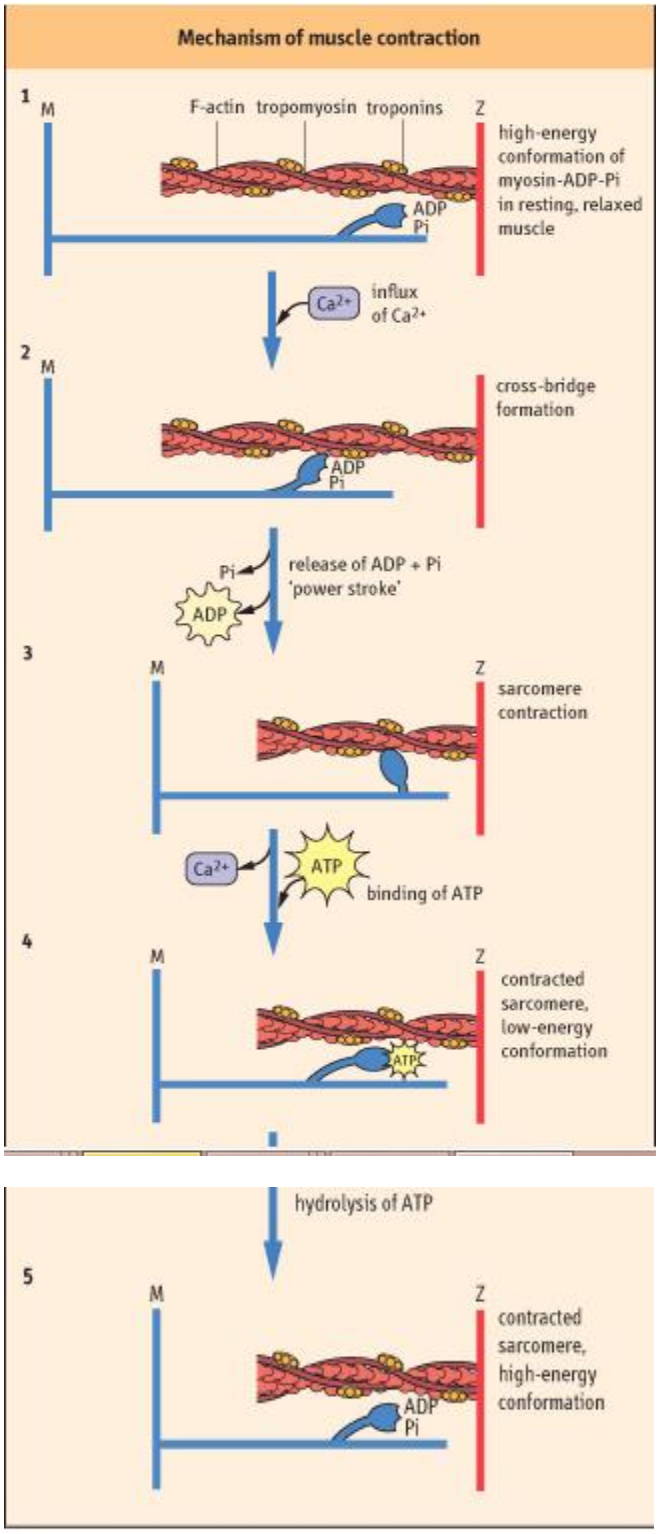


Figure 19.4 Proposed stages in *muscle contraction, according to the sliding-filament model.*

(1) In resting, relaxed muscle, calcium concentration is $\sim 10^{-7}$ mol/L. The head group of myosin chains contains bound ADP and Pi, and is extended forward along the axis of the myosin helix in a high-energy conformation. Although the myosin-ADP-Pi complex has a high affinity for actin, binding of myosin to actin is inhibited by tropomyosin, which blocks the myosin-binding site on actin at low calcium concentration.

(2) When muscle is stimulated, calcium enters the sarcoplasm through voltage-gated calcium channels (see Chapter 7). Calcium binding to Tn-C causes a conformational change in Tn-I, which is transmitted through Tn-T to tropomyosin. Movement of tropomyosin exposes the myosin-binding site on actin. Myosin-ADP-Pi binds to actin, forming a cross-bridge.

(3) Release of Pi, then ADP, from myosin during the interaction with actin is accompanied by a major conformational change in myosin, producing the 'power stroke', which moves the actin chain about 10 nm (100 Å) in the direction opposite the myosin chain, increasing their overlap and causing muscle contraction.

(4) The uptake of calcium from the sarcoplasm and binding of ATP to myosin leads to dissociation of the actomyosin cross-bridge.

(5) The ATP is hydrolyzed, and the free energy of hydrolysis of ATP is conserved as the high-energy conformation of myosin, setting the stage for continued muscle contraction in response to the next surge in Ca^{2+} concentration in the sarcoplasm.

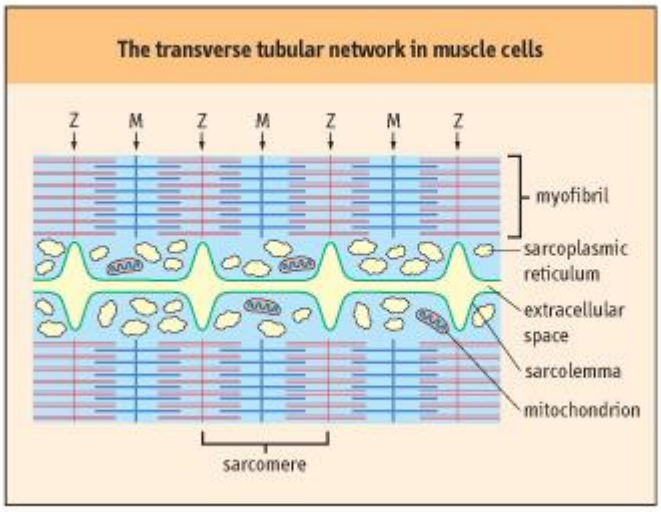


Figure 19.5 **Side-view of the transverse tubular network in skeletal muscle cells**. Transverse tubules are invaginations of the sarcolemma, which are in intimate contact with the sarcoplasmic reticulum (SR). The SR is a continuous, tubular compartment in close association with the myofibrils. The transverse tubules are extensions of the sarcolemma around the Z-line. They transmit the depolarizing nerve impulse to terminal regions of the SR, coordinating calcium release and contraction of the myofibril.

SARCOPENIA

Sarcopenia is defined as the loss of skeletal muscle mass with age. Sarcopenia is accelerated in humans after the fifth decade of life and can lead to frailty and loss of functional capacity. Besides the basic erosion of quality of life, loss of skeletal muscle mass also increases the risk of mortality and morbidity. The cause of sarcopenia appears to be related to both a biological program of muscle fiber loss and decreased physical activity. Muscle fiber innervation by spinal motor neurons is critical to both development and maintenance of the mature muscle phenotype. Spinal motor neurons decrease in number with advancing age, possibly because of cumulative oxidative damage to these post-mitotic cells. The loss of motor neurons induces muscle fiber loss and an increase in existing motor unit size, which decreases fine motor skill. Sarcopenia has also been linked to age-induced systemic changes to the endocrine, cardiovascular, and immune systems, whose functions are all critical for the maintenance of skeletal muscle mass.

Comment: The scientific evidence is clear that most elder individuals can increase muscle strength and mass with a regular resistance exercise program. Pharmaceutical treatments have also been examined for individuals who cannot regularly exercise. Currently there is no treatment for spinal motor neuron loss. Pharmaceutical treatments targeting muscle have had varying degrees of success, but are usually limited by side-effects. The treatments include endocrine interventions with male or female sex hormone replacement therapy, and growth hormone therapy. Anti-inflammatory medication is also employed to allow individuals to participate in physical activity programs. One of the best defenses from sarcopenia may be regular exercise in order to maintain muscle mass during middle age.

The calcium content of the muscle cytoplasm (sarcoplasm) is normally very low, 10^{-7} mol/L or less, but increases rapidly by ~100-fold in response to neural stimulation, leading to ATP hydrolysis and muscle contraction. Muscle cells have an adapted smooth endoplasmic reticulum organelle, known as the sarcoplasmic reticulum (SR), which serves as the site of calcium sequestration inside the cell. The plasma membrane of the myofiber cell, known as the sarcolemma, invaginates in and around the myofibrils at the Z-lines, forming a series of transverse

tubules that indirectly interact with the SR (Fig. 19.5). The muscle cell responds to motor nerve stimulation by initiating a wave of depolarization of the Na^+/K^+-gradient across the muscle plasma membrane, which is transmitted to the SR, causing a voltage-gated opening of SR Ca^{2+}-channels. Calcium is released from the SR, and the influx of Ca^{2+} into the muscle cytoplasm (sarcoplasm) triggers muscle contraction. In striated muscle, calcium interacts with troponin-C on the thin filament to allow actin-myosin interaction. Muscle contraction is mediated by changes in the conformations and interactions of actin and myosin. The actomyosin complex thereby transforms the chemical energy of ATP into the mechanical action of muscle.

Troponin protein is not expressed in smooth muscle. In this case, calcium triggers contraction by calmodulin binding. Calcium-calmodulin binding activates myosin light chain kinase. These events lead to myosin phosphorylation, which allows myosin-actin interaction.

Although muscle contraction is triggered by increased calcium, muscle relaxation is dependent on calcium being pumped back into the SR. The rate of muscle relaxation is directly related to SR-calcium ATPase activity. The SR is rich in a calcium-ATPase, which pumps calcium into the SR, maintaining cytosolic calcium in the muscle cell at submicromolar ($\sim 10^{-7}$ mol/L) concentrations. At the same time, the concentration of calcium in the SR is in the mmol/L range, comparable to that in the plasma compartment.

METABOLIC SYNDROME

Metabolic syndrome (Syndrome X), characterized by insulin resistance, hyperinsulinemia, hypertension, and hyperlipidemia, is a major, age-dependent risk factor for diabetes and cardiovascular disease. Loss of muscle mass and insulin sensitivity can dramatically influence the progression from Syndrome X to frank disease through their effect on blood glucose concentration. A half-hour of vigorous physical activity can increase skeletal muscle glucose uptake for up to 24 hours after the exercise period. Regular exercise also reduces the loss of muscle mass, improves muscle perfusion, and enhances the insulin sensitivity of muscle. There is clear evidence that obese people also benefit from regular exercise.

Muscle: An excitable tissue

Muscle has the ability to depolarize upon neural stimulation, and this depolarization is critical for the calcium release that triggers contraction. However, skeletal, cardiac, and smooth muscle receive this neural depolarization in different manners, and have different structural adaptations at the sarcolemma to account for depolarization propagation. Skeletal muscle contraction is volitional and fibers are innervated by motor nerve endplates that originate in the spinal cord. The neuromuscular junction is a special structural feature of skeletal muscle that is not found in cardiac or smooth muscle. Each individual fiber is innervated by only one motor nerve, and all the fibers innervated by one nerve are defined as a motor unit. Motor unit control and synchronization is the basis for coordinated whole muscle contraction.

Cardiac muscle is striated and contracts rhythmically under involuntary control. The general mechanism of contraction of heart muscle is similar to that in skeletal muscle, but the SR is less developed, and the transverse tubule network, an extension of the plasma membrane, is more developed in the heart. Thus, the heart is more dependent on, and actually requires, extracellular calcium for its contractile response. Lacking direct neural contact, cardiac myocytes propagate depolarization from a single node, the SA-node, throughout the myocardium. The depolarization is passed cell to cell along specialized membrane structures called intercalated disks. Cardiac muscle is also more responsive to hormonal regulation. For example, cAMP-dependent protein kinases phosphorylate transport proteins and Tn-I, mediating changes in the force of contraction in response to epinephrine .

Smooth muscle can respond to both neural and circulating factors for the stimulus of depolarization. Unlike skeletal muscle, neural input to smooth muscle innervates bundles of smooth muscle cells that cause both phasic (rhythmic) and tonic contractions of the tissue.

MALIGNANT HYPERTHERMIA

About 1 in 150 000 patients treated with halothane (gaseous halocarbon) anesthesia or muscle relaxants, responds with excessive skeletal muscle rigidity and severe hyperthermia with a rapid onset, up to 2°C (4°F) within 1 hour. Unless treated rapidly, cardiac abnormalities may be life-threatening; mortality from this condition exceeds 10%. This genetic disease results from excessive or prolonged release of Ca^{2+} from the SR, most commonly the result

of mutations in the Ca^{2+}-release channels within the SR. Excessive release of Ca^{2+} leads to a prolonged increase in sarcoplasmic Ca^{2+} concentration. Muscle rigidity results from Ca^{2+}-dependent consumption of ATP, and hyperthermia results from increased metabolism to replenish the ATP. As muscle metabolism becomes anaerobic, lacticacidemia, and acidosis may develop. The cardiac abnormalities result from hyperkalemia, caused by release of potassium ions from muscle; as supplies of ATP are exhausted, muscle is unable to maintain ion gradients across its plasma membrane. Treatment of malignant hyperthermia includes use of muscle relaxants, e.g. dantrolene, an inhibitor of the ryanodine-sensitive Ca^{2+}-channel , to inhibit Ca^{2+}-release from the SR. Supportive therapy involves cooling, administration of oxygen, correction of blood pH and electrolyte imbalances and also treatment of cardiac abnormalities.

MUSCLE ENERGY METABOLISM

Muscle consists of two types of striated muscle cells; fast-glycolytic and slow-oxidative fibers

Striated muscle cells are generally classified by their physiological contractile properties (fast versus slow) that are determined by the level of ATPase activity and the primary metabolic source of ATP synthesis (anaerobic vs. aerobic). The muscle type is closely related to muscle function. In skeletal muscle this comparison is easily seen with muscles whose contraction is necessary to continuously maintaining posture versus muscle contraction for infrequent-burst activities. The two striated muscle types are readily distinguished in skeletal muscle by coloring. Fast-glycolytic muscle is white in appearance because of less blood flow, lower mitochondrial density, and decreased myoglobin content than slow-twitch oxidative muscle, which is red. Fast-glycolytic fibers also have lower fat content and increased glycogen stores. The fast-glycolytic fibers, rely on glycogen and anaerobic glycolysis for short bursts of contraction when additional muscle force is required such as in the 'fight or flight' stress response. These muscle fibers are not capable of sustaining contraction for long periods, when compared to slow-oxidative fibers. In contrast, slow-oxidative fibers are well perfused with blood, rich in mitochondria (cytochromes) and myoglobin. This muscle type has the ability to sustain low-intensity contractions for long periods. Slow muscle uses fatty acid oxidation

for ATP synthesis, which requires mitochondria. Cardiac muscle, which is continuously contracting, has many contractile and metabolic characteristics that are similar to slow-oxidative skeletal muscle. Cardiac muscle is well perfused with blood, rich in mitochondria, and relies largely on oxidative metabolism of circulating fatty acids

METABOLISM AND MUSCLE CONTRACTION

Short-duration, high-intensity contractions

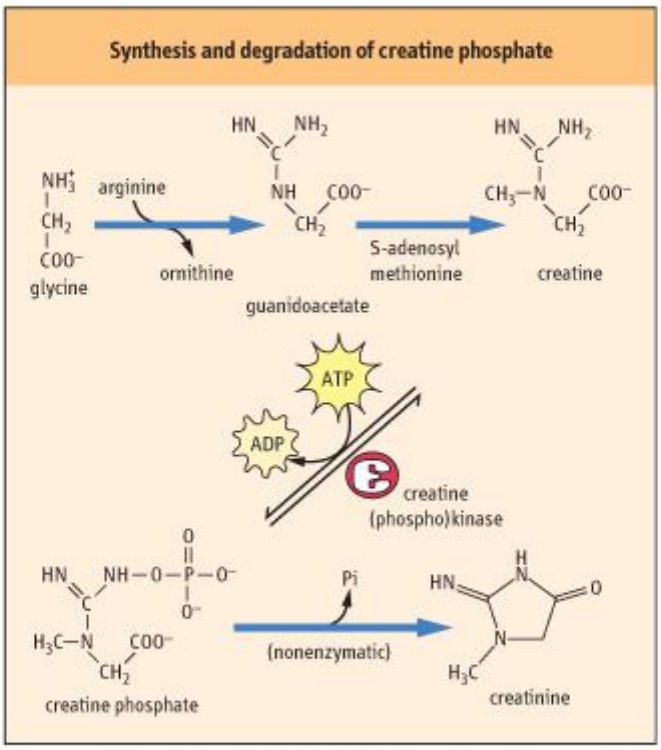


Figure 19.6 **Synthesis and degradation of creatine phosphate (creatine-P).** Creatine is synthesized from glycine[Rx] and arginine precursors. Creatine-P is unstable and undergoes slow, spontaneous degradation to Pi and creatinine, the cyclic anhydride form of creatine, which is excreted from the muscle cell into plasma and then into uri

ASSAY OF CREATININE TO ASSESS RENAL FUNCTION AND URINE DILUTION

Since creatine phosphate concentration is relatively constant per unit muscle mass, the production of creatinine is relatively constant during the day. Creatinine is eliminated in urine at a relatively constant amount per hour, primarily by glomerular filtration, and to a lesser extent by tubular secretion. Since its concentration in urine varies with

the dilution of the urine, levels of metabolites in random urine samples are often normalized to the urinary concentration of creatinine. Otherwise, a 24 h collection would be required to assess daily excretion of a metabolite. Normal creatinine concentration in plasma is about 20-80 mmol/L (0.23-0.90 mg/dL). Increases in plasma creatinine concentration are commonly used as an indicator of renal failure. The albumin/creatinine ratio in a random urine sample, an indicator of protein filtration selectivity of the glomerulus, is used as a measure of the microalbuminuria to assess the progression of diabetic nephropathy

For short bursts of energy, skeletal muscle relies on its ATP stores and an additional reserve of the high-energy storage compound, creatine phosphate (creatine-P), to regenerate ATP rapidly during the first minute as glycogenolysis is activated. Creatine is synthesized from arginine and glycine (Fig. 19.6), and is phosphorylated reversibly to creatine-P by the enzyme creatine (phospho)kinase (CK or CPK). CK is a dimeric protein and exists as three isozymes: the MM (skeletal muscle), BB (brain) and MB isoforms. The MB isoform is enriched in cardiac tissue

The level of creatine-P in resting muscle is several-fold higher than that of ATP (Table 19.3). Thus, ATP concentration remains relatively constant during the initial stages of exercise. It is replenished not only by the action of CK but also by adenylate kinase (myokinase) as follows

Creatine phosphokinase: creatine-P + ADP → creatine + ATP

Adenylate kinase: → 2 ADP → ATP + AMP

Table 19-3. Changes in energy resources in working muscle.

Changes in energy resources in working muscle			
Metabolite	Metabolite concentration (mmol/kg dry weight)		
	resting	3 minutes	8 minutes
ATP	27	26	
Creatine-P	78	27	
Creatine	37	88	
Lactate	5	8	
Glycogen	408	350	

Concentrations of energy metabolites in human leg muscle during bicycle exercise. These experiments were conducted during ischemic exercise, which exacerbates the decline in ATP concentra
They illustrate the rapid decline in creatine-P and the increase in lactate from anaerobic glycolysis of muscle glycogen. Data are adapted from Timmons JA *et al. J Clin Invest* 1998;101:79-85.

During the initial stages of exercise, muscle glycogenolysis, followed by both anaerobic and aerobic glycolysis, is the major source of energy. Calcium entry into muscle leads to formation of a Ca^{2+}-calmodulin complex, which activates phosphorylase kinase, catalyzing the conversion of phosphorylase b to phosphorylase a. AMP also allosterically activates muscle phosphorylase and phosphofructokinase-1, accelerating glycolysis from muscle glycogen

Low intensity, long duration contractions

Availability and utilization of oxygen in working muscle are the major limitations for maintaining continuous physical activity. At rest or at low-intensities of physical work, oxygen is readily available and the aerobic oxidation of lipid predominates as the main source of ATP synthesis. However, at higher work intensities oxygen availability and utilization can become limiting, and subsequently the work rate of the muscle decreases. One of the main adaptations to regular vigorous physical activity involves increasing muscle mass and oxygenation (perfusion).

During the first 15-30 minutes of exercise, there is a gradual shift from glycogenolysis and aerobic glycolysis to aerobic metabolism of fatty acids. Perhaps this is an evolutionary response to deal with the fact that lactate, produced by glycolysis, is more acidic and less diffusible than CO_2. In any case, the glycogen reserves in muscle are sufficient to support the energy needs of muscle during exercise for only about 1 hour. As exercise continues, epinephrine contributes to activation of hepatic gluconeogenesis, providing an exogenous source of glucose for muscle. Lipids gradually become the major source of energy in muscle during long-term exercise. The oxidative metabolism of lipids is supported by increased perfusion and delivery of oxygen

Long-term muscle performance (stamina) depends on levels of muscle glycogen

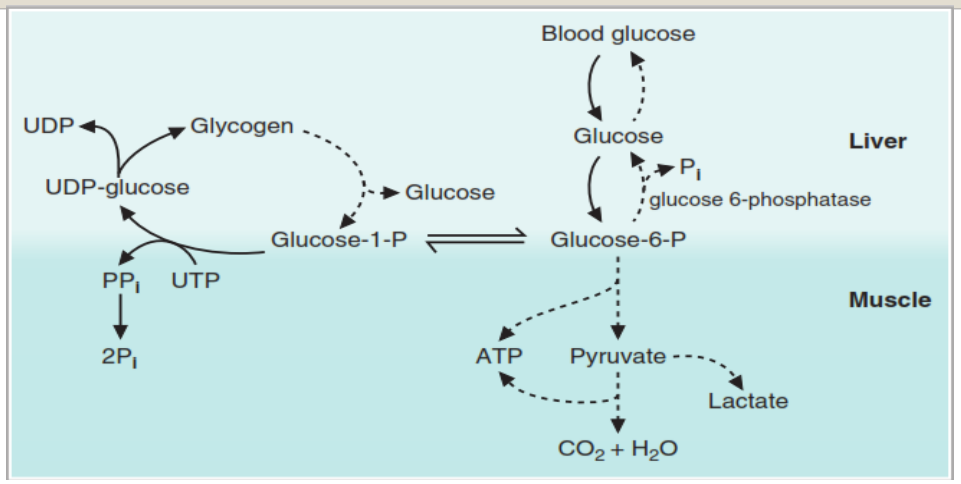
THE MEASUREMENT OF CARDIAC TROPONINS IS THE PRIMARY TEST TO DIAGNOSE MYOCARDIAL INFARCTION

Myocardial infarction (MI) is the result of blockage of blood flow to the heart. Tissue damage results in leakage of intracellular enzymes into blood. Among these are glycolytic enzymes, such as LDH ; however, measurements of myoglobin, total plasma CK and CK-MB isozymes are more commonly used for diagnosis and management of MI. Myoglobin is a small protein (17 000 kDa) and rises most rapidly in plasma, within 2 hours following MI. Although it is sensitive, it lacks specificity for heart tissue. It is cleared rapidly by renal filtration and returns to normal within 1 day. Since plasma myoglobin also increases following skeletal muscle trauma, it would not be useful for diagnosis of MI, e.g. following an automobile accident. Total plasma CK and the CK-MB

isozyme begin to rise within 3-10 hours following an MI, and reach a peak value of up to 25 times normal after 12-30 hours; they may remain elevated for 3-5 days. Total CK may also increase as a result of skeletal muscle damage but the measurement of CK-MB provides specificity for cardiac damage.

Comment. Enzyme-linked immunosorbent assays (ELISA) for the myocardial troponins are now recommended for the diagnosis and management of MI. These assays depend on the presence of unique isoforms of troponin subunits in the adult heart. Tn-T concentration in plasma increases within a few hours after a heart attack, peaks at up to 300 times normal plasma concentration, and may remain elevated for 1-2 weeks. An assay for a specific isoform in an adult heart, Tn-T_2, is essentially 100% sensitive for diagnosis of MI and yields fewer than 5% false-positive results. Significant increases in plasma Tn-T are detectable even in patients with unstable angina and transient episodes of ischemia in the heart. Troponins are commonly used as a component of an algorithm to differentiate high-risk from low-risk patients in terms of need for immediate invasive intervention.

Marathon runners typically 'hit the wall' when muscle glycogen reaches a critically low level. Glycogen is the storage form of glucose in skeletal muscle, and its muscle concentration can be manipulated by diet. Fatigue occurs when the requirement for ATP exceeds its rate of synthesis.



Overview of glycogen synthesis and degradation. Solid arrows, glycogen synthesis; broken arrows, glycogen degradation, glucose release in the liver, and glucose 6-phosphate utilization in the muscle

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For efficient ATP synthesis, there is a continuing requirement for a basal level of glycogen and carbohydrate metabolism in muscle, even when fats are the primary source of muscle energy. Carbohydrate metabolism is important as a source of pyruvate, which is converted to oxaloacetate

by the anaplerotic, pyruvate carboxylase reaction. Oxaloacetate is required to maintain the activity of the TCA cycle - for condensation with acetyl CoA derived from fats. Muscle glycogen can be spared and performance time increased during long-term vigorous physical activity by increasing the availability of circulating glucose , either by gluconeogenesis or by carbohydrate ingestion. Increased utilization of fatty acids is an important training adaptation to regular vigorous physical activity that can also serve to spare glycogen stores