Normal Bone Physiology

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Throughout life, the bones of the human skeleton are perpetually remodeled. Changes in biomechanical forces and removal and replacement of old damaged bone with new bone all contribute to this process. There are four categories of bones – irregular, flat, long, and short. These categories are made up of the appendicular skeleton which has 126 bones, the axial skeleton which consists of 74 bones, 6 auditory ossicles, and a variable number of sesamoid bones. This number is not set at birth; typically newborns have about 270 bones; however, this gradually decreases to 206 [1] in the skeletally mature adult. While the primary function of the skeleton is structural support, it also functions critically in movement by providing levers for muscles, maintains hematopoiesis and acid-base balance, and serves as a reservoir for minerals, cytokines, and growth factors.

In general, bones are made up of two different regions, an outer dense, solid, cortical region and an inner loose honeycomb-like trabecular region. The ratio of each differs from bone to bone, with vertebrae being the most trabecular and the diaphysis of long bones containing the most cortical bone [2]. Periosteum covers the outside cortical bone, and an endosteum lines the inside – both layers nourish cortical bone through a dense network of blood vessels. Osteons contained within both cortical and trabecular bone each have a slightly different structure. Cortical osteons are also known as haversian systems and make up the functional unit of cortical bone. Each one is 4–10 mm long and about 0.2 mm in diameter and consists of 5–15 concentric layers or lamellae of compact bone that surrounds a central haversian canal [3]. Each canal in turn contains central blood vessels which nourish each system. Lamellae, whose circumferential layers look like rings of a tree and whose collagen fibrils are arranged in an orthogonal pattern contain within them, contain spaces or lacuna. Inside each lacuna is an osteocyte, a differentiated mature osteoblast. Osteocytes, while grossly may appear to be isolated within each lacuna, actually contact each other via long thin cytoplasmic processes which traverse within transverse tunnels known as canaliculi [4]. Osteocytes, along with osteoclasts and osteoblasts, play a significant role in bone growth and remodeling. Prior to examination of bone formation and remodeling, a basic overview of aforementioned cell types will be discussed.

Osteoclasts

The only cell known to be capable of resorbing bone, osteoclasts, is truly unique with respect to bone remodeling. They are derived from

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mononuclear precursor cells that arise from a monocyte-macrophage lineage, and while found within many tissues, those which give rise to osteoclasts are thought to reside only in bone marrow [5]. Numerous transcription factors have been identified to play a role in osteoclast differentiation, many of which will be described below. PU.1 is an early transcription factor, which appears during myeloid differentiation and is essential for osteoclast development [5]. Likewise, c-Fos is essential for osteoclast development; mice lacking this transcription factor, like PU.1, develop osteopetrosis [6]. Interestingly, c-Fos-deficient mice still develop macrophages, while PU.1 knockout mice do not, implying c-Fos being secondary to PU.1 and PU.1 being necessary for early macrophage development [7]. Other transcription factors such as microphthalmia-associated transcription factor (MITF) and nuclear factor of activated T-cells cytoplasmic-1 (NFAT-1) are also required for osteoclast formation; however their roles are not as clearly defined [8]. RANKL and colony-stimulating factor-1 (CSF-1) are produced by osteoblasts and stromal cells of the bone marrow – these appear in both a cell surface and soluble form and play critical roles in mature osteoclast differentiation. Osteoprotegerin (OPG) functions to bind RANKL, thus leaving RANK receptors inactivated which decreases the maturation of osteoclasts. When mature, osteoclasts bind to bone matrix via integrin receptors to collagen, fibronectin, and laminin. This binding causes them to become polarized with their resorption surface developing the classic ruffled border, leading to the formation of vesicles containing cathepsin K and matrix metalloproteases. These vesicles are released into the extracellular space adjacent to the bone surface, and the acidic environment begins to digest organic matrix [9–11].

Osteoblasts

Osteoblasts develop from pluripotent mesenchymal stem cells and are controlled in part by the transcription factor RUNX2 – RUNX2 knockout mice have a complete lack of mineralized tissue [12]. The Wnt/Beta-catenin pathway has also been shown to be necessary for osteoblast formation with high expression being present within the embryonic skeleton. Osteoblasts form bone and play additional roles in the production of bone matrix proteins, bone mineralization, and the expression of osteoclastogenic factors [13]. They are a heterogeneous population – some respond one way to hormonal signals, while others have been shown to respond differently to similar signals within the axial and appendicular skeleton. When quiescent, osteoblasts exist in a flattened form which line both the endosteal surface as well as the undersurface of the periosteum. During bone remodeling they leave this state, become active and rounded, and move to areas of bone formation; they return to their flattened state once active bone growth is complete. Active forms secrete type I collagen and other matrix proteins and can be differentiated easily on microscopy due to their large single nucleus and prominent Golgi apparatus [14].

Osteocytes

Terminally differentiated osteoblasts are known as osteocytes and are found within lacunae inside bone matrix. Gap junctions allow for communication via filopodia and are required for osteocyte activity and survival. They function in mechanosensation and respond to various stresses placed on bone, a process which is thought to be mediated by cytoplasmic fluid flow [15]. Osteocytes live for decades, and the presence of empty lacunae in aging bone suggests apoptotic mechanisms, which has been shown to be regulated by estrogen deficiency. Estrogen treatment and bisphosphonates may function to prevent apoptosis and thus maintain bone health [16]. These three cell types are the most significant contributors to skeletal growth and remodeling – a brief overview will now be presented.

Bone Growth and Remodeling

Bone grows radially and longitudinally only during childhood and adolescence; however modeling occurs throughout life as bones make gradual

adjustments based on changes in applied forces [17]. Bones normally widen with age, as new bone is deposited just deep to the periosteum and resorbed from the endosteum. It also thickens in certain regions based on the increased forces, a concept known as Wolff's law [18]. Bone remodeling allows bone to maintain its strength and mineral homeostasis capabilities. Unlike growth and modeling, which serve to increase the overall net amount of bone, remodeling can be thought of as keeping the overall amount of bone in a steady state [19]. It should be noted however that remodeling does increase slightly in aging men and women – this process occurs at a faster rate in postmenopausal women [20]. The remodeling cycle happens in four stages: activation, resorption, reversal, and formation. These stages occur sequentially. Fractures will initiate the remodeling cascade, otherwise the sites at which remodeling is initiated are seemingly random [20, 21].

Activation involves the production and detection of initiating signals. These signals can be direct mechanical strain placed on bone, hormones such as estrogen and PTH, or small molecules from underlying exposed matrix. Recruitment of osteoclast precursors occurs in response to detection of these signals, and once they arrive at the area of interest, they fuse to form multinucleated preosteoclasts [22]. Preosteoclasts then bind to the bone matrix via integrins and form annular "sealing zones" where bone resorption will occur [23, 24].

Resorption is the next phase of bone remodeling. It normally lasts about 2–4 weeks during each remodeling cycle and is a complex process regulated by numerous factors including RANKL, OPG, IL-1 and 6, CSF-1, PTH, calcitonin, and 1,25-dihydroxyvitamin-D [25, 27]. These factors are released by osteoblasts, and while each subtly functions to increase or decrease osteoclast activity, the collective net effect is an increase, and subsequent resorption, of bone [26]. IL-1 and 6 have been shown to induce osteoclast differentiation [27, 28] to their ready form, while CSF-1 promotes proliferation and survival of osteoclasts as well as increased osteoclast motility and cytoskeletal reorganization. RANKL promotes differentiation to mature cells and also increases resorption activity. Various hormones will then increase or decrease osteoclast activity based on what is required at the time. The actual mechanism of resorption involves the secretion of hydrogen ions via H+-ATPase proton pumps and Cl channels found within the osteoclast cell membranes. The enzymatic pH is generally around 4–5, a level at which bone matrix can easily be mobilized [29, 30]. Cathepsin K, matrix metalloproteinase 9, and tartrate-resistant acid phosphatase then become released from lysosomes and digest organic matrix. Once the inorganic and organic substances have been removed, a characteristic shallow bowl-shaped Howship's lacuna remains on the surface [31]. Once done, osteoclasts undergo apoptosis leading to the next phase of remodeling.

The reversal phase was so named because it is during this stage that bone resorption is reversed, leading to subsequent bone formation. Although osteoclasts have undergone apoptosis and are no longer present at lacuna, mononuclear precursor cells, preosteoblasts, and liberated osteocytes remain and begin the process of reversal and preparation [32]. While the exact signals that trigger the initiation of reversal are not yet known, TGF-Beta, IGF-1 and 2, and BMPs are thought to play significant roles [33, 34]. These factors promote the final removal of undigested matrix and prepare for the final phase, formation.

As the name suggests, formation involves all the steps needed to deposit and mineralize new bone and takes approximately 4–6 months to complete [34]. It is during this phase that osteoblasts synthesize new matrix composed of type I collagen and deposit it within the previously formed lacuna. Proteoglycans, alkaline phosphatase small integrin-binding ligand (SIBLING) proteins, and lipids make up the remaining minority of organic substance [35]. The remaining step is hydroxyapatite secretion and incorporation into collagen, and while that exact mechanism is unknown, nonspecific alkaline phosphatase and nucleotide pyrophosphatase phosphodiesterase are thought to create the optimal extracellular environment to allow for this mineralization process [35].

With the formation of new bone, the remodeling process concludes. Osteoclasts undergo apoptosis, while osteoblasts either follow a similar fate

Table 1.1 The microscopic physiology and anatomy of bone

Cell type	Compound	Function	Mutations
Osteoclasts	PU.1	Early transcription factor, responsible for hematopoiesis. Implicated in osteoclast development	Osteopetrosis
	C-Fos	Transcription factor, requires for macrophage-osteoclast lineage	Osteopetrosis
	MITF	Required for osteoclast-specific membrane channels	Waardenburg syndrome type 2
	NFAT-1	Required for osteoclast formation, exact function unknown	Breast cancer
	CSF-1	Osteoclast differentiation	Osteopetrosis
	Osteoprotegerin	Binds RANKL, decreases maturation of osteoclasts	Juvenile Paget disease
Osteoblasts	RUNX2	Transcription factor, required osteoblast differentiation, known as the "master regulator of bone"	Cleidocranial dysostosis, osteosarcoma
	Sp7	Transcription factor, thought to interact with RUNX2 to promote osteoblast differentiation, induces Col1a1, osteonectin, osteopontin	Osteogenesis imperfecta
	DLX5	Transcription factor, interacts with RUNX2 and DLX5	Hand and foot malformation syndrome
	FGF	Promotes osteoblast differentiation	Chondrodysplasias
	FosB	Released by mechanical stress, increases osteoblast formation	Short-rib thoracic dysplasia
Osteocytes	PHEX	Involved in bone mineralization, osteopontin is the substrate for PHEX	X-linked hypophosphatemic rickets
	MEPE	Involved in integrin recognition, highly expressed in osteocytes	Osteomalacia, osteoporosis
	DMP1	Highly expressed by osteocytes, required for bone mineralization	Autosomal recessive hypophosphatemia
	E11/gp38	Promotes cytoplastic process formation	Unknown
	Sclerostin	BMP antagonist, has anti-anabolic effect on bone	Van Buchem disease

(about 50–70% of the total pool) and revert to the bone-lining phenotype or become embedded within matrix and differentiate to osteocytes. Osteocytes live within their lacuna and maintain a healthy environment. The appearance of this bone is now the characteristic osteon, made up of both organic and inorganic matrix which is the final description of the microscopic physiology and anatomy of bone (Table 1.1).

Organic Bone Matrix

Type I collagen makes up 85–90% of collagenous protein, with types III, IV, and fibril-associated collagen with interrupted triple helices (FACIT) making up the remainder. The latter proteins are non-fibrillary collagens that are thought to serve as bridges and help stabilize and organize extracellular matrices; these members include collagens IX, XII, XIV, XIX, and XXI [36]. Non-collagenous proteins, such as proteoglycans, phosphatases, and growth factors, help regulate cellular activity and matrix mineralization. As mentioned above, osteoblasts are responsible for the synthesis and secretion of both collagenous and non-collagenous proteins. Alkaline phosphatase is the principle glycosylated protein present in the extracellular matrix and is also found bound to osteoblast surfaces. The most prevalent non-collagenous protein however is osteonectin, also known as secreted protein acid which is rich in cysteine (SPARC), and is a basement membrane protein that is thought to play a role in collagen fibril assembly, procollagen processing, osteoblast growth, and profileration [37].

Inorganic Bone Matrix

The overall composition of bone is about 50–70% mineral, 20–40% organic matrix, and 5–10% water, and the remainder is lipid. The overwhelming majority of mineral is hydroxyapatite

$[Ca_{10}(PO_4)_6(OH)_2]$, with the rest being carbonate, magnesium, and acid phosphates. Unlike their geological cousin, bone hydroxyapatite is smaller by weight, poorly crystallized, and more soluble. Alkaline phosphatase, osteocalcin, osteopontin, and bone sialoprotein all regulate bone mineralization via the amount of hydroxyapatite that is formed. Minerals are first deposited in zones between the ends of collagen fibrils and then subsequently filled [38]. As bone matures, hydroxyapatite crystals purify and enlarge through aggregation and individual crystal growth. While not mentioned earlier, vitamin D plays an important role in stimulating the mineralization of unmineralized bone. After GI absorption or skin production, vitamin D is converted to its active form via the liver and kidneys, to 1,25-dihydroxyvitamin-D. It is this compound that is responsible for maintaining serum calcium and phosphorus levels allowing for the passive mineralization of bone matrix. This is accomplished by promoting intestinal absorption of these ions, as well as differentiation of osteoblasts and osteoblast expression of osteocalcin, osteonectin, OPG, and numerous other cytokines. The description above provides a brief and classic overview of bone physiology; below will describe some advances in molecular biology that have helped further the understanding and function of bone.

Updates on Bone Physiology

Osteoclast function is complex – much of their regulation and function is still unknown. In recent years, attention has focused on preosteoclasts, the cells that will eventually form multinucleated osteoclasts. Recent evidence has shown that preosteoclasts are mobilized to blood by sphingosine-1 phosphate (S1P) and sphingolipid, which are secreted by erythrocytes and platelets. Preosteoclasts and osteoclasts express S1P receptors and are attracted by this chemokine, possibly helping to promote the fusion of the mononucleated precursors to their more mature multinucleated form. Additionally, S1P expression is negatively regulated by cathepsin K, which may posit a future role for its inhibitors as a bone stimulating agent [39, 40]. S1P levels are also increased in the synovial fluid of rheumatoid arthritis, which may attract preosteoclasts to affected joints [39], and calcitonin has been shown to inhibit osteoclast activity by way of S1P [41]. While still early, this may prove important with respect to potential future pharmacotherapeutic agents.

G-proteins and regulators of G-protein (RGS) act to enhance the action of G-protein signaling and represent another example of complex regulation which has been implicated in osteoblast physiology. RGS2 has been shown to play a role in osteoblast differentiation via upregulation of forskolin and PTH. RGS5 may play a role in the osteoblastic response to extracellular calcium and Axin, a member of the RGS family, which negatively regulates bone mass (Axin knockout mice have increased bone density as compared to their wild-type controls) [42].

Neurohormonal regulation is another emerging area in bone physiology with serotonin, leptin, and neuropeptide-Y all having effects on bone. Brain serotonin has been shown to stimulate proliferation of osteoblasts and inhibit bone resorption [43]. Interestingly, gut-derived serotonin has the opposite effect, with genetically modified mice with low levels of duodenal serotonin having increased bone density. This is supported by some clinical studies which have shown that patients treated with SSRIs have decreased bone mass and increased risk of osteoporotic hip fracture, while adolescents taking SSRIs have significantly decreased bone mineral density. This may be explained in part by the presence of serotonin transporters in bone, although the exact mechanism is yet to be fully elucidated [44].

Leptin has been shown to increase proliferation of osteoblasts, presumably through its action on the beta-1-adrenergic system and IGF-1 system [45, 46]. Conversely, it suppresses bone formation via its activity on beta-2-adrenergic receptors and inhibits brain serotonin release, implicating a complex role for this hormone.

Neuropeptide Y (NPY) has been shown to be produced by osteoblasts and osteocytes [47] in a negative regulatory fashion as NPY overexpression slows formation of endo- and periosteal

bone, increases trabecular bone loss [48], and has been shown to be a modulator of leptin with respect to bone formation. Numerous other hormones, not typically associated with bone such as cannabinoids and norepinephrine, have also been shown to alter its physiology; however that is beyond the scope of this chapter and is merely mentioned to highlight the complexity of bone regulation.

Age-Related Changes in Bone

While peak bone age is achieved within relatively similar time frames, around 30 for both men and women, the point of maximal substantial bone loss differs significantly between the sexes. Cortical bone loss in women occurs in the years following menopause, while in men it occurs around 70–75 years of age. Trabecular bone loss by contrast occurs in both sexes at similar times with men experiencing 42% loss and women 37% loss by age 50 [64].

It was over 70 years ago that estrogen was first implicated in postmenopausal bone loss [49], and while initially unknown, it is now accepted that this mechanism acts through the RANKL/OPG system. Postmenopausal women have a threefold greater percentage of RANKL expressing cells as their premenopausal counterparts, and it seems that the reverse is true with the presence of estrogen suppressing bone resorption in both men and women [65]. Osteoporotic cortical and trabecular bone is thinner, although the mineral content per given area of tissue is actually increased, as is collagen linearity and carbonate content.

On a macroscopic scale, bone undergoes changes in shape throughout age in response to load, as described by Wolff's law. Additionally, it increases in cross-sectional area due to expansion of its outer diameter and thins at its cortical walls [50] – this pattern has been seen in both nonhuman and human models [51, 52]. With regard to trabecular bone, age-related loss is predominantly due to thinning of individual trabeculae in men, while in women, it is due to a loss of connectivity and a decreased complexity of networks [53]. Over time, resorption outweighs formation, and bone gradually thins – these macroscopic changes underlie the microscopic changes to individual populations of bone cells.

The major bone cell types have finite lifespans which are controlled by several external factors in addition to the replication cycles. It has been shown that osteoblast populations diminish due to decreases in their respective precursors [54]. A similar process happens in osteoclasts, with the number of hematopoietic precursor cells declining with age [55], while osteocytes are hypothesized to undergo apoptosis due to lack of mechanical stimulus or loss of canalicular networks [56]. At the next level of organization, proteins themselves undergo age-related changes.

There is evidence that bone's structural proteins undergo age-related changes as well, both in their modification and production, and perhaps the most important of these proteins is collagen. Appropriate function of collagen is essential for bone to maintain its strength, and if this is not maintained, bone can lose its integrity. A critical factor of collagen is its orientation and alignment with hydroxyapatite crystals. Collagen orientation becomes more linear with age (recall that it maintains its strength through its normally orthogonal orientation within lamellae), and this may have an effect on mineral crystallization as well as overall strength [56]. Enzymatic cross-linking of collagen is an important component of its posttranslational modification and adds to its strength; however nonenzymatic cross-linking has the opposite effect. There is evidence that nonenzymatic cross-linking of collagen increases with age and this leads to an overall decrease in bone's strength and toughness, which increases the risk of fracture [57, 58]. This type of cross-linking also affects the way collagen is mineralized which further alters its structural properties.

The mineral content of bone increases with age, and while this increases its breaking stress, it ultimately results in making it more brittle and decreasing toughness [59, 60]. Not only does the overall amount of inorganic substance change, but the composition changes as well. Hydroxyapatite crystals are their purest at around 25–30 years of age, over time, and they gain substitutions of carbonate for hydroxyl and phosphate within the

apatite surface [61]. This, along with a concomitant decrease in acid phosphate content [62, 63], is thought to be a factor contributing to the decreased toughness of aging bone. A better understanding of these processes may help to give more insight into the factors that lead to age-related bone loss.

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